Mammalian Fe-S proteins: definition of a consensus motif recognized by the co-chaperone HSC20

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Abstract

Iron-sulfur (Fe-S) clusters are inorganic cofactors that are fundamental to several biological processes in all three kingdoms of life. In most organisms, Fe-S clusters are initially assembled on a scaffold protein, ISCU, and subsequently transferred to target proteins or to intermediate carriers by a dedicated chaperone/co-chaperone system. The delivery of assembled Fe-S clusters to recipient proteins is a crucial step in the biogenesis of Fe-S proteins, and, in mammals, it relies on the activity of a multiprotein transfer complex that contains the chaperone HSPA9, the co-chaperone HSC20 and the scaffold ISCU. How the transfer complex efficiently engages recipient Fe-S target proteins involves specific protein interactions that are not fully understood. This mini review focuses on recent insights into the molecular mechanism of amino acid motif recognition and discrimination by the co-chaperone HSC20, which guides Fe-S cluster delivery.

Introduction

The chemical properties unique to transition metal ions enable them to participate in several processes that are essential to life by engaging directly in biochemical reactions. A central functional property of transition metals is the propensity to undergo one-electron transfer processes under physiological conditions. This property arises from the mid-range, one-electron reduction potentials of metals such as iron, copper, cobalt, nickel, manganese, and molybdenum. Iron-sulfur (Fe-S) clusters are ancient, ubiquitous cofactors composed of iron and inorganic sulfur. The combination of the chemical reactivity of iron and sulfur, together with many variations of cluster composition, oxidation states and protein environments, enables Fe-S clusters to participate in numerous biological processes (Figure 1A). The entire energy metabolism of the cell depends on electron transfer processes fulfilled by Fe-S clusters. Long-range electron transfers through distances of 10–14 Å link Fe-S centers of multimeric complexes involved in electron transfer pathways that catalyze ATP synthesis and processes essential to the global biogeochemical cycles of N, C, O, S, and H, some of which involve highly elaborate Fe-S prosthetic groups, such as those found in the molybdenum (Mo), vanadium (V), and iron only (Fe) nitrogenases that fix atmospheric N2 into ammonia[1], in the [Ni–Fe] and [Fe–Fe] hydrogenases, responsible for hydrogen

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production and consumption[2], and in the [Ni–Fe] and [Mo-Fe] carbon monoxide dehydrogenases (CODH) that carry out a reversible, biological water-gas shift reaction[3–5].

In 1951, researchers reported on the first Fe-S cluster dependent biochemical reaction: reduction of methaemoglobin by the crude or unwashed preparations of chloroplasts from a variety of plants[6]. In the following years, the use of electron paramagnetic resonance (EPR) imaging techniques enormously advanced the field, and EPR signals emanating from nonheme iron were first obtained for succinate dehydrogenase (SDH) in 1960[7, 8]. By the mid-1960s, additional Fe-S proteins of bacterial origin became the subject of intensive studies, and they were shown to contain complexes of iron and acidic-labile sulphide in the form of two- and four-iron clusters[9].

Underpinning the requirement of Fe-S proteins in the most crucial biological processes is the ability of Fe-S cofactors to undergo redox reactions, which are significantly influenced by the proteinaceous environment surrounding the cluster. Generally, the stability of the cluster decreases as its overall oxidation level increases. In addition to their capacity to undergo one-electron redox reactions, Fe-S clusters also carry out Lewis acid-based catalysis, such as in aconitase[10–12]. The cytosolic isoform of aconitase is a bifunctional enzyme (iron regulatory protein 1, IRP1/ cytosolic aconitase, ACO1), involved in regulation of cellular iron homeostasis[13–15]. Moreover, an astonishing array of diverse and biochemically challenging reactions is catalyzed by the radical S-adenosylmethionine (SAM) enzymes, which are involved in thermodynamically unfavorable reactions and require redox-active [Fe4–S4] conserved clusters for activity[16]. Several nucleic acid processing enzymes, such as DNA polymerases, glycosylases, helicases and primases require Fe-S clusters for their function[17]. Moreover, Fe-S clusters are present in DNA repair enzymes (Fanconi anemia group J protein, FANCJ, and Xeroderma pigmentosum group D protein, XPD)[18, 19].

The inherent reactivity of Fe-S clusters towards a variety of redox-active species, including small redox-active compounds, reactive oxygen and reactive nitrogen species has been exploited during the evolution of bacterial sensors, which utilize the reactivity of Fe-S clusters to detect biologically relevant molecules (e.g., drugs, O2, NO)[20, 21] and coordinate the cell's response to oxidative and nitrosative stress conditions. However, this also means that Fe-S clusters are prone to degradation. Any of the reactions in which Fe-S cluster cofactors are involved may result in a change in the redox state of the cluster, cluster conversion, or even complete cluster loss. Moreover, interaction of Fe-S cofactors with strongly coordinating species may destroy the cluster by abstracting iron/sulfide or displacing thiolate ligands from the cluster[22, 23].

While the cell usually strives to protect its complement of iron–sulfur proteins from the destructive effect of reactive species, Fe-S cofactors become particularly prone to damage when removed from the protective environment of the cell, a step that is necessary to characterize properties and functions of Fe-S proteins, but that increases the probability of Fe-S cluster destruction before characterization. It is, in fact, possible that previous failures to detect Fe-S clusters in numerous mammalian enzymes may have resulted from difficulties with overexpression or purification of intact cofactors[24]. Therefore, progress in the field of Fe-S protein biogenesis depends critically on the availability of methods for investigating

structures and chemical properties of Fe-S enzymes, while preserving the integrity of their essential prosthetic groups[25]. An array of techniques and methods has been developed to enable researchers to obtain intact and biologically active Fe–S proteins in pure and stable forms. In addition, methods for the generation of highly concentrated samples containing Fe-S clusters isotopically labelled with ^{57}Fe for Mössbauer and related spectroscopies have enabled researchers to identify novel mammalian Fe-S proteins, and to uncover new biological functions for Fe-S clusters in cellular metabolism[26].

