Minireview

Ferredoxins of the third kind

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Received 22 August 2001; revised 30 September 2001; accepted 8 October 2001

First published online 29 October 2001

Edited by Richard Cogdell

Abstract Most low-potential ferredoxins (Fds) are of the well-known [2Fe–2S] plant or [4Fe–4S] bacterial type. Yet, an additional class of [2Fe–2S] Fds has been recognized on the basis of sequence and spectroscopic idiosyncrasies. A recent crystal structure has confirmed the uniqueness of this third kind of Fd, and shown that these proteins display an unexpected structural similarity to thioredoxin. The properties of these thioredoxin-like [2Fe–2S] Fds are summarized, and hypotheses concerning their function are discussed.

Key words: Iron–sulfur; Electron transfer; Complex I; Hydrogenase; Thioredoxin; Evolution

1. Introduction

Ferredoxins (Fds) are small soluble iron–sulfur (Fe–S) proteins that were discovered nearly 40 years ago [1,2]. Most Fds are low-potential electron carriers that were long believed to consist of but two phylogenetically distinct families. The first one encompasses relatives of the small (ca. 55 residues) 2[4Fe–4S] Fds first isolated from anaerobic bacteria [1]. These ubiquitous proteins may differ in cluster type (3Fe or 4Fe), number (one or two), and length of polypeptide chain (up to over 100 residues). They nevertheless share a common structural core similar to the small 2[4Fe–4S] Fd framework [3]. The second family is likewise widely distributed and consists of monomeric (ca. 90–130 residues) proteins containing one [2Fe–2S] cluster. It includes plant and algal Fds that function as electron carriers in photosynthesis [4], as well as Fds that transfer electrons in redox chains feeding hydroxylases and oxygenases in a wide range of organisms (from bacteria to mammals) [5]. Because of these functional differences, which are reflected in moderate structural discrepancy [5], the plant and mammalian type Fds are regarded as two related subfamilies of the so-called plant and mammalian type [2Fe–2S] Fds.

In addition to their existence as small soluble electron carriers, the [4Fe–4S] and [2Fe–2S] protein folds also occur in domains and subunits of numerous redox enzymes and complexes [6–9].

High-potential iron proteins ([10] and references therein) are electron carriers containing [4Fe–4S]$^{3+/2+}$ clusters. Their sequences and structures qualify them as a Fd class distinct from the two previous ones. By tradition, however, the name Fd has been used for low-potential proteins only [11].

The existence of a third family of low-potential Fds was initially suggested by the primary structure of a [2Fe–2S] protein from *Clostridium pasteurianum* [12,13], and recently confirmed by the crystal structure of a homologous protein from *Aquifex aeolicus* ([14], Fig. 1). Rather unexpectedly, the latter [2Fe–2S] protein displays a fold similar to that of thioredoxin [14]. The main properties, distribution, and putative functions of these thioredoxin-like [2Fe–2S] proteins are outlined hereafter.

2. Discovery and occurrence

The first thioredoxin-like [2Fe–2S] Fds were isolated from the aerobe *Azotobacter vinelandii* [18] and the anaerobe *C. pasteurianum* [19]. Spectroscopic studies of both proteins led to significant advances in the understanding of [2Fe–2S] clusters [18,20,21]. The presence of a similar protein in the photosynthetic bacterium *Chlorobium tepidum* was also reported [22]. Investigations on this type of Fds subsequently slowed down, presumably because of their relative scarcity and lack of clear function. Somewhat later, the *C. pasteurianum* protein was shown to be a homodimer [23] endowed with a unique primary structure [12]. Cloning [24,25] and expression in *Escherichia coli* [25] of the encoding gene allowed the development of a molecular engineering program which unveiled a number of idiosyncrasies of the polypeptide chain and [2Fe–2S] cluster [26–33]. More recently, genes encoding similar proteins from *A. vinelandii* [34] and *A. aeolicus* [35] were also cloned and expressed in *E. coli*, thus providing a set of well characterized members of this family of Fds, including a hyperthermophilic one [35].

