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How Is Fe-S Cluster Formation Regulated?

Erin L. Mettert, **Patricia J. Kiley**

Department of Biomolecular Chemistry, School of Medicine and Public Health, University of Wisconsin–Madison

Abstract

Iron-sulfur(Fe-S)clustersarefundamentaltonumerousbiologicalprocesses in most organisms, but these protein cofactors can be prone to damage by various oxidants (e.g., O_2, reactive oxygen species, and reactive nitrogen species) and toxic levels of certain metals (e.g., cobalt and copper). Furthermore, their synthesis can also be directly influenced by the level of available iron in the environment. Consequently, the cellular need for Fe-S cluster biogenesis varies with fluctuating growth conditions. To accommodate changes in Fe-S demand, microorganisms employ diverse regulatory strategies to tailor Fe-S cluster biogenesis according to their surroundings. Here, we review the mechanisms that regulate Fe-S cluster formation in bacteria, primarily focusing on control of the Isc and Suf Fe-S cluster biogenesis systems in the model bacterium *Escherichia coli*.

Keywords

iron-sulfur cluster; Fe-S; regulation; homeostasis; Isc pathway; Suf pathway

THE SIGNIFICANCE OF REGULATING FE-S CLUSTER FORMATION

Present in nearly all organisms, iron-sulfur (Fe-S) clusters serve as protein cofactors and thus are an integral part of their respective protein functions. Because of their intrinsic chemical properties, Fe-S clusters are extremely versatile cofactors, as reflected by their involvement in diverse biological processes (e.g., respiration, photosynthesis, nitrogen fixation, DNA replication and repair, RNA modification, and gene regulation). Composed of different combinations of inorganic iron and sulfur, Fe-S clusters can exist in various forms, the most common types being [4Fe-4S] and [2Fe-2S] clusters in the oxidized (2+) or reduced (1+) redox state. As electrons are delocalized over both Fe and S ions, Fe-S clusters are well suited for redox sensing, catalysis, or electron transfer (6, 41, 61).However, although these properties are important for versatility, the reactive nature of Fe-S clusters is also the source of their sensitivity. Solvent-exposed clusters are susceptible to oxidation by O_2 and reactive O_2 species (ROS), potentially leading to cluster conversion or complete cluster loss (53–55). Fe-S clusters are also prone to damage by nitric oxide (NO) and toxic levels of certain metals, like cobalt and copper (27, 72, 104). In addition, iron limitation

elmettert@wisc.edu.

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undoubtedly influences the amount of iron available for building Fe-S clusters. Consequently, prokaryotes and eukaryotes not only possess multiprotein systems dedicated to Fe-S cluster assembly but also have evolved strategies to control the activity of these systems according to their surroundings. These regulatory strategies enable microorganisms to promote Fe-S biogenesis when the need for clusters is heightened (e.g., during oxidative or nitrosative stress) or limit Fe-S formation when cluster demand is low (e.g., during anaerobic growth), thus preserving iron and sulfur for other cellular pathways. Given the vital roles of Fe-S clusters and their prevalence in nature, understanding the mechanisms by which Fe-S biogenesis pathways are regulated has been a major research objective. Here, we review the mechanisms involved in controlling Fe-S cluster synthesis, focusing primarily on the regulation of the Isc and Suf Fe-S biogenesis pathways present in the model bacterium *Escherichia coli*. The major factors that are known or proposed to be involved in this regulation are listed in Table 1.

BACTERIAL SYSTEMS RESPONSIBLE FOR FE-S BIOGENESIS

Three major Fe-S cluster assembly pathways in bacteria have been identified: the Isc, Suf, and Nif systems (recently reviewed in 10, 15, 85, 100, 106). However, the number and type of Fe-S biogenesis systems vary substantially among bacterial species. Highly conserved among prokaryotes and eukaryotes, the Isc and Suf systems are capable of providing Fe-S clusters to a wide range of apo-protein substrates. In eukaryotes, the Suf system is mainly localized in chloroplasts, whereas the Isc system is found in mitochondria and is distinct from cluster assembly proteins that facilitate Fe-S maturation in the cytosol. In contrast, the Nif system, initially discovered in the nitrogen-fixing bacterium *Azotobacter vinelandii*, is specifically dedicated to the maturation of nitrogenase, which fixes atmospheric nitrogen to produce ammonia. Accordingly, expression of this system is primarily controlled by factors that sense nitrogen availability (reviewed in 73).

Present in *E. coli* are the *iscRSUA-hscBA-fdx-iscX* (*isc*) and *sufABCDSE* (*suf*) operons, which respectively encode for the Isc and Suf machinery that function in Fe-S biogenesis; components of these pathways are listed in Table 2. Fe-S cluster assembly involves acquisition of sulfur from L-cysteine by cysteine desulfurase enzymes IscS or SufSE and its subsequent donation to the respective scaffold proteins IscU or SufB, upon which Fe-S clusters are transiently assembled. From the scaffold, nascent clusters are transferred either directly to apo-protein substrates or to the carrier proteins IscA and SufA, which in turn deliver clusters to specific substrates. The ATP-hydrolyzing HscBA complex of the Isc pathway facilitates this transfer; a similar function for the ATPase SufC of the Suf pathway has been proposed. Also participating in this process is Fdx, a ferredoxin that provides electrons for the reduction of sulfur on IscS and/or the reductive coupling of two [2Fe-2S] clusters to generate [4Fe-4S]. Furthermore, additional proteins not encoded by the *isc* or *suf* operons have known or implicated roles in Fe-S biogenesis (listed in Table 2) (10, 15, 85, 100, 106). It should be noted that the source of iron for cluster assembly by either pathway is uncertain. Although IscA, SufA, CyaY, and IscX display iron-binding properties and have been proposed to act as iron donors for cluster assembly in vivo (13, 33–35, 62, 64, 71, 81, 93, 94, 135), additional studies argue that iron delivery is not the primary function of these proteins; the roles of IscA and SufA as Fe-S carriers are well established (127, 128), and as

discussed below, evidence suggests that CyaY and IscX serve as allosteric regulators of IscS (1, 20, 52, 62, 98).