Bioinformatics approaches have dramatically expanded biological knowledge and enabled investigators to focus on particular candidates in their experimental work. Sophisticated bioinformatics tools were applied to the radical S-adenosylmethionine (SAM) superfamily to overcome the obstacles in the analysis of such a large number of enzymes which link different biosynthetic pathways. Radical SAM enzymes contain [Fe4-S4] cluster cofactors and are involved in an astonishing array of reactions that impact numerous cellular processes, including transcription, translation, gene regulation, the biosynthesis of several essential metabolites and complex metallo-cofactors, and signal transduction[16, 27, 28]. The term radical SAM was coined in a powerful bioinformatics approach, which used iterative profile methods to identify high homology and distinguishing features among the five founding members of the superfamily (biotin synthase, lipoyl synthase, lysine 2,3-aminomutase, and the activases for pyruvate-formate lyase and class III ribonucleotide reductases) and over 600 other uncharacterized proteins[29]. Iterative profile methods can greatly extend the power of sequence homology searches[30, 31]. While a strong match in a BLAST search can be used to infer protein homology, the weaker similarity detectable by an iterative profile method may uncover a more distant relationship and provide evidence for a conserved fold in the protein structure[30]. The most remarkable feature of radical SAM enzymes is a Cx3Cx2C motif, which ligates a [Fe4-S4] cluster. The fourth iron of the cluster was later found to be coordinated by the amino and carboxyl groups of SAM[32, 33], allowing an electronic configuration that favors cleavage of SAM into methionine and 5'-deoxyadenosyl radical[34, 35]. More recently, a bioinformatics analysis conducted by the Structure Function Linkage Database (SFLD) expanded the number of radical SAM enzymes to almost 114,000 unique sequences in all organisms and over 60 distinct enzymatic reactions[36]. These approaches have linked functionally diverse enzymes based on conserved functional attributes such as a step or part of the reaction that they catalyze, creating the exciting possibility that the roles of novel Fe-S enzymes will be elucidated in the chemistry of numerous biological pathways[36]. Several other methods can facilitate identification of novel Fe-S proteins in mammalian cells.

This minireview focuses on recent advances achieved on the identification of molecular features that guide selection of specific subsets of nascent Fe-S recipient proteins by the co-chaperone HSC20. Short interaction modules, known as peptide motifs, determine biomolecular interactions in several cellular processes. Studies conducted in our lab allowed the identification of the tripeptide Leu-Tyr-Arg (LYR) as the molecular signature that mediates direct binding to the co-chaperone HSC20 and guides delivery of nascent clusters to succinate dehydrogenase subunit B, SDHB[37]. Here, we discuss the potential relevance of LYR motifs in proteins that were found to directly interact with HSC20, some of which are essential for the assembly and function of mitochondrial respiratory complexes I-III[37, 38].

Fe-S cluster biogenesis: the assembly step

Biogenesis of Fe-S clusters is an elaborate process that involves formation of large dedicated multimeric complexes. In the 1980's the synergistic work of chemists, biophysicists, biochemists and geneticists helped characterize the cellular mechanisms involved in in vivo assembly of Fe-S clusters in bacteria, and subsequent studies revealed that the basic components of the Fe-S biogenesis machinery are highly conserved throughout evolution, often functioning in large supra-molecular complexes[39–41]. Many details of how Fe-S cofactors are assembled and ligated to cellular proteins in the nucleus, cytosol and mitochondria of mammalian cells remain unclear.

In bacteria, the Fe-S biogenesis machinery supplies Fe-S clusters to recipient proteins in a single cellular compartment[20, 42], whereas in mammalian cells the synthesis and distribution of Fe-S clusters are more complicated. The mammalian proteins involved in initial assembly of Fe-S clusters have been detected not only in mitochondria, but also in the cytosol and nucleus[43, 44]. In contrast, most of the early components of the biogenesis pathway have not been found in the cytosolic or nuclear compartments of the model organism *Saccharomyces cerevisiae*, in which the original building blocks of Fe-S clusters for cytosolic and nuclear proteins are proposed to derive from the mitochondrial machinery[45]. An additional level of complication arises from the presence of Fe-S clusters of different nuclearities that need to be delivered to specific recipients by a set of diverse intermediate carriers[46]. Most frequently, Fe-S proteins contain the rhomboid [Fe_2-S_2], the cuboidal [Fe_3-S_4], and the cubane [Fe_4-S_4] clusters[5]. However more complicated forms have been characterized that also harbor other metal ions[5]. Cysteine residues of the polypeptide chain typically ligate Fe-S clusters, but other amino acids, such as serine, arginine, and histidine, may also function as ligands. For instance two cysteines and two histidines coordinate the [Fe_2-S_2] cluster of the Rieske protein of respiratory chain complex III. Central to the initial assembly of Fe-S clusters is a cysteine desulfurase (NFS1 in mammals, Nfs1 in yeast, and IscS in bacteria), which abstracts sulfur from free cysteine and forms a persulfide intermediate on its active site cysteine (Figure 2). Two distinct conformational changes have been suggested to assist this initial step in Fe-S cluster biogenesis[47, 48]. One change exposes the buried substrate-binding sites thereby promoting cysteine binding, and it depends upon binding to NFS1 of the allosteric regulator of the cysteine desulfurase activity, frataxin (FXN). A second change that brings the bound substrate cysteine and the active site cysteine into close proximity for persulfide formation has been proposed to be mediated by binding of a small accessory protein, ISD11 (also known as LYRM4 in humans)[49] to NFS1. Subsequently, the persulfide sulphur is transferred from NFS1 to the main scaffold protein ISCU, which provides the cysteine ligands to coordinate the nascent cluster[50]. In the bacterial IscU/IscS co-crystal structure, the ISCU ortholog binds to the cysteine desulfurase so that it orients the cysteine-rich active site in close proximity to the persulfide delivery loop of NFS1[51]. Tight structural coupling of NFS1 and ISCU seems reasonable to preserve the efficiency of the pathway and to curb the toxicity that release of free sulphide would cause within the cell[52, 53]. A recent characterization of the thermodynamic, structural and electronic properties of Fe(II) bound to the scaffold protein Isu1 from *S. cerevisiae*, showed that the C-terminal helix of the ISCU scaffold protein, with its large number of conserved, surface exposed acidic and basic

residues, is a potential candidate for the site of initial iron binding to ISCU[52]. The X-ray crystallographic structure of the *E. coli* apoIscU–IscS complex shows that each IscU molecule interacts with one subunit of the IscS dimer, leading to a 2:2 stoichiometry[51], whereas, to date, no eukaryotic structures of the core complex are available. Using a co-expression approach, the mammalian recombinant ternary complex formed by NFS1, ISD11 and ISCU was biochemically and biophysically characterized, and a quaternary complex that formed when frataxin and two key substrates, iron and cysteine were added was analysed[54]. The mass of the ternary complex suggested formation of a homodimeric complex in which each unit had a 1:2:1 NFS1:ISD11:ISCU stoichiometry. Frataxin, which was shown to interact with the preformed ternary complex[55], was proposed to bind in a ratio of 1:2:1:1 in the quaternary complex (NFS1:ISD11:ISCU:FXN)[54]. The source of iron for the nascent cluster remains to be identified, but proposed donors include frataxin[56, 57], which has acidic surface patches to which iron binds with low affinity, or a complex of glutathione and glutaredoxin[58]. Electrons, needed to achieve the final electronic configurations of Fe-S clusters, are provided by ferredoxins (FDX1/2) and ferredoxin reductase (FDXR)[59, 60]. Ferredoxin has also been shown to facilitate reductive coupling of two distinct [Fe2-S2] clusters into a single [Fe4-S4] cluster on bacterial IscU in vitro[61].