Genomes of several bacteria contain genes potentially encoding thioredoxin-like [2Fe–2S] Fds. While in most cases the data remain to be finalized and published (see http://www.tigr.org, http://www.sanger.ac.uk, or http://www.ncbi.nlm.nih.gov), translations of seven putative genes have been aligned in Fig. 2A with those of the three isolated and characterized proteins. One of the two putative proteins from *C. tepidum* (only one of them is included in Fig. 2) should be expected to be the previously isolated one [22]. The alignment reveals a small number of fully conserved residues. These include the four cysteine ligands of the [2Fe–2S] cluster and a few other ones which are for the most involved in the dimer interface

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0014-5793/01/$20.00
PII: S0014-5793(01)03049-6

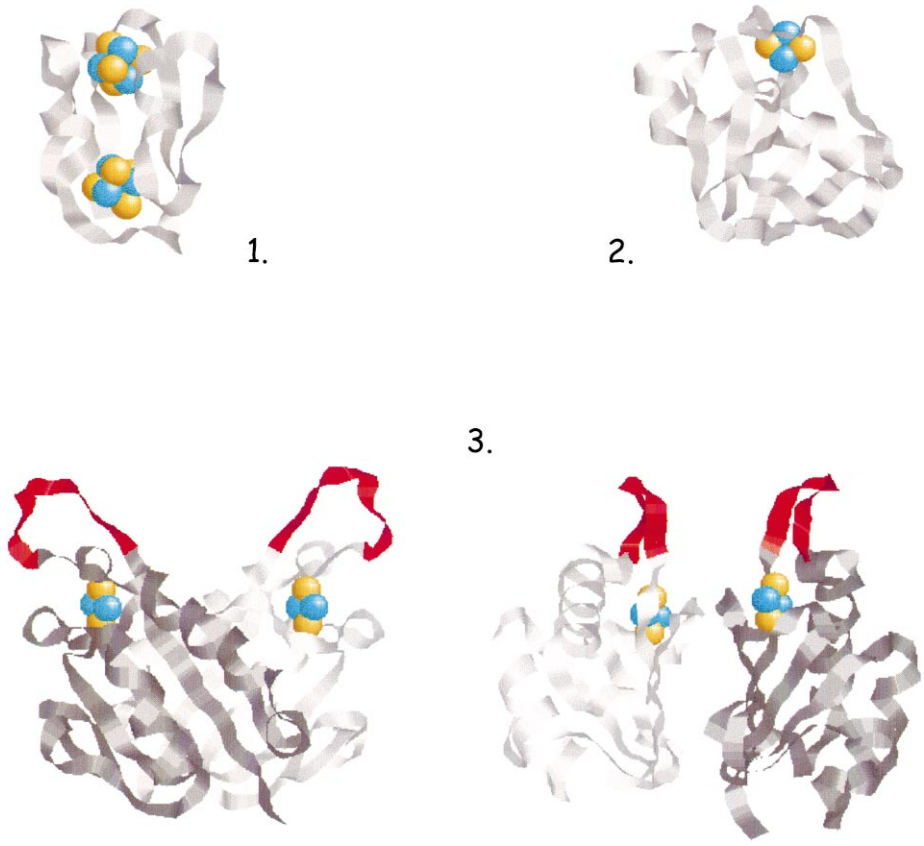

Fig. 1. Protein folds of the three kinds of low-potential Fds. The structures shown are: **1**: the 2[4Fe–4S] Fd from *Clostridium acidurici* ([15], PDB entry 2FDN), **2**: the [2Fe–2S] Fd from *Anabaena* PCC7119 ([16], PDB entry 1QT9), and **3**: two perpendicular views (rotated around a vertical axis) of the [2Fe–2S] Fd from *A. aeolicus* ([14], PDB entry 1F37). The structures were drawn using RASMOL [17]. The polypeptide chains are shown as gray ribbons, and the inorganic atoms of the Fe–S clusters as colored spheres (iron in cyan and sulfur in yellow). The two subunits of *A. aeolicus* Fd are in different shades of gray and the loops near the Fe–S clusters are shown in red.

[14]. The cysteine ligands of the [2Fe–2S] cluster display a pattern unique to these sequences: C-x(10,12)-C-x(29,34)-C-x(3)-C. At the time of this writing, thioredoxin-like Fd sequences have been found in bacteria only.

3. Structure

The properties of this novel class of Fd, as disclosed by various approaches over the years, are unique in many respects. We shall consider in turn the overall structure, the [2Fe–2S] active site, and the unusual versatility of the interaction between the polypeptide chain and the Fe–S cluster.