As the above description implies, proteins of the Isc and Suf pathways display functional redundancy. Consistent with this notion, *E. coli* mutants lacking either pathway are viable, whereas a strain lacking both Isc and Suf is not (116). Nevertheless, individual components of the Isc and Suf systems do not appear to be interchangeable; deletion of any of the *suf* genes, with the exception of *sufA*, was shown to be synthetically lethal with the Δ*iscS* allele (86). Furthermore, the Isc and Suf machineries exhibit some mechanistic variation in cluster formation (reviewed in 15, 102, 106) and display ≥10-fold difference in their estimated copy number per cell during standard, aerobic growth (Table 2) (69). These characteristics may in part account for their distinct physiological roles in *E. coli*. Under standard growth conditions, the majority of cluster formation is due to Isc, which is considered the housekeeping Fe-S biogenesis pathway and whose cellular protein levels are relatively abundant (Table 2) (50, 126). Although Suf protein levels are much lower than those of the Isc machinery under standard growth conditions, they are predicted to be elevated during oxidative stress or iron limitation given that these stresses induce *suf* transcription (11, 56, 65–67, 86, 136, 142). It is under these conditions that Suf is thought to be the favored pathway for Fe-S biogenesis, because these stresses limit the function of the Isc machinery (32, 56). Although resistance of the Suf system to stress is not fully understood, recent studies have identified characteristics of the Suf machinery that may be advantageous for Fe-S cluster assembly during stress. For example, the [2Fe-2S] cluster on the SufB scaffold is less sensitive to destabilization by hydrogen peroxide (H_2O_2), O_2, and the iron chelator ethylenediaminetetraacetic acid (EDTA) than the [2Fe-2S] cluster on IscU in vitro (9). Also, the specific activity of SufSE in vitro is higher than that of IscS alone or in complex with IscU at low L-cysteine concentrations and after exposure to H_2O_2 (31). Finally, the Suf pathway may be well suited to acquire iron when it is scarce; SufD and the ATPase activity of SufC were required for proper iron delivery to SufB in vivo, and the $SufBC_2D$ complex isolated from *E. coli* contains $FADH_2$, capable of reducing ferric iron for Fe-S assembly (108, 133). Despite these features, even high levels of the Suf machinery failed to fully mature some Fe-S proteins in the absence of the Isc pathway (37). Thus, on one hand, although Suf may function more efficiently than Isc during stress, it may meet only the minimal Fe-S biogenesis requirements by providing clusters to essential Fe-S proteins. On the other hand, the broadened specificity of the Isc pathway makes this an ideal system for housekeeping Fe-S biogenesis. Below, we discuss how *E. coli* coordinates regulation of the Isc and Suf pathways to maintain Fe-S homeostasis in response to stress.

ALLOSTERIC REGULATION OF FE-S CLUSTER ASSEMBLY

By directly associating with one or more components of the Fe-S biogenesis machinery, allosteric regulatory proteins may promote or hinder cluster formation. This mechanism would provide a rapid means for fine-tuning cluster assembly based on cellular needs. Below, we highlight the major findings that have implicated CyaY and IscX as potential allosteric regulators in this process.

The Influence of CyaY on In Vitro and In Vivo Fe-S Formation

E. coli CyaY has gained attention as an allosteric regulator of the Isc pathway. A similar function has been demonstrated for frataxin, the CyaY ortholog in eukaryotes whose reduced expression causes increased mitochondrial iron levels, decreased Fe-S protein activity, and, in humans, the neurodegenerative disease Friedreich ataxia (23, 24, 80, 88, 89, 92). In vitro, frataxin promotes Fe-S synthesis by binding the Isu-Nfs1-Isd11 complex (in which Isu and Nfs1 are IscU and IscS homologs, respectively) to stimulate cysteine desulfurase activity, sulfur transfer from Nfs1 to Isu, and/or L-cysteine binding by Nfs1 (19, 20, 25, 87, 90, 122). Whereas CyaY directly interacts with IscS from *E.coli*, CyaY appears to function in a manner opposite of frataxin in that it inhibits in vitro Isc-mediated cluster formation (1, 52, 64, 98). Interestingly, experiments in which analogous Fe-S biogenesis proteins were interchanged revealed that activation or inhibition of Fe-S formation by the frataxin ortholog is dictated by the identity of the cysteine desulfurase (20). For example, CyaY stimulated Nfs1-Isd11 activity, whereas frataxin suppressed cluster assembly by the IscS-IscU complex. Thus, these findings suggest that evolution of the cysteine desulfurase may have reversed the mode of allosteric control exhibited by frataxin. The difference in intrinsic activity displayed by IscS and Nfs1 may be the underlying reason for this frataxin role reversal; although these enzymes share 60% sequence identity, IscS exhibits a k_{cat}/K_M value for cysteine desulfurase activity that is 10–70-fold higher than that of the Nfs1-Isd11 complex. However, in the presence of frataxin and Isu, the k_{cat}/K_M of Nfs1-Isd11 is similar to that of IscS (20, 122). Because IscS also provides sulfur to several sulfur-utilizing pathways in addition to Fe-S biogenesis (Figure 1), including thiamine and molybdopterin synthesis and tRNA modification (48), it is tempting to speculate that the high intrinsic activity of IscS may be vital for its multiple cellular roles in *E. coli* and that CyaY may influence the pathway in which IscS participates.

The mechanism by which CyaY imparts its negative role on Isc-mediated cluster formation in vitro is unclear. CyaY, IscS, and IscU can form a ternary complex, and CyaY hinders Fe-S formation by inhibiting IscS activity (52, 98). However, when different conditions and assays were used, CyaY had little effect on IscS activity, or IscS activity was even slightly increased when in complex with IscU (1, 20). Furthermore, another study found that in contrast to earlier observations, CyaY, IscU, and Fdx all compete for overlapping binding sites on IscS (63, 134). Therefore, the mutually exclusive binding of these proteins to IscS is an alternative mechanism by which CyaY could affect Fe-S cluster assembly in vitro.

Although further work is needed to elucidate this mechanism, these biochemical properties of CyaY must also be linked with its physiological function. *E. coli* Δ*cyaY* mutants exhibit modest defects in maturation of the Fe-S proteins IscR, FNR, and respiratory complexes I and II, suggesting that like frataxin, CyaY promotes Fe-S biogenesis in vivo (96, 107). Similar defects in Fe-S protein activity have also been reported for the *Salmonella enterica* serovar Typhimurium Δ*cyaY* mutant (118, 129). CyaY specifically contributes to cluster biogenesis via the Isc pathway and not the Suf pathway, reinforcing the similarities between CyaY and eukaryotic frataxin. These findings are also consistent with the co-occurrence of *cyaY* and genes of the *isc* operon, particularly *hscB* and *hscA*, in *Proteobacteria* genomes (51, 107). However, in contrast to frataxin depletion, deletion of *cyaY* in *E. coli* does not

result in changes in bacterial growth, cellular iron content, and survival after oxidant exposure (68); *S.* Typhimurium strains lacking CyaY also grow normally but have been shown to have some increased sensitivity to H_2O_2 (125).

Recent studies highlight some additional differences between the eukaryotic and prokaryotic Isc machinery that affect frataxin function. In the yeast *Saccharomyces cerevisiae*, mutation of a single residue in the Isu1 scaffold protein bypassed the need for the frataxin ortholog Yfh1 in Fe-S biogenesis. Specifically, substitution of Met at position 107 with Ile, Cys, Leu, or Val restored alterations in mitochondrial iron levels, Fe-S enzyme activity, and Nfs1-Isd11 cysteine desulfurase activity in cells depleted of Yfh1 (138, 139, 140). In addition, phylogenetic analysis revealed that whereas Met at this position in Isu1 is conserved among eukaryotes, this position is occupied by Ile, Cys, Leu, or Val in most prokaryotes (139, 140). Indeed, *E. coli* IscU contains Ile at the analogous position, which upon substitution with Met renders Isc-mediated Fe-S biogenesis more CyaY dependent (105). Together, these findings suggest that the identity of the residue at this position influences the level of dependence on frataxin for Fe-S biogenesis and indicate an intrinsic difference in the frataxin dependency between the eukaryotic and prokaryotic Isc machinery. Perhaps these differences also partially explain the dissimilarities in growth phenotype for mutants lacking frataxin/CyaY/Yfh1. Clearly, more research is required to reconcile the physiological function of CyaY with its biochemical activities.