Fe-S cluster biogenesis: transfer to recipient proteins

A specialized chaperone/co-chaperone system has evolved in bacteria (HscA/HscB) and in fungi (Ssq1/Jac1) to facilitate transfer of Fe-S clusters, newly assembled on the main scaffold ISCU, to Fe-S recipient proteins or to intermediate carriers, which then target specific clients[62]. Most eukaryotes utilize a multifunctional mitochondrial HSP70 (mtHSP70) that works together with a specialized co-chaperone, the ortholog of HscB/Jac1[63, 64]. In vivo studies using a dominant negative mutant of the bacterial scaffold IscU (IscU[D39A] in *Azotobacter vinelandii*, or IscU[D35A] in *Archaeglobus fulgidus*), which remained trapped in a complex with the sulfur donor IscS, and contained a nascent Fe-S cluster[65, 66], showed that genetic approaches could be used to uncouple cluster assembly and release steps. Fe-S cluster release and transfer is an ATP dependent process[67], which occurs upon binding to IscU of the dedicated chaperone/co-chaperone system.

In mammalian cells, the cochaperone known as HSC20[68] (HscB in bacteria[63], and Jac1 in S. cerevisiae[69]) binds to the scaffold protein ISCU, and forms a complex with its chaperone partner, HSPA9 (HscA in bacteria, and Ssq1 in S. cerevisiae), a member of the HSP70 heat shock protein family[37, 70]. HSP70 homologs use the energy released by hydrolysis of ATP to drive conformational changes and refolding of target proteins[71]. The C-terminal domain of the cochaperone is directly responsible for binding the scaffold protein ISCU, with three highly conserved non-contiguous hydrophobic residues being of crucial importance for the HSC20-ISCU interaction[46, 72–74]. The Fe-S cluster transfer machinery is highly conserved among different organisms[75], and cochaperones dedicated to Fe-S cluster biogenesis share common features[73, 76, 77]. A conserved structural core consists of two domains arranged in a L-shaped fold. The N-terminal J-domain, which contains an invariant histidine, proline, aspartate (HPD) motif, is responsible for stimulating the ATPase activity of the HSP70 cognate chaperone[37, 76, 78, 79], whereas the C-terminal domain forms a three-helix bundle. The N-terminus of the human co-chaperone shows distinctive features compared to the

specialized DnaJ type III proteins of bacteria and fungi, in that it contains, downstream of the mitochondrial targeting sequence (residues 1–26[80]), an additional domain, which harbors two CxxC modules (C41/C44 and C58/C61) that were found to coordinate a zinc ion in vitro[77]. The physiological relevance of the unique N-terminal domain of HSC20 remains to be elucidated, though it may facilitate dimerization of HSC20.

Recent studies with the purified components of the bacterial Fe-S transfer complex (HscA, HscB, IscU) have provided important insights into the molecular mechanism by which modulation of the ATPase activity of the chaperone HscA by the co-chaperone HscB is coupled to Fe-S cluster transfer from the scaffold protein IscU to a recipient Fe-S apo-protein[81]. Common to all HSP70 proteins is a highly conserved nucleotide-binding domain (NBD) that binds to and hydrolyzes ATP[82, 83]. The specialized substrate-binding domain (SBD) of HscA uniquely recognizes a central LPPVK motif in IscU as a substrate[84–87]. Similar to other HSP70s, nucleotide binding to the NBD of HscA allosterically regulates substrate binding affinity in the SBD[67]. The co-chaperone HscB binds to and delivers [Fe2-S2]-IscU to the SBD of HscA; together, IscU and HscB synergistically enhance HscA's ATPase activity nearly 1000-fold[63, 67]. Two slowly interconverting states of IscU were shown to preferentially interact with HscA or HscB[88–90]: HscB binds preferentially to the more structured (S) state of IscU, which harbors a [Fe2-S2] cluster, whereas HscA binds preferentially the disordered (D) state of IscU, which forms after release of the cluster. Activation of the ATPase activity of HscA by the co-chaperone HscB, leads to hydrolysis of ATP to ADP in the NBD of HscA, driving a conformational change in the SBD of HscA through the HscA's interdomain linker. The substrate-binding domain of HscA encloses the LPPVK peptide of IscU, which stabilizes the interaction and facilitates the transition of IscU from the S- to the D-state, which may promote release of the [Fe2-S2] cluster from holo-IscU[81, 88]. A similar mechanism has been proposed to assist biogenesis of Fe-S clusters in mammalian cells[70]. Inactivation of the *HscA* gene leads to diminished cellular growth rates and activities of Fe-S proteins, including SDH and aconitase[91, 92], and inhibition of the ATPase activity of HscA via an ATPase deficient mutant, HscA^{T212V}, abolishes [Fe2-S2] cluster transfer in vitro[93]. Mutations in the co-chaperone HSC20 and in its orthologs cause defects in Fe-S protein activities[37], mitochondrial iron accumulation, and reduced mitochondrial respiration in human cell lines[68], and multiple model systems, including yeast[79, 94] and fly[95]. Additionally, recent studies in yeast confirm the central role of energy release coupled to conformational changes for Fe-S cluster biogenesis, as ATP consumption facilitates transition from the initial assembly of an Fe-S cluster on the main scaffold protein Isu by the core complex (NFS1-ISD11-FXN-ISCU), to the chaperone-cochaperone assisted transfer of the cluster to recipient proteins[96].

