The Yeast Nbp35-Cfd1 Cytosolic Iron-Sulfur Cluster Scaffold Is an ATPase*

Eric J. Camire, John D. Grossman, Grace J. Thole1, Nicholas M. Fleischman, and Deborah L. Perlstein2

From the Department of Chemistry, Boston University, Boston, Massachusetts 02215

Background: Nbp35 and Cfd1 are iron-sulfur cluster scaffolds with an NTPase domain of unknown function. Results: Nucleotide binding and hydrolysis assays paired with mutagenesis demonstrate ATP hydrolysis by these cluster scaffolds.

Conclusion: Nbp35 and the Nbp35-Cfd1 complex are ATPases.

Significance: This first demonstration of ATPase activity enables future investigation of how nucleotide influences cluster biogenesis by this large family of proteins.

Nbp35 and Cfd1 are prototypical members of the MRP/Nbp35 class of iron-sulfur (FeS) cluster scaffolds that function to assemble nascent FeS clusters for transfer to FeS-requiring enzymes. Both proteins contain a conserved NTPase domain that genetic studies have demonstrated is essential for their cluster assembly activity inside the cell. It was recently reported that these proteins possess no or very low nucleotide hydrolysis activity in vitro, and thus the role of the NTPase domain in cluster biogenesis remains uncertain. We have reexamined the NTPase activity of Nbp35, Cfd1, and their complex. Using in vitro assays and site-directed mutagenesis, we demonstrate that the Nbp35 homodimer and the Nbp35-Cfd1 heterodimer are ATPases, whereas the Cfd1 homodimer exhibited no or very low ATPase activity. We ruled out the possibility that the observed ATP hydrolysis activity might result from a contaminating ATPase by showing that mutation of key active site residues reduced activity to background levels. Finally, we demonstrate that the fluorescent ATP analog 2′/3′-O-(N′-methylanthraniloyl)-ATP (mantATP) binds stochiometrically to Nbp35 with a KD = 15.6 μM and that an Nbp35 mutant deficient in ATP hydrolysis activity also displays an increased KD for mantATP. Together, our results demonstrate that the cytosolic iron-sulfur cluster assembly scaffold is an ATPase and pave the way for interrogating the role of nucleotide hydrolysis in cluster biogenesis by this large family of cluster scaffolding proteins found across all domains of life.

Iron-sulfur (FeS) clusters are vital enzyme cofactors required for numerous fundamental biochemical pathways. The growing number of DNA replication and repair enzymes requiring an FeS cluster for their function and the recent discovery that defects in de novo cluster biogenesis result in genomic instability have sparked interest in elucidating how FeS enzymes located within the nucleus acquire their cofactor (1, 2). Currently, eight proteins have been identified as components of the eukaryotic cytosolic iron sulfur cluster assembly (CIA) pathway required for maturation of cytosolic and nuclear, but not mitochondrial, FeS enzymes (3). For many of the proteins in the CIA pathway, our molecular understanding of their function is in its infancy.

One long standing question has been the role of the NTPase domain possessed by the CIA cluster scaffolding proteins. These scaffolding proteins are responsible for the assembly of nascent FeS clusters that are ultimately inserted into the active sites of apo FeS enzymes localized to the cytosol and nucleus (3–7). In yeast, the CIA scaffold is comprised of two homologous proteins called Nbp35 and Cfd1 that can form a complex capable of scaffolding an FeS cluster at the Nbp35-Cfd1 interface (8). Genetic studies have demonstrated that conserved residues within the NTPase domains are required for cluster biogenesis in vivo (4, 8). However, the inability to detect nucleotide binding or hydrolysis by Nbp35 and Cfd1 in vitro has hampered progress toward a molecular understanding of the scaffolding mechanism.

Nbp35 and Cfd1 are prototypical members of the MRP/Nbp35 family of cluster scaffolding NTPases, a large and widely distributed family of cluster biosynthesis proteins found in all kingdoms of life (9). Although several MRP/Nbp35 family members from bacteria, archaea, and eukaryotes can assemble and rapidly transfer an FeS cluster to apoprotein recipients in vitro, only the Salmonella enterica ApbC cluster scaffold has been demonstrated to possess ATPase activity in vitro (10–15). Furthermore, it was recently reported that Nbp35 and Cfd1 do not display detectable affinity for nucleotide (<0.05 mol mol−1), nor do they possess measurable hydrolysis activity (<10 nmol min−1 mg−1) (8). The dearth of information about the nucleotide hydrolysis activity of the MRP/Nbp35 family of enzymes has made it challenging to develop a molecular understanding of the essential role nucleotide hydrolysis plays in cluster biogenesis.