IscX Displays Behavior Similar to That of CyaY

Another allosteric regulator of *E. coli* Isc-mediated Fe-S biogenesis is IscX (also called YfhJ), encoded by a gene in the *isc* operon. Like CyaY, initial in vitro studies revealed that IscX binds iron and that it directly associates with IscS, suggesting that IscX likely plays a role in cluster assembly (62, 93, 112, 119). Indeed, IscX inhibits in vitro Fe-S formation by binding to IscS and reducing its cysteine desulfurase activity when in complex with IscU (62). Thus, although CyaY and IscX both negatively regulate cluster assembly, they may do so by different mechanisms. In addition, because CyaY, IscX, and Fdx compete for overlapping binding sites on IscS, it was proposed that CyaY and IscX have distinct roles (62, 134). Yet, like CyaY mutants, an Δ*iscX* mutant lacks an obvious growth phenotype and exhibits only minor defects in Fe-S protein activities (44, 107, 120). Furthermore, as in the case of CyaY, not all Isc-containing bacteria encode IscX (93). However, strains lacking both CyaY and IscX showed greater defects in Fe-S protein activity than the single mutant, suggesting that CyaY and IscX have an overlapping function in promoting Fe-S biogenesis in vivo (107). Consistent with this notion, *iscX* was found to have a weaker genetic link with *cyaY* than with other genes of the *isc* operon among *Proteobacteria*. Additionally, although the presence of IscX in eukaryotes appears to be restricted to a specific group of protozoa, including those of the genus *Plasmodium*, these particular eukaryotes do not contain homologs of CyaY, thus raising the possibility that IscX and CyaY replace each other among eukaryotic genomes (93). Further research is needed to test this hypothesis.

Interestingly, a separate interaction between *E. coli* IscX and IscU has been identified through nuclear magnetic resonance spectroscopy and copurification experiments (62). Structural evidence indicates that residues of IscU that interact with IscX are distinct from

those that interact with IscS. As binding of Fe^{2+} by IscX appeared to stabilize its interaction with IscU in vitro, it has been hypothesized that in addition to its allosteric regulatory role, IscX may have a second function as the iron donor for Fe-S biogenesis (62). According to the proposed model, [2Fe-2S] assembly would be initiated by binding of Fdx to IscS for sulfur reduction. Subsequent to IscU accepting reduced sulfur and iron from IscS and IscX, respectively, IscX would be available to bind IscS and inhibit cysteine desulfurase activity. The second cycle in the assembly process would be initiated by Fdx-mediated displacement of IscX from IscS. Although the lack of a growth phenotype for the Δ*iscX* mutant seems to disagree with an iron donor role, additional work is needed to characterize the IscX-IscU interaction and to understand its relevance in vivo.

Perspective on Allosteric Regulation

Despite findings pointing to a role of CyaY and IscX as allosteric regulators of Fe-S cluster synthesis in vitro, determining the physiological function of these regulators in vivo has been a challenge. This complexity may be due to *E. coli* having two functionally redundant Fe-S biogenesis systems. For example, an alteration in Isc activity that results from mutating a putative allosteric regulator may be masked through compensation by the Suf pathway. In addition, functional redundancy may exist between these two allosteric regulators. Consistent with the role of IscS in multiple sulfur-utilizing pathways (Figure 1), biochemical and global proteomic approaches revealed that IscS interacts with nine or more binding partners, four of which (CyaY, IscX, Fdx, and TusA) are known to compete for overlapping binding sites on IscS (4, 22, 30, 62–64, 112, 134). This emphasizes the complexity of IscS regulation in vivo and suggests an additional possibility—that CyaY and IscX promote Fe-S biogenesis by limiting IscS involvement in other sulfur-utilizing pathways. In support of this notion, the Δ*cyaY* Δ*iscX* double mutant was found to be hypersensitive to λ phage infection, a process that requires TusA-mediated sulfur acquisition from IscS for tRNA thiolation (75, 76, 107). In summary, current evidence has set the initial framework for understanding how Fe-S biogenesis may be allosterically regulated, but more research is needed to understand its physiological impact.

REGULATION OF FE-S CLUSTER DELIVERY

Although the delivery of nascent clusters to apo-protein substrates occurs downstream of Fe-S formation, studies have indicated that one or more carrier proteins can participate in this step, depending on growth conditions and/or the identity of the substrate (70, 127, 128). Thus, cluster delivery may provide an additional point of regulation that ultimately influences Fe-S protein maturation. In *E. coli*, cluster delivery is carried out by A-type carrier proteins IscA, SufA, and ErpA. Although these proteins function similarly in vitro, genetic analyses indicate that they have distinct and overlapping roles in vivo (127, 128). Because it is difficult to follow the fate of Fe-S clusters in vivo, dissecting these routes has required genetic approaches involving gene disruption(s) and ectopic expression of the carrier protein in question. Characterization of the Fe-S cluster maturation pathways for several substrate proteins (e.g., IspG/H and NsrR) under aerobic growth conditions revealed that subsequent to Fe-S formation on the IscU scaffold, the cluster is transferred first to IscA, then to ErpA, and finally to the apo-protein target. However, under anaerobic

conditions or stress, alternate routes may be used in which the cluster travels from the IscU or SufB scaffold to IscA or SufA, respectively, and then directly to the substrate (127, 128). Furthermore, substrates may acquire clusters from different routes. IscR, for example, can receive its cluster in a carrier-mediated manner, involving IscA and ErpA but not SufA, or directly from the IscU or SufB scaffold (128). Taken together, cluster delivery by carrier proteins is highly versatile, ensuring efficient Fe-S maturation under numerous growth conditions. Furthermore, as the specific route(s) of cluster delivery may depend on intrinsic features of the apo-protein target that are not yet known, it is possible that *E. coli* has evolved a hierarchy by which substrates receive clusters according to their cellular function such that essential Fe-S proteins receive clusters most efficiently. It has also been proposed that carrier proteins may provide a mechanism to distinguish which substrates receive [4Fe-4S] clusters versus [2Fe-2S] clusters (128). Further experiments are needed to explore these hypotheses.

Growing evidence suggests that several non-A-type proteins, such as NfuA, also participate in cluster delivery. NfuA is a two-domain protein, consisting of an Nfu domain responsible for binding and transferring Fe-S clusters and a degenerate A-type carrier domain that promotes interaction of NfuA with its targets (3, 101). As NfuA was found to be important for cellular growth during oxidative stress and iron limitation, the two-domain arrangement is proposed to be advantageous for Fe-S maturation during these stresses (3).

The monothiol glutaredoxin GrxD may also play a role in Fe-S maturation, given that mutants lacking *grxD* and components of the *isc* operon are inviable, similar to that of mutants lacking *isc* and *suf* (21). GrxD is highly conserved in prokaryotes and eukaryotes and can bind Fe-S clusters as a homodimer and as a heterodimer with BolA, whose function in *E. coli* is not well understood (26, 137). Both of these complexes can transfer their Fe-S cluster to a substrate protein in vitro, an activity reminiscent of the *S. cerevisiae* heterodimer Grx3/4-Fra2 (homologous to GrxD-BolA), which transfers its Fe-S cluster to Aft2, a transcription factor that regulates iron uptake (97, 137). The physiological function of GrxD in *E. coli* is unknown; however, a recent study showed that GrxD and NfuA are important for activity of the Fe-S cluster-containing enzyme MiaB, involved in tRNA modification. Interestingly, although both GrxD and NfuA directly associate with MiaB, only NfuA was capable of transferring a cluster to MiaB in vitro, whereas GrxD was proposed to function in MiaB cluster repair (14).