In multiple cellular processes, co-chaperones are thought to determine the substrate specificity of their cognate chaperones[64, 71, 73]. The C-terminal domains of co-chaperones can selectively bind target substrates[97, 98], facilitate refolding of denatured proteins, and enhance cell viability[99, 100]. Cochaperones serve a dual function in Fe-S cluster biogenesis: they guide ISCU to the substrate binding domain of the HSP70 cognate chaperone, and activate the ATPase activity of the chaperone, thereby driving a conformational change that might facilitate cluster release from ISCU and delivery to the final acceptor apoprotein or to intermediate carriers, which ultimately donate their clusters to specific recipients[62, 63]. The

mechanism by which recipient Fe-S proteins are selected by the transfer machinery from amongst the full complement of human protiens has recently been partially characterized[37]. Interestingly, clinical and biochemical investigations of several newly described human diseases caused by mutations in *NFU1*, *BOLA3*, *IBA57* or *ISCA2* suggest that transfer of Fe-S clusters downstream of the holo-ISCU/chaperone/co-chaperone complex depends on selective pathways. The distinctive phenotypes associated with the various disease gene mutations reveal our lack of knowledge about how discrete Fe-S proteins are targeted in order to incorporate their vital prosthetic groups[46, 101–111].

Recent progress: identification of molecular features that guide selection of recipient Fe-S proteins by the Fe-S transfer complex

Recent studies were conducted to try to elucidate how iron sulfur clusters are transferred from the initial scaffold protein, ISCU, to Fe-S clients, focusing on the human co-chaperone HSC20[37] for two main reasons. Most eukaryotes, including humans, have a single multifunctional mitochondrial chaperone of the HSP70 family, which participates in diverse cellular functions by virtue of its ability to interact with an array of different J proteins (also known as HSP40s or co-chaperones) (for reviews, see[71, 112–114]). Therefore, HSPA9, a human HSP70 family member, would have not been the ideal prey to use to screen the human proteome for potential interacting Fe-S proteins, due to its promiscuous ATP-dependent substrate binding recognition activity. Co-chaperones are thought to play the crucial role of driving the functional specificity of HSP70s[64, 71, 73], mostly through their C-terminal domains[97–100], which makes the HSC20 co-chaperone a perfect candidate to use to search for specific interacting partners. Importantly, there is only one highly conserved HSC20 homologue in the human genome[68], which also made HSC20 a good candidate to use in screening for target Fe-S recipient proteins. Since Fe-S clusters are highly sensitive to degradation by oxidants, the role of the co-chaperone may be to facilitate protected transfer of the cluster downstream of the holo-ISCU scaffold by directly binding recipient Fe-S apo-proteins. Studies in our lab identified several HSC20 binding partners in a stringent yeast two-hybrid (Y2H) screen, a method used to reveal direct molecular interactions between pairs of proteins[37]. Notably, multiple individual HSC20 interacting clones identified in the screen encoded succinate dehydrogenase subunit b (SDHB), which is the Fe-S cluster containing subunit of complex II. The interaction between HSC20 and SDHB was further validated in vivo in mammalian cells[37]. SDHB contains three clusters of different nuclearities; $[Fe_2-S_2]$, $[Fe_4-S_4]$ and $[Fe_3-S_4]$[115], which are deeply buried within the mature protein and that likely need to be inserted during folding. Complex II is situated at the nexus of two essential energy-generating processes of the cell, the citric acid cycle and mitochondrial oxidative phosphorylation. In eukaryotes, mutations in each of the four nuclear-encoded succinate-coQ oxidoreductase (SQR) genes (SDHA, SDHB, SDHC, SDHD) or associated accessory factors (SDHAF1, SDHAF2) account for a variety of clinical phenotypes, including optic atrophy, myopathy, encephalopathy[116, 117], and tumor formation[118–120]. Assembly of CII is a multistep process that depends upon incorporation of several prosthetic groups, including flavin adenine dinucleotide (FAD), covalently attached to SDHA, three Fe-S clusters coordinated by SDHB and an heme b moiety bound by the SDHC/SDHD membrane anchors[121].

To identify the potential motifs that mediate Fe-S cluster transfer through binding to the co-chaperone HSC20, the SDHB primary sequence was subdivided into multiple peptides, which were cloned into Y2H prey constructs to screen 50 to 100 subclones of varying sizes for their ability to interact with HSC20. In recent years, investigators have focused increasingly on molecular interactions mediated by compact modules that are typically less than ten amino acid residues in length and are often found within intrinsically disordered regions of polypeptides. On average, binding motifs are 6–7 amino acids in length, and only 3–4 core positions confer specificity[122]. Because the binding surface area is small, these motifs bind with low affinity and engage in interactions that are transient and readily modulated. It has been suggested that over one million such motifs are present in the human proteome, and that they extend the functional capabilities of eukaryotes well beyond known structural domains[123], influencing protein processing, localization, degradation, and transient participation in multiprotein complexes. Three independent binding sites for HSC20 were identified on SDHB, and two of these were iterations of the LYR motif. One SDHB peptide that interacted with HSC20 (residues 238–258 of SDHB) contained the distinctive motif, LYR, while the other (residues 35–52 of SDHB) contained a closely related sequence, IYR. Notably, the two native L(I)YR consensus sequences in SDHB are located in unstructured loops of the crystal structure. One of the L(I)YR motifs appears in SDHB near the N-terminus, proximal to the first cysteines that ligate the [Fe2-S2] cluster, whereas the second is closer to the C-terminus and the cysteinyl ligands of the [Fe4-S4] and [Fe3-S4] clusters, in positions where binding of the chaperone-cochaperone transfer apparatus can guide release of the cluster from holo-ISCU into the distal Fe-S binding sites of SDHB. Mutagenesis of the LYR or IYR tripeptide eliminated binding of motif-containing peptides to HSC20[37]. Interestingly, the amino acid residues of the two L(I)YR motifs were highly conserved in SDHB throughout evolution, including bacteria and yeast[37]. Homology searches indicated that the key features of this distinctive sequence, which mediated binding to HSC20, included having a hydrophobic residue in position 1, followed by an aromatic residue (Y or F) at position 2, and a positive residue, R or K, at position 3. Substitution of Arg46 into Gln in the IYR sequence was identified as a critical cause of renal cancer[124], and substitutions of the IYR, LYR and cysteines important in ligating Fe-S clusters were identified as frequent causal mutations in rare tumors such as paragangliomas and gastrointestinal stromal tumors.