With a mass of 25 kDa [19,20], the thioredoxin-like [2Fe–2S] Fds are significantly larger than other low-potential Fds (6–18 kDa). However, this results from their being dimeric [23], and the polypeptide chains (100–120 residues, Fig. 2A) have sizes similar to those of plant and mammalian type Fds. The crystal structure of *A. aeolicus* Fd [14] at 2.3 Å resolution confirmed the absence of relationship with other Fds as previously inferred from sequence data [12,24]. While this protein fold is novel among Fe–S proteins, it quite surprisingly assumes a strong resemblance with that of thioredoxin: the rmsd between 76 Cα atoms is 2.6 Å, despite only marginal (7%) sequence similarity. The [2Fe–2S] cluster is located near the surface of the protein, at a site corresponding to the redox-active disulfide bridge in thioredoxin. Other noteworthy features of the structure are the presence of a protruding loop near the Fe–S cluster (highlighted in red in Fig. 1) and a strong dimer interaction (surface area 810 Å^2) between the subunits [14]. While the crystal structure has provided important information regarding this class of proteins and Fe–S proteins at large, the present resolution of 2.3 Å does not provide access to metric details of the [2Fe–2S] cluster.

The presence of a [2Fe–2S] cluster in thioredoxin-like Fds was indicated by early spectroscopic data which also detected minor differences with the [2Fe–2S] cluster of plant type Fds [19,20]. These differences were later confirmed by resonance Raman spectroscopy [23]. Subsequent spectroscopic studies provided detailed information on the Fe–S cluster in its oxidized and reduced levels [42], and disclosed similarities with the NuoE subunits of complex I ([43], see Section 4).

Heterologous expression in *E. coli* of the gene encoding the [2Fe–2S] protein from *C. pasteurianum* [25] initiated the production of numerous molecular variants of that protein, some of which (Cys56Ser and Cys60Ser) having serine-ligated [2Fe–2S] clusters [26,27]. In their reduced $[2Fe–2S]^+$ level, these mutated forms were shown by electron paramagnetic resonance and magnetic circular dichroism to assume a delocalized mixed-valence state resulting in an $S = 9/2$ ground spin state [28]. This novel property was further substantiated [29] and analyzed in detail [33] by Mössbauer spectroscopy.

Molecular variants of the *C. pasteurianum* Fd were also prepared with the aim of identifying the four (among the five present in the sequence [12,24]) cysteine ligands of the