Finally, Mrp may also serve as a carrier protein. Although its function in *E. coli* is not known, it exhibits sequence similarity to the eukaryotic cytosolic Fe-S assembly proteins Cfd1 and Nbp35, which bind Fe-S clusters individually as homodimers and as a heterotetrameric complex (83). Indeed, the Mrp ortholog in *S. enterica*, ApbC, has been shown capable of binding and transferring Fe-S clusters in vitro and is required for Fe-S-dependent enzyme activity in vivo (16, 17, 113). Interestingly, ApbC may be a unique carrier in that it exhibits ATPase activity (113). Although this activity is dispensable for Fe-S cluster transfer in vitro, it is needed for ApbC function in vivo (18).

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL REGULATION OF FE-S BIOGENESIS MACHINERY

In several bacteria, the cellular concentration of Fe-S assembly proteins is regulated at transcriptional and posttranscriptional levels. In *E. coli*, IscR is well characterized as the master regulator of maintaining Fe-S homeostasis, controlling transcription of both the Isc and Suf pathways according to the Fe-S demand. However, additional factors also play a role in modulating expression of Fe-S biogenesis pathways, such as the Fe^{2+}-sensing regulator Fur, whose regulon also includes many genes involved in iron uptake. Below, we first describe the transcriptional and posttranscriptional mechanisms by which the Isc and Suf pathways are individually regulated. We then describe how expression of both pathways is highly integrated to ensure preservation of Fe-S homeostasis under a variety of stress conditions.

Regulation of Isc Expression Is Multifaceted

Given that the housekeeping Isc pathway is predicted to supply over 150 different proteins with Fe-S clusters in *E. coli*, physiologically it makes sense that levels of the Isc machinery are relatively abundant under standard growth conditions (Table 2) (100). Nevertheless, it is well known that expression of the Isc pathway is intimately coupled with the cellular Fe-S demand. This occurs primarily through transcriptional repression of the *isc* operon by IscR (encoded by the first gene in the operon), in which IscR uses the occupancy status of its own Fe-S cluster to alter *isc* transcription (44). In addition, Fur indirectly regulates Isc expression through the small RNA RyhB, which differentially destabilizes the *isc* transcript when iron is limiting (32). Finally, components of the Isc pathway are substrates for degradation by the ClpXP and FtsH proteases (40, 132). Collectively, these regulatory mechanisms suggest that tight control of Isc expression is important for Fe-S homeostasis (Figure 2).

IscR Adjusts *isc* Transcription to Accommodate the Cellular Fe-S Demand

In addition to *isc*, IscR directly or indirectly controls expression of over 40 other genes in *E. coli*, in which direct regulation involves specific binding of IscR to either one of two distinct DNA motifs, referred to as type 1 and type 2 sites (45). These sites are differentially regulated by IscR (44, 82). Studies to dissect the mechanism by which IscR controls *isc* transcription have provided the initial framework for understanding how IscR regulates genes whose promoters contain type 1 sites, e.g., the promoters of *isc*, *erpA*, and *nfuA* (45). Additionally, the findings from these studies, summarized below, have been critical to understanding how *E. coli* sustains Fe-S homeostasis.

Several genetic and biochemical analyses have revealed that IscR regulates *isc* transcription through an autoregulatory feedback loop that depends on IscR Fe-S cluster ligation. In vivo, IscR acquires a [2Fe-2S] cluster primarily from the Isc machinery, and in its cluster-containing form (holo-IscR), IscR is capable of binding to the *iscR* promoter (P_{iscR}) region to repress transcription (44, 45, 109). While holo-IscR binds up to three sites within P_{iscR} in vitro, regulation of *isc* in vivo requires holo-IscR binding to only two of these sites, both of which are type 1 sites (44). These sites overlap with the promoter elements responsible for recruiting RNA polymerase (RNAP). Because binding of IscR and RNAP to P_{iscR} is

mutually exclusive (79), IscR hinders *isc* transcription by occluding association of RNAP with P_{iscR}. In contrast to holo-IscR, clusterless apo-IscR does not bind P_{iscR} with high affinity in vitro, and IscR cluster binding mutants are defective in repressing *isc* transcription in vivo (44). Together, these findings imply that IscR uses the ligation state of its own cluster to sense the cellular Fe-S status and, accordingly, adjusts *isc* expression so that the need for Fe-S biogenesis may be met.

Indeed, this homeostatic regulation has been shown to accommodate changes in Fe-S demand that result from fluctuations in O_2 tension. For example, there is approximately 3- to 5-fold less *isc* repression under aerobic conditions, when some Fe-S clusters are known to be destabilized by O_2 and ROS, compared with anaerobic conditions, when clusters are generally more stable. Furthermore, this derepression of *isc* occurs despite aerobic IscR protein levels being approximately 10-fold higher (44). To account for this O_2-mediated regulation of *isc*, it is predicted that differences in IscR [2Fe-2S] cluster occupancy exist between aerobic and anaerobic conditions. Because it is challenging to assess in vivo cluster occupancy directly, this hypothesis was tested by quantifying the amount of *isc* repression over a similar range of IscR protein levels in aerobic and anaerobic cells. Because half-maximal *isc* repression required approximately 9-fold more IscR protein under aerobic conditions relative to anaerobic conditions, this result supported the notion that IscR [2Fe-2S] cluster occupancy is lower under aerobic conditions (44). The elevated need for Fe-S biogenesis under aerobic conditions likely creates competition between IscR and other substrate proteins for the Isc machinery, leading to decreased IscR [2Fe-2S] cluster occupancy and derepression of *isc*. In contrast, the low Fe-S demand in the absence of O_2 would diminish this competition, resulting in increased IscR cluster occupancy, and thus, *isc* repression. This homeostatic model proposes that IscR may be a poor substrate for the Isc machinery, thus ensuring that cluster acquisition by other Fe-S proteins is satisfied prior to IscR receiving its [2Fe-2S] cluster. As the IscR [2Fe-2S] cluster is coordinated by an atypical $(Cys)_3(His)_1$ cluster ligation scheme, it is possible that this feature makes IscR a less efficient competitor for the Isc machinery compared with other substrates with the more common $(Cys)_4$ cluster ligation (39).

While work is ongoing to address this question, some insight into the mechanism of IscR regulation has been obtained from the recently solved apo-IscR crystal structure (103). Despite the weak electron density of residues encompassing the cluster-binding region, its location indicates that this region is solvent exposed, perhaps allowing for easy access to the [2Fe-2S] cluster. Additionally, the cluster-binding site from one IscR monomer is proximal to the DNA-binding domain of the other monomer, raising the possibility that cluster ligation induces a conformational change within IscR that influences DNA binding. This may involve rearrangement of glutamate 43 in the DNA-binding domain that was found to be a critical residue for discriminating between type 1 and type 2 sites (103). Efforts to solve the [2Fe-2S]-IscR crystal structure to provide further support for this model are in progress. Determining how cluster ligation promotes DNA binding to both type 1 and type 2 motifs will have a significant impact on understanding of how IscR globally regulates Fe-S formation.