The LYR motif family is an annotated family present only in eukaryotes (Conserved Domains Accession: cl05087)[125], which lists eleven proteins in humans characterized by having the LYR tripeptide close to their N-terminus. Members of the LYR family are SDHAF1 (LYRM8), a bona fide succinate dehydrogenase complex assembly factor[117], ISD11 (LYRM4), a component of the initial Fe-S assembly complex[126], LYRM7, a complex III assembly factor, and two subunits of respiratory chain complex I known as NDUFA6 and NDUFB9 (also annotated as LYRM6 and LYRM3, respectively)[127] (Figure 1B). Several LYR motif proteins do not have a known function, such as LYRM2, LYRM5, and LYRM9, whereas others have been only partially characterized in yeast, such as ACN9 (acetate non-utilizing protein 9)[128], or FMC1 (formation of mitochondrial complexes protein 1)[129]. Interestingly, human ISD11 and LYRM7 share high sequence homology with their yeast orthologues, Isd11 and Mzm1, respectively, and they perform similar functions[126, 130],

whereas human SDHAF1 has low similarity to the yeast ortholog Sdh6 and failed to restore respiration in a Sdh6 null background (Sdh6) yeast strain[117].

SDHAF1, a member of the LYR motif family, assists Fe-S cluster incorporation into SDHB

SDHAF1 (also known as LYRM8) was shown to be important for SDH activity and for assembly of the holo-complex in fibroblasts[117]. Homozygous mutations in *SDHAF1* cause a distinctive early-onset leukoencephalopathy in which accumulations of lactate and succinate in the white matter are associated with selective loss of SDH activity[117, 118, 131]; however the molecular role of *SDHAF1* mutations in disease pathogenesis remained for some time undefined. A recent investigation used biochemical and functional approaches on cell lines derived from patients with *SDHAF1* mutations to characterize the role of this accessory factor in the assembly of Complex II[38]. SDHAF1 was shown to recruit the Fe-S transfer complex to the C-terminus of SDHB through direct binding of its N-terminal LYR motif to the co-chaperone HSC20. The region L53-R65 of SDHAF1, enriched in arginine residues, was found to be involved in the interaction with SDHB at three binding sites containing multiple aromatic amino acids. Binding of SDHAF1 to SDHB and recruitment of the HSC20-HSPA9-holo-ISCU complex by its first LYR motif was shown to facilitate Fe-S cluster incorporation into SDHB. In vivo iron radiolabeling of SDHB showed defective Fe-S cluster incorporation into the protein synthesized in SDHAF1-deficient patient-derived cells. SDHB levels were drastically reduced in patient cells, where lack of functional SDHAF1 led to impaired biogenesis of SDHB, which was rapidly degraded by the mitochondrial protease, LONP1[38].

Potential role of LYR motif proteins in Fe-S cluster biogenesis

The functional significance of the common LYR motif was completely unknown when the Pfam clan Complex1_LYR-like superfamily (CL0491) was built by P. C. Coggill[132]. Based on bioinformatics analyses, the motif was defined as a tripeptide in which the first position was generally an aliphatic hydrophobic amino acid such as isoleucine or leucine, the second was an aromatic amino acid such as tyrosine or phenylalanine, and the third was a positively charged arginine or lysine. The tripeptide was followed by a conserved phenylalanine separated from LYR by about twenty-five residues[127]. The LYR motif was present in several other proteins selected in the Y2H screen as interacting partners of HSC20 (i.e. EPRS, HELZ, SPC25, GTF2E2, ETFA), and also in LYRM7, the Rieske Fe-S protein chaperone of complex III[130] and an annotated member of the LYR family[127]. Overall, co-immunoprecipitation and Y2H studies indicated that LYR motifs present in known recipient Fe-S proteins (i. e. SDHB) or in accessory factors (i. e. SDHAF1, LYRM7) mediated an interaction with the co-chaperone HSC20, and guided insertion of Fe-S clusters into the Fe-S subunit of respiratory chain complex II, and, possibly, into complex III[37]. In fact, LYRM7, which has been characterized as a complex III assembly factor, binds UQCRFS1, the Rieske Fe-S protein of complex III[130], and engages the HSC20- HSPA9-ISCU complex[37], suggesting that the LYRM7-HSC20 interaction might guide the insertion of the [Fe2-S2] cluster into UQCRFS1. LYR containing subunits are present in mitochondrial complex I.

The role of the complex I subunit LYRM6 (also known as NDUFA6 and as NB4M in the yeast *Yarrowia lipolytica*) has been recently characterized[133]. Chromosomal deletion of NB4M in *Y. lipolytica* or mutagenesis of the LYR tripeptide and of a conserved downstream phenylalanine into alanines caused loss of the [Fe4-S4] N2 cluster, which is the last Fe-S center in the ascending electron transfer sequence of the matrix arm of complex I with the highest redox potential, and totally abrogated the ubiquinone reductase activity of complex I, despite the fact that all the central subunits of the complex were intact. This finding suggests that NB4M (NDUFA6) is required for proper incorporation of the N2 [Fe4-S4] center, and for the function of complex I. Notably, NDUFS8, which ligates two [Fe4-S4] clusters, contains a conserved LYR motif in a non-canonical position, at its C-terminus. As only 14 of the 44 complex I subunits have a catalytic function, some of the accessory or supernumerary subunits may contribute to the assembly and stability of the complex, and the LYR-containing subunits may aid insertion of some of the Fe-S clusters.

A second consensus sequence, KKx(6–10)KK, was identified in the screening of the SDHB peptides that interacted with HSC20 through its C-terminal domain. Interestingly, human glutaredoxin 5 (GLRX5) has a similar pattern of lysines at its C-terminus (K139K140(x10)K151K152) and was found to interact with HSC20 in vivo[37]. The yeast ortholog Grx5 interacts with Ssq1, the mitochondrial chaperone dedicated to Fe-S cluster biogenesis[134].

Molecular features of peptides containing the LYR motif that affect binding to HSC20

The function of the LYR and KKx(6–10)KK motifs may be very dependent on their molecular context and structural location[37]. Other features may define which LYR proteins engage HSC20 and the transfer apparatus, and these features will likely be defined as more LYR and Fe-S proteins are studied. Interestingly, there are two other examples in which a small peptide motif functions in the Fe-S transfer complex. The first is the well-known role of the tripeptide His, Pro, Asp (HPD) of HSC20 in activating the ATPase activity of its partner chaperone[79]. A second example is the role of the tripeptide Pro, Val, Lys (PVK) of ISCU in binding to the chaperone as part of the activation of the Fe-S transfer cycle [85, 87, 135]. Experimental work will be needed to establish what other features are required for LYR motif-containing proteins to engage in binding with the Fe-S transfer complex. Upon inspection of the two motifs in SDHB and of the LYR motifs in SDHAF1 and LYRM7 that mediate an interaction with HSC20 (as assessed by co-immunoprecipitation of endogenous human proteins), it can be noticed that all four of these motifs appear in a peptide context in which a large hydrophobic residue, Phe, or two smaller hydrophobic amino acids, Val and Leu, are present just upstream of the LYR. For SDHB, the sequence F-X-IYR and F-X-LYR are highly conserved for the first and second LYR motifs, respectively. In LYRM7, the sequence VL-X-LFK is highly conserved, and in SDHAF1 (LYRM8), the sequence VLSLYR is highly conserved. Thus, it is possible that a small hydrophobic patch near the N-terminal side of LYR enhances binding to HSC20. A combination of informatics analyses and experimental tests will likely elucidate all the features of a functional LYR motif. In the years ahead, it will be interesting to see whether

identification of LYR motifs through informatics can lead to discovery of unrecognized human Fe-S proteins[24].