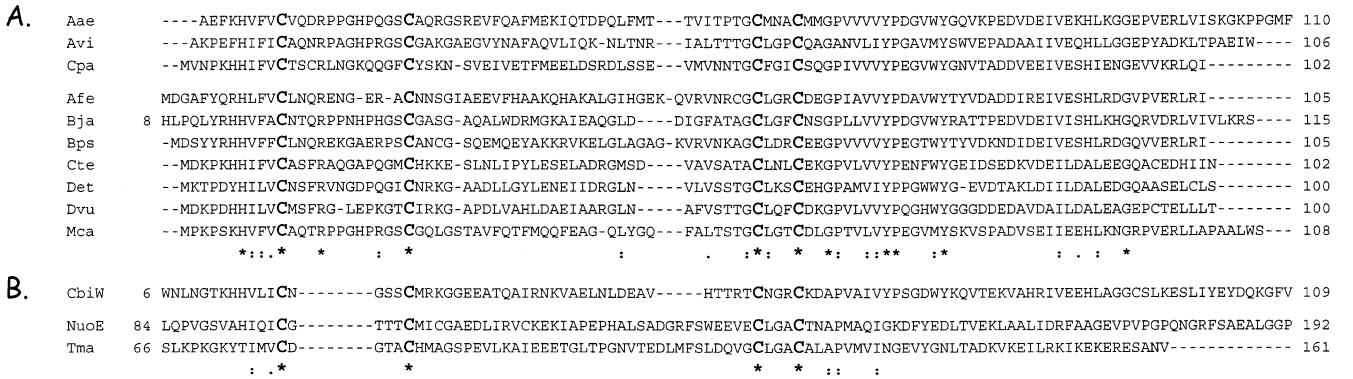

Fig. 2. Alignments of protein sequences from thioredoxin-like [2Fe–2S] Fds and homologues. A: Alignment of Fd sequences. The three upper sequences are those of the characterized proteins from *A. aeolicus* (Aae) [35], *A. vinelandii* (Avi) [34], and *C. pasteurianum* (Cpa) [12,24]. Those below are translated sequences from *Acidithiobacillus ferrooxidans* (Afe) (Tigr/DOE), *Bradyrhizobium japonicum* (Bja) [36], *Burkholderia pseudomallei* (Bps) (Sanger Centre/Beowulf Genomics), *C. tepidum* (Cte) (Tigr/DOE), *Dehalococcoides ethenogenes* (Det) (Tigr/DOE), *Desulfovibrio vulgaris* (Dvu) (Tigr/DOE), and *Methylococcus capsulatus* (Mca) (Tigr/DOE). Unpublished sequences were retrieved from the following web sites: http://www.tigr.org/tdb/mdb/mdbinprogress.html and http://www.ncbi.nlm.nih.gov/microb_blast/unfinishedgenome.html. All sequences are shown in full length, except for seven N-terminal residues of the *B. japonicum* one. B: Alignment of representative homologues of the thioredoxin-like [2Fe–2S] Fds. The CbiW protein (total length 127 residues, GenBank CAA04306) from *B. megaterium* is encoded by a gene belonging to the cobalamin biosynthesis operon (*cob*) [37]. The NuoE subunit (total length 239 residues) of complex I is from *Paracoccus denitrificans* ([38], GenBank A40296). The HydC (or HndA [9]) subunit (total length 161 residues) of hydrogenase is from *Thermotoga maritima* ([39], GenBank AAC02684). Sequences were retrieved using the TBLASTN program [40] with the *C. pasteurianum* sequence as bait, and aligned with the CLUSTALW program [41]. Symbols below alignments: stars are for identities, semicolons for high similarities, and dots for low similarities. Symbols in part B of the figure (last line) refer to the combined alignment of all 13 sequences of parts A and B.

[2Fe–2S] cluster [26,27]. This led to the unexpected finding that one of the cysteine ligands (cysteine 24) could be moved along a segment encompassing residues 14–26 of the polypeptide chain. Providing a cysteine ligand was maintained in that region, up to 12 residues could be removed without deleterious effects on the protein or [2Fe–2S] cluster [30]. This dispensable region of the polypeptide chain eventually turned out to be the protruding loop unveiled by the crystal structure [14]. The cysteine 60 ligand was also shown to be exchangeable [32]. The crystal structure of the *A. aeolicus* Fd [14] shows that cysteines 24 and 60 of the *C. pasteurianum* Fd bind to different Fe atoms, but both occur on the outermost side of the [2Fe–2S] cluster. In contrast, the non-exchangeable cysteines (11 and 56) are located nearer to the hydrophobic core and subunit interface of the protein. This cysteine ligand swapping is remarkable in at least two ways. First, the presence of a [2Fe–2S] cluster in all investigated variants shows that the protein framework is rigid enough, despite local flexibility, to specifically stabilize this particular type of metal site. Second, the crystal structure of the *A. aeolicus* Fd shows that residues which are distant (ca. 15 Å, near the tip of the loop [14], see Fig. 1) from the metal site in the wild-type protein can be drawn to this site when cysteine residues are introduced in these positions. Such experiments demonstrate that the affinity of Fe–S clusters for cysteine ligands [11] has the potential to drive significant structural rearrangements of the polypeptide chain [30,32]. The latter property is an interesting counterpart of previously observed polypeptide chain driven structural modifications of an Fe–S cluster [44].

4. Homologous proteins and domains

Proteins homologous to thioredoxin-like [2Fe–2S] Fds enjoy a wide distribution in living organisms and biological functions. A significant example is NADH-ubiquinone oxidoreductase (complex I) of mitochondrial and bacterial respiratory chains [8]. The NuoE (or NQO2, or 24 kDa) subunit of this complex displays sequence similarities, particularly with respect to the cysteine distribution pattern [38], and shares spectroscopic idiosyncrasies with thioredoxin-like [2Fe–2S] Fds [43]. The main differences are the larger size of NuoE (180–240 residues), mostly due to the presence of a ca. 80-residue N-terminal extension, and the shorter segment (four vs. 10–12 residues) separating the two first cysteine ligands in the sequence (Fig. 2B).