Stress-Induced Regulation of *isc* Expression

Although the homeostatic model for *isc* expression proposes that the amount of competition between IscR and other substrates for the Isc machinery influences the level of IscR [2Fe-2S] cluster occupancy, it does not exclude the possibility that holo-IscR may directly respond to other signals that destabilize Fe-S clusters or hinder their formation, such as ROS, NO, iron depletion, or toxic levels of cobalt or other metals. In fact, these stresses are known to promote *isc* transcription (Figure 3) (11, 38, 59, 67, 86, 99, 128, 136, 142). Nevertheless, studies have shown that under certain stress conditions, the Isc machinery is either not functional or not present at sufficient levels to satisfy the cellular requirements for Fe-S biogenesis. For example, despite the induction of *isc* transcription during oxidative stress, the Isc machinery is inactivated by submicromolar levels of H_2O_2, most likely through oxidation of Fe-S clusters formed on IscU (56). Furthermore, the *isc* transcript is differentially destabilized by the small RNA RyhB when iron is limiting (32). Expression of *ryhB* is repressed by Fur under iron-replete conditions; upon iron depletion, this repression is relieved because Fur requires its Fe^{2+} cofactor for DNA binding (74). In turn, this results in elevated levels of RyhB. Binding of RyhB to the complementary sequence that overlaps the *iscS* ribosomal-binding site promotes cleavage of the downstream portion of the transcript through the actions of the RNA chaperone Hfq and the RNA degradosome. In contrast, the portion containing *iscR* remains relatively stable, presumably because of a secondary structure within the transcript that shields it from degradation (32). Taken together, these regulatory mechanisms limit activity or translation of the Isc machinery while simultaneously allowing the accumulation of apo-IscR, which is capable of activating expression of the alternate Suf Fe-S biogenesis pathway (discussed below).

Control of Isc Levels Through Proteolysis

Investigations to identify novel protease substrates revealed that components of the Isc machinery are targeted for degradation. For example, IscS is a substrate of the essential protease FtsH, whereas both IscS and IscU are proteolyzed by ClpXP (40, 132). As oxidation of Fe-S clusters by O_2 or ROS could coincidentally result in oxidative damage to proteins, proteolysis of these Isc components may simply maintain protein quality control. Interestingly, however, evidence suggests that posttranslational control of the eukaryotic IscU homolog (Isu) has a direct role in regulating Fe-S biogenesis in *S. cerevisiae*. Whereas Isu is degraded by the Lon-type protease Pim1 in wild-type cells, increased stability of Isu was observed in a subset of mutant strains lacking mitochondrial Fe-S assembly factors. Furthermore, this increased stability required direct interaction between Isu and the Nfs1 cysteine desulfurase, possibly because of Nfs1 blocking Pim1 recognition of Isu (2, 115). Although elevated Isu levels prolonged the lifespan of cells during stationary phase, they had deleterious effects on cell growth and heme-containing enzyme activity under iron-limiting conditions, presumably because of sequestration of available iron by high levels of Isu (115). Thus, by modulating its degradation rate, Isu may be maintained at appropriate amounts according to cellular iron levels, providing a mechanism to spare iron for essential pathways when it is limiting. Studies are needed to establish whether Isc protein degradation also plays a direct role in regulating Fe-S biogenesis in *E. coli*.

Coordinate Regulation of *suf* Expression

Like the Isc pathway, expression of the stress-induced Suf Fe-S biogenesis system is tightly controlled through transcriptional regulation. However, unlike *isc* transcription, which appears to be regulated in *E. coli* solely by IscR, transcriptional regulation of the *suf* operon involves the coordinate action of multiple regulatory proteins (Figure 3). This coordinate regulation ensures that levels of Suf machinery are elevated in response to environmental stresses that increase the Fe-S demand but limit Isc function.

In contrast to the Isc proteins, the Suf machinery is maintained at relatively low levels under nonstress conditions (Table 2), consistent with Isc being the primary Fe-S biogenesis pathway during standard growth. This is mainly due to the action of Fur, which represses transcription of *suf* and whose target site within the *sufA* promoter (P_{sufA}) overlaps with promoter elements recognized by RNAP (65, 67, 86, 95). In agreement with the Suf pathway functioning primarily under conditions of stress, transcription of *suf* is strongly induced in response to iron limitation and exposure to various oxidants (11, 59, 65–67, 86, 99, 136, 142). Iron depletion is well known to lead to Fur inactivation, but ROS and NO—through oxidation and nitrosylation, respectively, of its Fe^{2+} cofactor—also inactivate Fur (28, 29, 47, 66, 67, 123). Thus, these stresses relieve Fur binding to P_{sufA}. However, stress-mediated increases in *suf* transcription are not simply due to loss of Fur binding. Rather, full induction of *suf* expression requires the combined action of OxyR, IHF, and IscR (66, 67, 136).

Upon sensing H_2O_2, the OxyR regulatory protein activates transcription of *suf*, in addition to several antioxidant genes (142). Atypical of other OxyR-dependent promoters, the OxyR binding site within P_{sufA} is located relatively far upstream of the *sufA* start codon, suggesting that alteration in DNA architecture may be necessary for *suf* induction (142). Indeed, OxyR-dependent activation of *suf* requires the DNA-bending protein IHF, which upon binding P_{sufA} presumably brings the distant OxyR target site close to the RNAP recognition elements (66, 86). It is worth noting that the OxyR regulon (in addition to the oxidant-responsive SoxRS regulon) includes the gene encoding Fur (141). As Fur inactivation by ROS would relieve Fur-dependent repression of iron uptake pathways, increased iron uptake during oxidative stress may promote formation of hydroxyl radicals that cause substantial damage to cellular components. To circumvent this problem, OxyR upregulates *fur* to restore repression of iron uptake pathways (123, 141). This regulation may also be a mechanism for reestablishing Fur-mediated repression of *suf* following adaptation to peroxide stress.

Like OxyR and IHF, IscR has a major role in upregulating the Suf pathway by activating *suf* transcription in response to oxidative stress and iron limitation (136). Furthermore, this regulation does not require the cluster bound form of IscR, unlike regulation of the *isc* promoter (82, 136). Underlying this differential regulation is the presence of a type 2 IscR–binding site within P_{sufA} that is required for IscR-mediated induction (45, 82). In contrast to the type 1 sites of P_{iscR}, type 2 sites are bound with high affinity by holo- or apo-forms of IscR (82). Indeed, wild-type [2Fe-2S]-IscR and a clusterless IscR variant (IscR-C92A) are both capable of activating *suf* in vivo and in vitro (43, 77, 82, 136). Interestingly, the type 2 site within P_{sufA} overlaps the Fur target site, and binding of IscR and Fur to P_{sufA} are mutually exclusive (67). As Fur exhibits a higher binding affinity for P_{sufA} under standard

growth conditions, Fur serves as an antiactivator by blocking IscR from binding P_{sufA}, in addition to preventing RNAP recognition. On the other hand, stresses that inactivate Fur also hinder Isc-mediated Fe-S biogenesis, resulting in the accumulation of apo-IscR, which in turn further activates *suf* transcription.

Together, these findings demonstrate how *suf* expression is highly coordinated through the activity of four transcription factors—Fur, OxyR, IHF, and IscR. Furthermore, preliminary evidence suggests that additional factors may contribute to the complexity of *suf* transcriptional regulation. For instance, the NO-responsive transcription regulator NsrR may repress *suf*. Chromatin immunoprecipitation experiments showed binding of NsrR to P_{sufA} in vivo (91). In addition, the stationary phase σ factor RpoS was observed to have a negative effect on *sufA* induction; further work is needed to address the mechanism (65).