Intermediate carriers and secondary scaffolds that deliver Fe-S clusters to recipient proteins

A crucial although still largely uncharacterized process is the transfer of Fe-S clusters downstream of the main scaffold protein ISCU through secondary carriers. The co-chaperone HSC20 also binds to glutaredoxin 5[37], which was shown to interact with Ssq1, the chaperone dedicated to Fe-S cluster biogenesis in *S. cerevisiae*[134]. Glutaredoxin 5 has been characterized as an intermediate carrier involved in the transfer of Fe-S clusters downstream of the scaffold protein ISCU, in yeast[134, 136–138], zebrafish[139], and humans[140–142]. In eukaryotes, monothiol glutaredoxins localize to mitochondria or chloroplasts and have been implicated in Fe-S cluster biogenesis, whereas multidomain Glrxs (i.e. yeast Grx3/4 and human GLRX3) localize to the cytosolic/nuclear compartment and are proposed to play dual roles in iron trafficking and regulation as well as in Fe-S cluster biogenesis[143–147]. The crystal structure of human glutaredoxin 5 revealed the presence of a [Fe2-S2] cluster coordinated by a glutaredoxin homodimer and two glutathione molecules, which were held in place by non covalent interactions within the binding pocket of each GLRX5 monomer and by covalent linkages to the cluster[148]. Moreover, in this structure, two [Fe2-S2]-bridged homodimers interacted to form a tetramer. The current working model to describe the role of glutaredoxins in Fe-S cluster biogenesis proposes that they transiently accept a [Fe2-S2] cluster and engage the chaperone/co-chaperone transfer complex in order to facilitate insertion of the cluster into final acceptor apoproteins[134, 149]. An alternative cluster transfer mechanism hypothesizes that a [Fe2-S2] cluster coordinated by four glutathione molecules is exchanged with the cellular Fe-S cluster biogenesis components, including glutaredoxins[150]. Glutaredoxins have been linked by bioinformatics analyses, Y2H and affinity purification studies to another family of widely distributed proteins, the BolA-like proteins, both in prokaryotes and in eukaryotes, including plants[151–155]. BolA proteins were initially identified in *E. coli* with the observation that their overexpression promoted a switch from rod-shaped to rounded cell morphologies[156]. The Grx3/Grx4 proteins are involved in iron regulation in yeast by forming a complex with the BolA-like protein, Fra2, and ligating a [Fe2-S2] cluster coordinated by the Grx active site cysteine of Grx3/Grx4, a conserved histidine in Fra2 (His103), and a cysteine from glutathione[144, 157, 158]. Mammals have three BOLA orthologs, named BOLA1, BOLA2, and BOLA3, of unknown functions. Recently, mutations in human BOLA3 have been discovered to cause multiple mitochondrial dysfunctions syndrome 2[101, 104], caused by defects in the biogenesis of the Fe-S cluster enzyme lipoic acid synthase (LIAS). Human BOLA3 has two isoforms that result from alternative splicing; both are predicted to localize to mitochondria (NM_001035505.1 and NM_212552.2) and isoform 2 differs because it lacks exon 3.

Human NFU1 has been recently characterized as a late acting factor specifically required for biogenesis of lipoic acid synthase, rather than as an alternative scaffold acting in parallel to ISCU, based on the fact that mutations in NFU1 are associated mainly with defective lipoic acid (LA) metabolism[101, 102, 104], whereas other Fe-S proteins, such as aconitase, are not

affected. The A-type scaffolds are thought to support the biogenesis of a distinct subset of Fe-S proteins. The isc and suf operons of bacteria encode two A-type proteins IscA and SufA, respectively, which were found to be involved in the biogenesis of some [Fe4-S4] proteins, such as aconitase and LIAS[50, 159]. An additional member of this family, ErpA, is specifically involved in isoprenoid biosynthesis[160]. The human genome encodes two A-type proteins, named ISCA1 and ISCA2, related to S. cerevisiae Isa1 and Isa2, respectively, thought to be necessary for biogenesis of several [Fe4-S4], but not [Fe2-S2], enzymes[161]. Consistent with the proposed role of ISCA1/ISCA2 and IBA57 in the biogenesis of [Fe4-S4] clusters for a subset of mitochondrial proteins, a mutation affecting the stability of IBA57 was found in two patients with severe myopathy and encephalopathy associated with compromised activities of the lipoic acid dependent enzymes, indicating defective maturation of LIAS[109]. A loss of function mutation in *IBA57* was recently identified in a patient affected by infantile leukodystrophy with lack of protein lipoylation and decreased complex I and II activities[162]. In most cases, the conclusion that an intermediate carrier protein has specific downstream recipients is based on broad inferences rather than on direct experimental data. It should always be considered that mutations in secondary Fe-S cluster carriers that affect putative recipients may not directly interact with the affected Fe-S protein, and Fe-S proteins are sensitive to several cellular conditions that could indirectly affect their function.

A potential error in assigning roles of proteins in Fe-S cluster delivery may derive from giving a protein the function of an intermediate Fe-S cluster carrier solely based on the fact that its dysfunction leads to a defect in an Fe-S protein or protein complex. For instance, the mitochondrial P-loop NTPase IND1 (Fe-S protein required for NADH dehydrogenase) was proposed to function as an Fe-S cluster carrier for mitochondrial complex I, based on the fact that its deletion caused decreased complex I activity[163, 164]. Recent studies of IND1 in *Arabidopsis thaliana* (INDH) were able to unravel primary from secondary phenotypes caused by inactivating mutations in INDH, and demonstrated that INDH has a primary role in mitochondrial translation[165]. Impairment of complex I likely was due to the fact that seven of its subunits are encoded by the mitochondrial genome and translated in the mitochondrial matrix. In summary, it appears that virtually all mammalian Fe-S clusters are initially assembled on the main scaffold protein ISCU. Recipient Fe-S proteins can then acquire their clusters directly from ISCU in a chaperone/co-chaperone mediated transfer process, or indirectly from secondary carriers, which likely acquired their initial clusters from ISCU and that then deliver these clusters to only a subset of Fe-S proteins with which they specifically interact. Perhaps, the use of co-immunoprecipitation techniques may help to identify direct interactions between secondary carriers and Fe-S clients, although interpretation of these results can still be confounded by the presence of large multimeric complexes, in which two proteins can be found to interact even though they do not directly bind to one another. While the early steps in the biogenesis of Fe-S proteins are nearly universally shared, later steps may confer great specificity, and several molecular processes based on specific interactions allow for proper incorporation of Fe-S clusters into the correct recipient. One such mechanism is based on selection of substrates among the human proteome by the co-chaperone HSC20 which specifically recognizes a short interaction

module in Fe-S apo-proteins[37, 38]. An additional level of specificity arises from the presence of secondary carriers, which seem to selectively target subsets of Fe-S recipient proteins.