Numerous proteins or domains similar to thioredoxin-like [2Fe–2S] Fds also occur in hydrogenases (reviewed in [9]). Many of them are small subunits [45,46] most akin to the NuoE subunit of complex I (Fig. 2B). Others are domains of larger subunits [39,47–49]. In a few of the latter ones, the sets of four putative cysteine ligands of the [2Fe–2S] cluster either display distribution patterns different [49] from the canonical one shown in Fig. 2, or are incomplete [47].

In the operon (*cob*) encoding the cobalamin synthesis enzymes of *Bacillus megaterium*, the *cbiW* gene [37] encodes a putative protein of 127 residues that resembles thioredoxin-like [2Fe–2S] Fds (Fig. 2B). A very similar gene occurs in the genome of *Bacillus halodurans*, but not within a *cob* operon [50]. These sequences display a cysteine distribution pattern closely similar to that of the NuoE subunits of complex I (Fig. 2B), but their size is more like that of the Fds (Fig. 2). None of the products of these *Bacillus* genes has been isolated yet.

All homologues of thioredoxin-like [2Fe–2S] Fds discussed in this section differ from the Fds mainly by having a C-x(4)-C spacing instead of C-x(10,12)-C between the first two cysteine residues (Fig. 2). A remarkable exception to this rule occurs in a putative sucrolytic enzyme from potato (*Solanum tuberosum*), where the central part of the sequence resembles that of *C. pasteurianum* [2Fe–2S] Fd [51]. However, while the overall sequence similarities with thioredoxin-like [2Fe–2S] Fds are clear, the putative sucrolytic protein possesses a cysteine pattern nearly identical to that of plant type Fds: C-x(8)-C-x(2)-C-x(25)-C [51]. This observation has triggered the design of molecular variants of the *C. pasteurianum* [2Fe–2S]

Fd that disclosed novel aspects of cysteine ligand swapping in Fe–S proteins [32].

Protein domains assuming the thioredoxin-like Fd fold are widespread in nature. In that respect, this novel class of Fd is not unlike the other two kinds of low-potential Fds. It is worth noting that all three of these protein folds are present in complex I [8,9]. Furthermore, some hydrogenases accommodate all of the three Fd folds in a single polypeptide chain [9,39,49].

5. Function

This is by far the least understood property of thioredoxin-like [2Fe–2S] Fds. While homologous domains in large redox enzymes (e.g. complex I and hydrogenases) most likely function as electron carriers [8,9], the role of the Fds themselves is largely unknown.

In the first publication on the *C. pasteurianum* [2Fe–2S] Fd, this protein was reported to be more abundant in N_2-fixing cells that in ammonia-grown cells [19]. Recently, a specific interaction of the Fd with the MoFe component of nitrogenase was demonstrated by affinity chromatography experiments involving percolation of soluble *C. pasteurianum* extracts through a column carrying covalently bound Fd [52]. The interaction was shown to be mostly electrostatic, and was further substantiated by cross-linking reactions implementing the purified proteins, including several molecular variants of *C. pasteurianum* Fd [52]. In a subsequent study involving a complete set of surface charge modified variants of the Fd, a map of the Fd interaction surface with the MoFe protein was derived [53], based on the structural model of *A. aeolicus* Fd [14].

Further indications that thioredoxin-like Fds might be involved in dinitrogen fixation are provided by the genomic context of the encoding genes. For instance, the *C. pasteurianum* Fd-encoding gene, though monocistronic, is surrounded by two genes which have counterparts in *nif* regions of other bacteria (e.g. *A. vinelandii*) [24]. The gene encoding the *A. vinelandii* Fd belongs to the main *nif* gene cluster [34,54]. Similar observations can be made in currently sequenced bacterial genomes. Out of the seven sequences aligned in the lower part of Fig. 2A, five are translations of putative genes that neighbor *nif* genes (Fig. 3). These indications of a possible involvement in dinitrogen fixation are nevertheless to be taken with caution, as there is no evidence that any of these Fd-encoding genes are co-transcribed with *nif* genes. Furthermore, a significant discrepancy with the trend suggested by Fig. 3 is presented by *A. aeolicus*, of which the genome indicates that it does not possess the ability to fix dinitrogen [55]. A possible explanation may be that *A. aeolicus* Fd has a function different from that of its homologues in other organisms. Alternatively, the assumption that most of these proteins are involved in dinitrogen fixation may have to be revised: they might have a role in a more general part of nitrogen metabolism, rather than in the specialized dinitrogen fixation reaction. Many uncertainties thus remain, even regarding the most basic aspects of the function. Electron transfer would seem likely, but no supporting evidence has been reported so far. A totally different type of activity, perhaps a regulatory one, may be suggested by the very stable dimeric structure of these proteins, which is quite unusual among electron carriers. Furthermore, the protruding and flexible loops ([14] and Fig. 1), which are absent from the Fd-like subunits of redox complexes (Fig. 2 and Section 4), are attractive candidates for a regulatory interaction with a partner molecule, be it DNA or protein. The vicinity of the loops and [2Fe–2S] clusters, together with the demonstrated versatility of the polypeptide chain in that region [30,32], might then suggest the possibility of a redox switch regulation.