Regulation of the Isc and Suf Pathways Is Integrated

As described above, IscR and Fur are key factors in coupling expression of the Isc pathway with that of the Suf pathway. This integrated regulation enables *E. coli* to maintain Fe-S homeostasis under a variety of growth conditions. Critical to this process is the unique ability of IscR to be constitutively active as a transcription factor but differentially regulate genes depending on the occupancy of its [2Fe-2S] cluster. This was recently emphasized through genetic analyses demonstrating that IscR-dependent activation of *suf* is essential inmutants deprived of the Isc pathway because strains lacking *iscSUA-hscBA-fdx* and either IscR or an intact type 2 binding site within P*sufA* are not viable (77). In addition, the high IscR protein levels that occur in the absence of the Isc machinery are responsible for the increased *suf* transcription exhibited by a Δ*iscSUA-hscBA-fdx* mutant (44, 78). Interestingly, this mutant also exhibits residual [2Fe-2S]-IscR activity, presumably due to apo-IscR acquiring its [2Fe-2S] cluster from elevated levels of Suf machinery (44). In support of this notion, when the Δ*iscSUA-hscBA-fdx* allele was combined with a deletion of *fur*, *suf* expression was even further enhanced, as was [2Fe-2S]-IscR activity (77). Together, these findings affirm the essential role of IscR in coordinating expression of the two Fe-S biogenesis pathways. However, they also bring to light the potential challenge in interpreting the phenotypes of relevant mutants because defects in Isc-mediated Fe-S biogenesis coincide with an upregulation of *suf*. In turn, elevated levels of the Suf machinery may compensate for the lack of Isc function. Thus, future studies to thoroughly investigate potential factors involved in Fe-S biogenesis will require in vivo and in vitro analyses.

REGULATION OF FE-S CLUSTER REPAIR

In addition to studies dedicated to understanding how Fe-S formation is regulated, several studies have focused on the mechanisms by which damaged Fe-S clusters are repaired and how this process is controlled. Although many factors have been implicated in this process (glutathione, YgfZ, YggX, RIC, and iron storage proteins such as Bfr, FtnA, FtnB, and Dps), only RIC (formerly known as YtfE) has been demonstrated to have a functional role in cluster repair both in vitro and in vivo (8, 36, 42, 46, 60, 114, 117, 124, 125). The gene encoding RIC was initially discovered to be highly induced by exposure to NO and was later shown to be a direct target of the NO-responsive transcription factor NsrR (12, 59, 91, 99).

An *E. coli* mutant lacking RIC displayed increased sensitivity to NO, H_2O_2, and iron limitation and exhibited defects in the repair of Fe-S clusters damaged by NO and H_2O_2 for the enzymes aconitase and fumarase (57–59). Further, addition of purified RIC to cell lysates of this mutant restored the activities of these enzymes. Because this restoration required the presence of a di-iron center within RIC, it has been proposed that RIC likely repairs clusters by providing them with iron (58). Indeed, holo-RIC was recently shown to be capable of donating iron for in vitro Fe-S cluster assembly on apo-protein forms of IscU and spinach ferredoxin in the presence of IscS and L-cysteine (84). Decreased aconitase and fumarase activities in cells lacking RIC under standard growth conditions raise the possibility that RIC also acts as an iron donor for Fe-S maturation in the absence of stress (57). Further analysis is needed to test this hypothesis and to understand how other factors may function in Fe-S cluster repair.

OUTLOOK

Undeniably, the regulation of Fe-S cluster formation is crucial for bacteria and other organisms to adapt to changes in Fe-S demand that go hand in hand with living in dynamic environments. As reviewed here, Fe-S formation is controlled at many levels by several integrated mechanisms, including allosteric control of Fe-S machinery, variations in cluster delivery by carrier proteins, and regulation of Fe-S assembly protein expression at transcriptional and posttranscriptional levels. Although much progress has been made in dissecting these mechanisms, more research is decidedly needed to gain further insight into the roles of additional players in this process. Indeed, recent studies using genetic screens (49) have not only identified many novel candidates but also provided supporting evidence for the involvement of previously implicated proteins in *E. coli* Fe-S homeostasis. For example, a genetic interaction between Isc pathway components and the highly conserved, folate-binding protein YgfZ was recognized (21). The observation that a Δ*ygfZ* mutant displays defects in Fe-S–dependent enzyme activity and sensitivity to oxidative stress led to the proposal that YgfZ is needed for Fe-S homeostasis, with its folate cofactor possibly serving as an electron donor (130). Consistent with this hypothesis, genes encoding folate synthesis enzymes were similarly found to have a genetic interaction with the Isc pathway (*folB*), or when they were deleted, a phenotype similar to that of the Δ*ygfZ* mutant was observed (*folE* and *folP*) (21, 130). Also identified was a genetic interaction between genes of the ferric enterobactin transport system and *csdA*, encoding the third cysteine desulfurase in *E. coli* (5). Overexpression of CsdA was found to restore viability of the Δ*iscSsufS* double mutant and, thus, Fe-S–dependent activity through functional interactions with the SufBCDE proteins (121). This raises the possibility of a hybrid CsdA-Suf system participating in cluster assembly under certain growth conditions in which ferric iron is available. Together, these findings underscore the intricacy of regulating Fe-S biogenesis in *E. coli*.

Additionally, prospective studies are required to further characterize how known factors impart their regulatory function on Fe-S formation. For example, given that IscR plays a major role in coordinating expression of both the Isc and Suf systems, determining the [2Fe-2S]-IscR crystal structure and comparing it with that of the apo-protein form will be critical to understanding how cluster ligation confers differential regulation of these

pathways. Furthermore, as IscR is highly conserved among *Proteobacteria* and particularly prevalent in many pathogenic bacteria, discerning the mechanistic features that contribute to the extreme versatility of this transcription factor will have a significant impact (79).

Finally, studies of other bacteria may shed light on different strategies by which Fe-S formation is regulated. For example, cyanobacteria utilize the SufR transcription factor to regulate expression of the *sufBCDS* operon, encoding the primary Fe-S biogenesis pathway of these bacteria (110, 111, 131). Likewise, in some *Rhizobia* species, the transcription factor RirA appears to tailor transcription of the *sufS2BCDS1XA* operon according to iron availability (7). Given that the number and type of Fe-S biogenesis systems vary among bacterial species, further analysis will likely reveal additional mechanisms by which bacteria regulate Fe-S formation.

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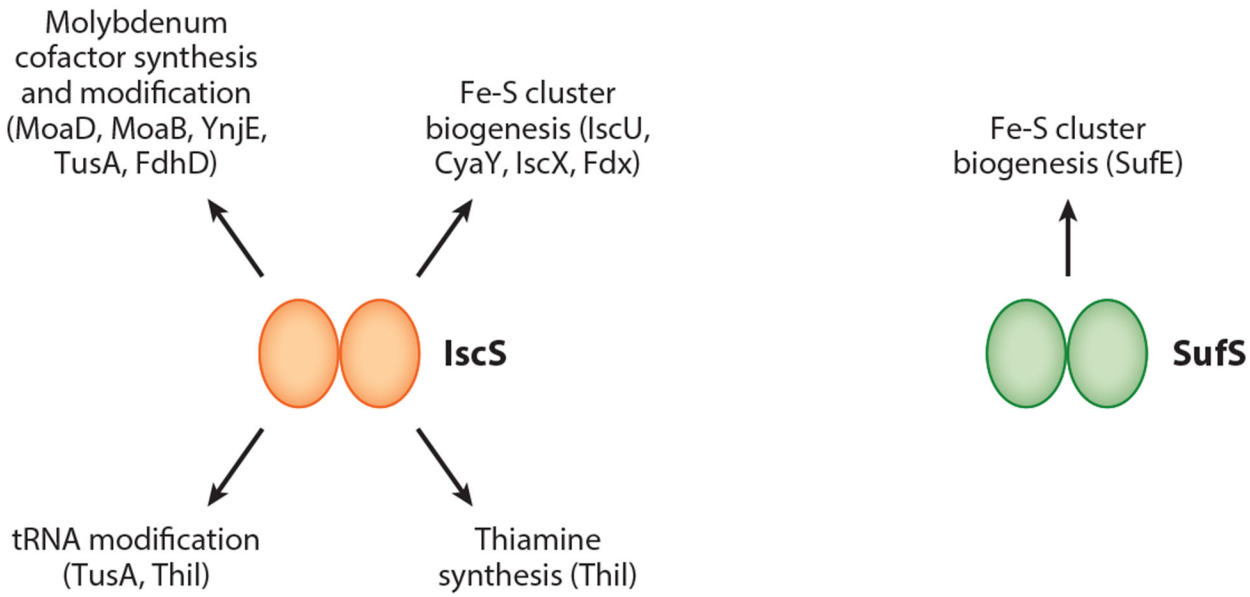

Figure 1.