Conclusions

During the last decade, Fe-S proteins have been identified at the core of ribosomal function and translation as well as of DNA metabolism. These discoveries were unexpected, and they were made possible by a combination of different approaches. The anaerobic preparation of crystals allowed detection of two [Fe4-S4] clusters in the bacterial ABCE1, an ABC cassette protein with a central although incompletely defined role in mRNA translation and ribosomal function[166]. Recently, an alternative approach for detecting Fe-S proteins through their physical interaction with the cytosolic Fe-S biogenesis machinery was developed, which linked essential enzymes involved in DNA metabolism and repair to MMS19, a protein that when mutated in yeast causes DNA instability, and to the cytosolic Fe-S biogenesis machinery[167, 168]. Bioinformatics approaches have accelerated identification of novel Fe-S proteins based on homology studies[36]. An alternative method was to identify specific motifs that are recognized by the Fe-S transfer machinery[37]. The cochaperone HSC20 binds to LYR motifs present in Fe-S recipient proteins or their binding partners. The discovery of the role of the LYR motif in engaging the Fe-S transfer complex has the potential to enhance the identification of novel Fe-S proteins. There are probably many more mammalian Fe-S proteins that have not been recognized so far because of the instability of Fe-S clusters and the previous lack of identifying sequence elements.

Acknowledgments

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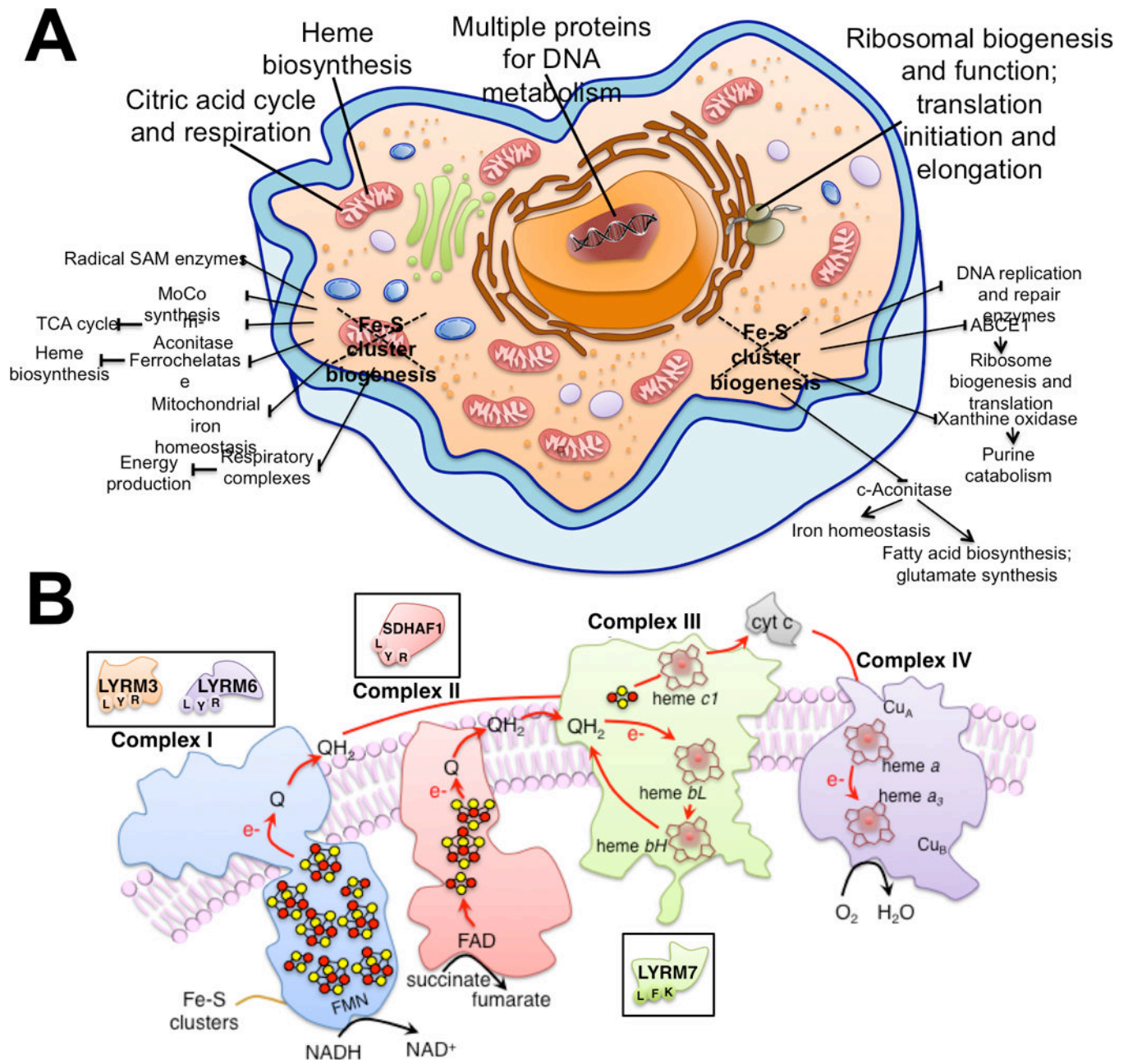
Figure 1. Fe-S proteins are critical to several metabolic pathways