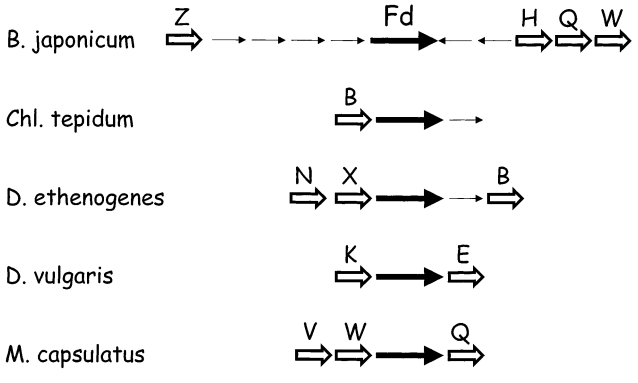

Fig. 3. Genomic regions surrounding genes potentially encoding thioredoxin-like [2Fe–2S] Fds. The DNA fragments shown are from the five bacteria, among the seven listed in the lower section of Fig. 2A, which have Fd-encoding genes in *nif* regions. References to sequences are as in the legend to Fig. 2. Thick black arrows represent putative Fd-encoding genes. Thick white arrows are for *nif* genes (with names indicated), thin arrows for other genes. Genes and intergenic regions are not drawn to scale.

6. Conclusions and prospects

The existence of a novel type of thioredoxin-like [2Fe–2S] Fd is now well founded on structural grounds. While the protein fold of these Fds is unique among Fe–S proteins, it quite unexpectedly resembles that of thioredoxin. This first observation of a possible evolutionary link between Fe–S proteins and dithiol/disulfide oxidoreductases deserves further investigation.

Thioredoxin-like [2Fe–2S] Fds are endowed with unique structural properties that have been disclosed by a combined use of molecular engineering, spectroscopy, and X-ray crystallography. One of their remarkable features is the unusual flexibility of parts of the polypeptide chain which may undergo large movements driven by the affinity of the [2Fe–2S] cluster for cysteine ligands. The novel electronic and magnetic properties of some molecular variants having serine-ligated [2Fe–2S] clusters are also worth noting. These idiosyncrasies raise questions and sometimes provide answers bearing on the structure and function of Fe–S proteins at large.

The distribution of thioredoxin-like [2Fe–2S] Fds in nature appears as yet to be scantier than that of the two other classes, namely the plant type [2Fe–2S] and bacterial type [4Fe–4S] Fds. However, like the two latter ones, thioredoxin-like [2Fe–2S] polypeptide folds are widely distributed among redox enzymes and complexes (e.g. respiratory chains and hydrogenases).

The most intriguing question remains the function(s) of the thioredoxin-like [2Fe–2S] Fds. Initial leads suggesting an involvement in dinitrogen fixation have been substantiated to some extent, but other tracks are to be explored. Even basic questions remain standing: do all these proteins have the

same function, or different ones depending on the host organism? Is this function electron transfer, or a regulatory one instead? Answers to both questions are expected in the near future, given the number of these proteins and the tools at hand. It may be anticipated that the function(s) will be no less surprising than the structure.

Acknowledgements: The contributions of C. Chatelet, J. Fujinaga, and M.-P. Golinelli to the research summarized in this review have been essential. The development of this work has also greatly benefited from collaborations with J. Gaillard, M. Lutz, J.-M. Moulis and the groups of M. Bruschi, R. Cammack, M.K. Johnson, E. Münck, and D.C. Rees.

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