In *Escherichia coli*, the cysteine desulfurase IscS plays a major role in providing sulfur for several pathways. In contrast, the cysteine desulfurase SufS appears to be specifically dedicated to Fe-S cluster biogenesis under conditions of stress. Indicated by arrows are known examples of proteins within sulfur-utilizing pathways that make direct protein-protein interactions with IscS or SufS.

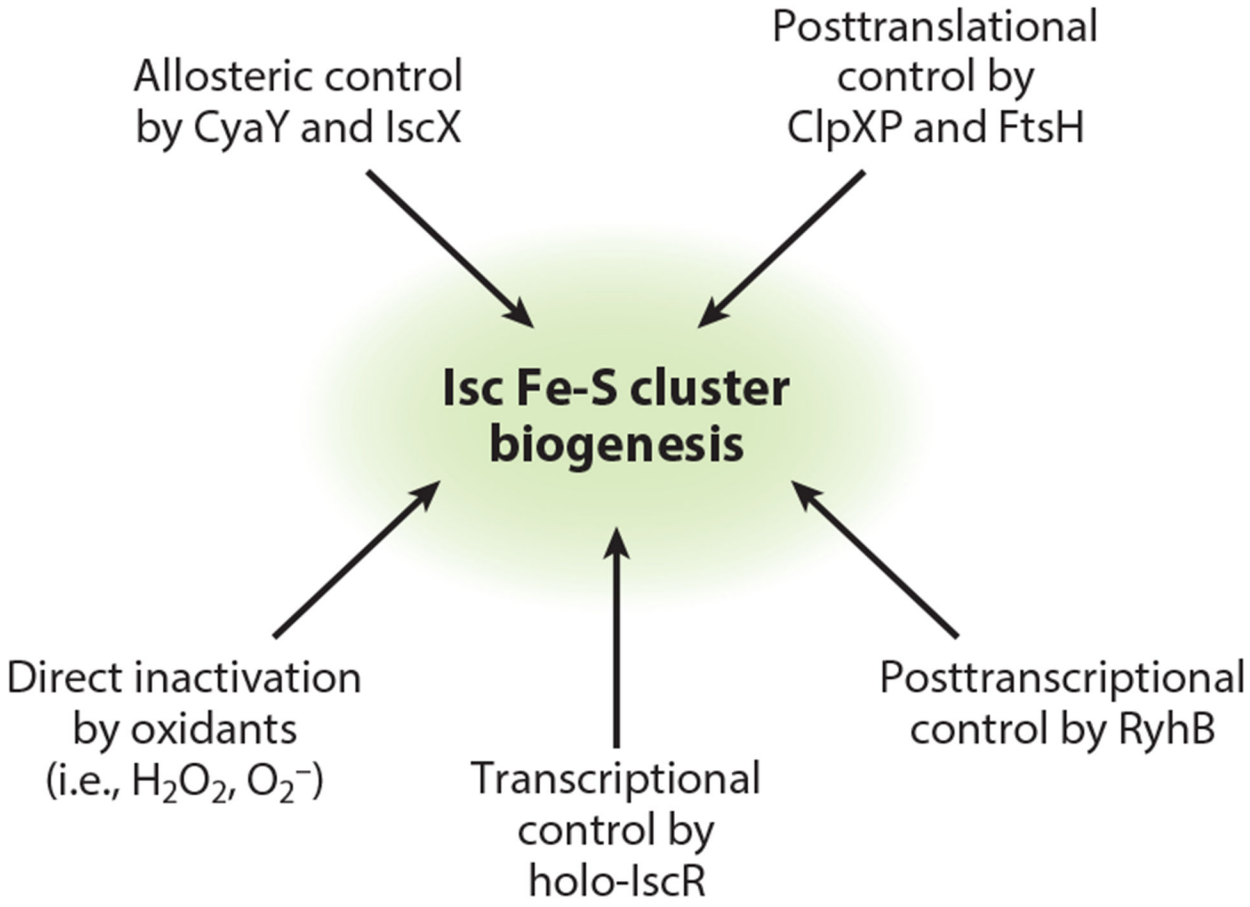

Figure 2.
Regulation of Isc-mediated Fe-S cluster biogenesis occurs at multiple levels.