While the importance of the Fe-S enzymes mitochondrial aconitase and succinate dehydrogenase for the citric acid cycle has long been known, as well as the dependence of respiratory chain complexes I–III on the presence of Fe-S cofactors, the recent identification of new Fe-S proteins has revealed novel roles of Fe-S clusters in ribosomal biogenesis and in translation, DNA metabolism and repair (Panel A). (B) Schematic representation of mitochondrial respiratory chain complexes I-IV. LYR motif proteins are involved in the assembly of complex II, namely SDHAF1, and III (LYRM7). Two subunits of complex I are also LYR motif proteins, LYRM3 and LYRM6.
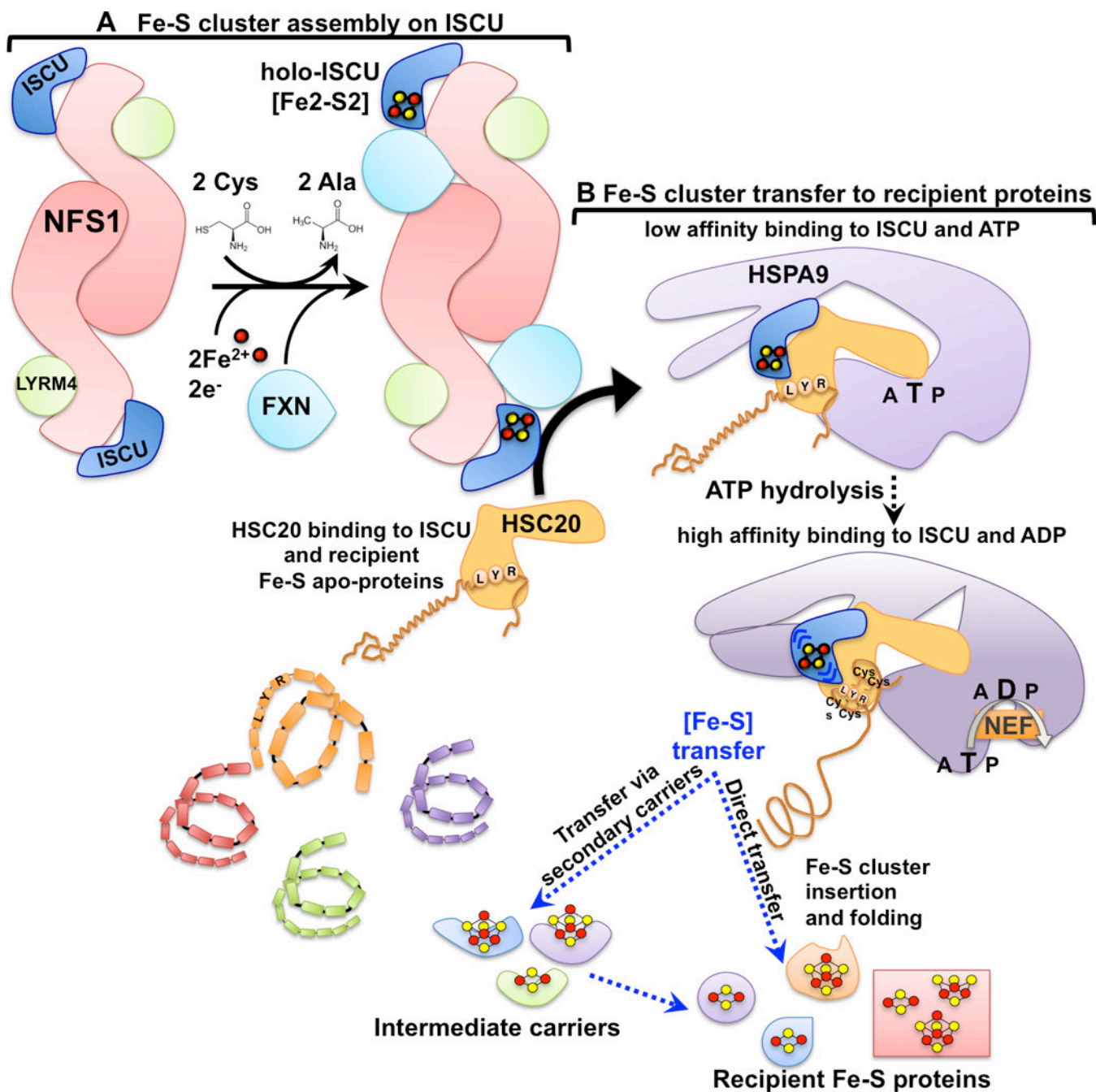
Figure 2. Fe-S cluster biogenesis in mammalian cells: a schematic of the main steps
Nascent Fe-S clusters are initially assembled on the main scaffold protein ISCU. A cysteine desulfurase NFS1 forms a dimer to which monomers of the primary scaffold ISCU are proposed to bind at either end. LYRM4 is a structural component of the core complex in eukaryotes and it is required for the activity of NFS1, which, aided by its cofactor pyridoxal phosphate, provides sulfur, removed from cysteine, for the nascent cluster. Frataxin (FXN) is part of the core complex, potentially binding in a pocket-like region between NFS1 and ISCU. The cluster assembles upon ISCU when iron is provided together with the reducing equivalents needed to generate the final electronic configuration of the cluster. Cluster

transfer from ISCU to recipient apoproteins is assisted by a dedicated chaperone/ cochaperone (HSPA9/HSC20) system that facilitates cluster release from the primary scaffold ISCU and transfer to recipient apoproteins or to intermediate carriers, which then target specific recipients. The cochaperone HSC20 interacts with ISCU through a patch of hydrophobic amino acid residues in the C-terminus, and with the chaperone HSPA9 through the N-terminal domain (J-domain). The J-domain of HSC20 contains a conserved His (H), Pro (P), Asp (D) tripeptide essential for activation of the ATPase activity of HSPA9. The energy derived from activation of HSPA9 drives a conformational rearrangement in the substrate-binding domain of the chaperone, which is proposed to propagate a conformational change to its binding partner, ISCU. The conformational change is thought to facilitate release of the cluster from the main scaffold, ISCU, and allow protected transfer directly or via intermediate carriers to specific subsets of Fe-S recipient proteins. A nucleotide exchange factor (NEF) exchanges ADP with ATP and completes the ATPase cycle of the chaperone. The Leu-Tyr-Arg (LYR) motif present in recipient Fe-S proteins (i.e. SDHB) is a binding site for HSC20, which selects LYR-containing proteins from amongst the full spectrum of proteins in the proteome and forms a complex with holo-ISCU and the chaperone HSPA9. Upon hydrolysis of ATP, and concomitant with release of the cluster from ISCU, the Fe-S cluster is transferred into the domain of the recipient protein, which contains the Fe-S cluster cysteinyl ligands that can rapidly tether and protect the cluster, while driving folding of the primary peptide sequence.