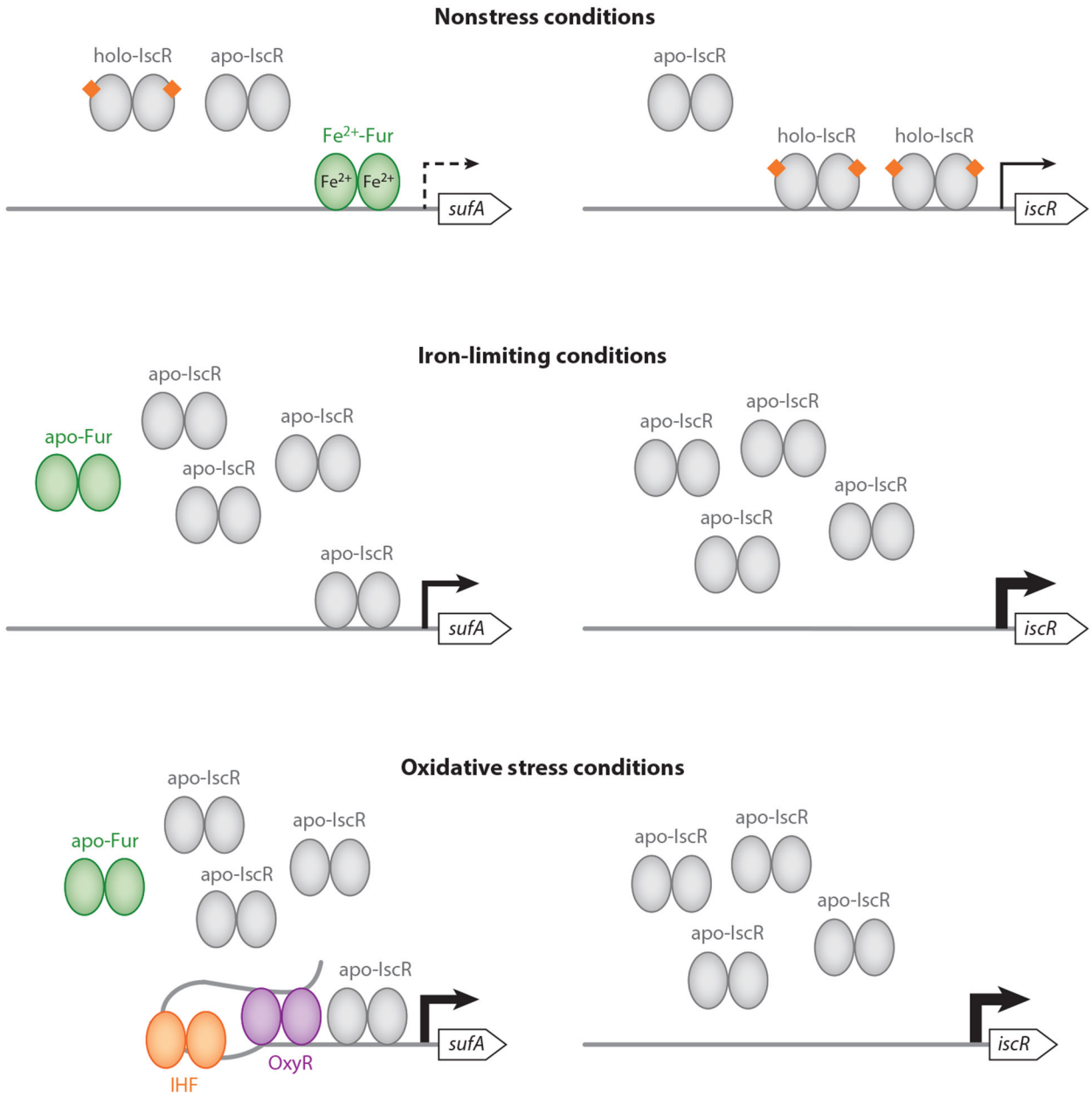

Figure 3.

Transcriptional regulation of the *suf* and *isc* operons under various growth conditions. Under nonstress conditions, transcriptional repression of the *suf* and *isc* operons is mediated by Fe^{2+}-Fur and holo-IscR, respectively. In contrast, under conditions of iron limitation or oxidative stress, Fur and IscR are predicted to be primarily in their apo-protein forms. This results in derepression of the *isc* operon, which in turn results in elevated levels of apo-IscR. As apo-Fur is inactive for DNA binding, this allows apo-IscR to bind the *suf* promoter region to activate transcription. Additionally, oxidative stress leads to activation of OxyR, which in conjunction with the DNA-bending protein IHF promotes *suf* transcription. Although not depicted here, the Isc pathway is also subject to additional forms of regulation,

such as differential degradation of the *isc* mRNA transcript by RyhB when iron is limiting and direct inactivation of the Isc machinery by various oxidants (Figure 2).

Table 1

Factors known or proposed to regulate *Escherichia coli* Fe-S cluster assembly

Type of regulation	Gene product	Relevant function	Estimated protein copies per cell[a]	
			MOPS complete	MOPS minimal
Allosteric	CyaY	Regulates Isc-mediated cluster assembly	4,479	963
	IscX	Regulates Isc-mediated cluster assembly	4,185	1,140
Transcriptional	IscR	Global Fe-S-containing transcriptional regulator that represses and activates *isc* and *suf* transcription, respectively	5,551	3,324
	Fur	Global Fe^{2+}-containing transcriptional regulator that represses *suf* and *ryhB* under Fe-replete conditions	6,426	2,619
	OxyR	H_2O_2-responsive transcriptional regulator that activates *suf* transcription along with IHF	1,867	726
	IHF (A and B subunits)	DNA-bending protein that mediates OxyR-dependent activation of *suf*	11,148/15,238	5,115/12,357
	NsrR	NO-responsive transcription factor that may regulate *suf* transcription	426	168
	RpoS	Stress-responsive σ factor that may regulate *suf* transcription	2,254	10,222
	FNR	Anaerobic-responsive Fe-S-containing transcription factor that activates *fnrS*	6,551	2,582
Posttranscriptional	RyhB	Small regulatory RNA that targets the *iscSUA-hscBA-fdx-iscX* transcript for degradation under Fe-limiting conditions	NA	
	FnrS	Small regulatory RNA that may regulate the *iscRSUA-hscBA-fdx-iscX* transcript under anaerobic growth conditions	NA	
	FtsH	Essential, membrane-bound protease that degrades IscS	4,550	1,124
	ClpXP	Protease that degrades IscS and IscU	5,857/6,334	2,475/3,256

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; NA, not applicable.

[a] Determined by Li et al. (69), using aerobic cultures grown to exponential phase in either MOPS-complete or MOPS-minimal media.

Table 2

Escherichia coli proteins known or proposed to be involved in Fe-S cluster assembly, delivery, and repair

System	Protein	Relevant function	Estimated protein copies per cell[a]	
			MOPS complete	MOPS minimal
Isc pathway	IscS	Cysteine desulfurase that provides sulfur for cluster assembly	13,340	5,500
	IscU	Scaffold protein upon which clusters are built	13,785	7,019
	IscA	A-type carrier that receives and delivers clusters	5,912	1,974
	HscBA	Cochaperone/chaperone system that hydrolyzes ATP for cluster release from IscU	1,461/2,256	416/542
	Fdx	Ferredoxin that supplies electrons for cluster assembly	6,027	1,374
Suf pathway	SufA	A-type carrier that receives and delivers clusters	146	359
	SufB	Scaffold protein upon which clusters are built; forms a complex with SufC and SufD	75	143
	SufC	ATPase proposed to function in cluster release from SufB or iron delivery to SufB	117	226
	SufD	Proposed to function in iron delivery to SufB	82	152
	SufS	Cysteine desulfurase that provides sulfur for cluster assembly	63	122
	SufE	Enhances SufS activity; relays sulfur from SufS to SufB	76	117
Non-Isc, non-Suf proteins that may function with either pathway or individually	ErpA	A-type carrier that receives and delivers clusters	17,438	4,534
	NfuA	Non-A-type carrier that receives and delivers clusters, possibly during stress	15,911	4,329
	GrxD	Proposed carrier that binds and transfers clusters as a homodimer or as a heterodimer with BolA	20,731	8,964
	BolA	Forms a heterodimer with GrxD that may function to bind and transfer clusters	1,945	2,040
	Mrp	Proposed carrier that also displays ATPase activity	5,277	1,391
	RIC (YtfE)	Functions in cluster repair, possibly by providing iron	70	11
	YggX	Proposed to function in cluster repair	7,132	2,665
	YgfZ	Folate-binding protein implicated in cluster repair	5,476	1,708
	FtnA	Iron storage protein ferritin A that may be involved in cluster repair	6,077	2,719
	FtnB	Iron storage protein ferritin B that may be involved in cluster repair	333	170
	Bfr	Iron storage protein bacterioferritin that may be involved in cluster repair	192	4,062
	Dps	Ferritin-like DNA-binding protein that may provide iron for oxidative damage protection and repair	2,131	13,008

System	Protein	Relevant function	Estimated protein copies per cell[a]	
			MOPS complete	MOPS minimal
	GshA/B	Enzymes involved in glutathione synthesis	1,300/2,739	502/1,288
	CsdA	Cysteine desulfurase that shares similarity to SufS	307	72
	CsdE	Enhances CsdA activity; shares similarity to SufE	813	181

Abbreviation: MOPS, 3-(*N*-morpholino)propanesulfonic acid.

[a]Determined by Li et al. (69), using aerobic cultures grown to exponential phase in either MOPS-complete or MOPS-minimal media.