*This work was supported by Boston University. The authors declare that they have no conflicts of interest with the contents of this article.

1 Supported by the Boston University Undergraduate Research Opportunities Program.

2 To whom correspondence should be addressed: Dept. of Chemistry, Boston University, 590 Commonwealth Ave., Rm. SCI299, Boston, MA 02114. Tel.: 617-358-6180; E-mail: dperl@bu.edu.

The abbreviations used are: CIA, cytosolic iron sulfur cluster assembly; mantATP, 2′/3′-O-(N′-methylanthraniloyl)-ATP; mantADP, 2′/3′-O-(N′-methylanthraniloyl)-ADP.
**The CIA Scaffold Is an ATPase**

**A)** structure of MRP/Nbp35 protein from *A. fulgidus* (Protein Data Bank code 3KB1) bound to ADP showing the nucleotide sandwich dimer. Zinc (gray sphere) is in the cluster scaffolding site. ADP (sticks) is bound at the dimer interface, one in each ATPase site. The deviant Walker A sequence is colored orange, and the side chains of the canonical lysine (Lys-32) and the deviant lysine (Lys-27) are shown. The DxD motif is pink with the aspartate side chains shown. The proposed magnesium ion site (blue sphere) was identified by overlaying 3KB1 with the structure of NifH bound to MgADP-AlF$_4$ (Protein Data Bank code 1M34) (16). C. consensuses: GGhGhGK(ST)X$_{20-22}$DxD $\begin{array}{llllll}3KB1 & K27 & K32 & D54 & D56 \\ Nbp35 & K81 & K86 & D109 & D111 \\ Cfd1 & K26 & K31 & D53 & D55 \end{array}$

**B)** consensus sequence of 3KB1, K81, K86, and the side chains of the D and K residues in 3KB1, K81, K86, and D109, D111, K27, and K31. D53, D55, D54, and D56.

**Figure 1.** A, structure of MRP/Nbp35 protein from *A. fulgidus* (Protein Data Bank code 3KB1) bound to ADP showing the nucleotide sandwich dimer. Zinc (gray sphere) is in the cluster scaffolding site. ADP (sticks) is bound at the dimer interface, one in each ATPase site. B, the nucleotide binding site of 3KB1. The deviant Walker A sequence is colored orange, and the side chains of the canonical lysine (Lys-32) and the deviant lysine (Lys-27) are shown. The DxD motif is pink with the aspartate side chains shown. The proposed magnesium ion site (blue sphere) was identified by overlaying 3KB1 with the structure of NifH bound to MgADP-AlF$_4$ (Protein Data Bank code 1M34) (16). C, consensus sequence of 3KB1, Nbp35, and Cfd1.

Some information about the possible function of the NTPase domain can be gained from analyzing the sequences of the MRP/Nbp35 family as well as the structure of an archaeal homolog (Protein Data Bank code 3KB1) (Fig. 1). These data demonstrate that Nbp35, Cfd1, and their homologs form a distinct subfamily in the signal recognition particle, MinD, and BioD (SIMIBI) family of nucleotide hydrolases harboring a deviant Walker A motif, GGhGhGK(ST) (h is hydrophobic residue) (9). Deviant Walker A NTPases possess widely divergent functions and are distributed into at least eight subfamilies, including the Get3 subfamily for insertion of tail-anchored membrane proteins, the nitrogenase iron protein NifH subfamily for nitrogenase metallocofactor assembly and catalysis, and the MinD subfamily, which regulates bacterial cell division (9, 17, 18). As first demonstrated for NifH and subsequently observed for several other deviant Walker A enzymes, these proteins form a dimeric nucleotide sandwich structure with the NTPase site at the dimer interface, making the dimer the minimal functional unit for this family of enzymes. In the presence of ATP, the deviant lysine (underlined in the signature sequence and throughout text) reaches across the dimer interface to interact with the nucleotide in the active site of the other protomer (19–22). This interaction serves to couple protein conformational or oligomerization changes in response to nucleotide binding and hydrolysis. It is unknown whether the MRP/Nbp35 family of cluster scaffolds also undergoes similar nucleotide-driven conformational changes observed for other deviant Walker A enzymes and if so what role this might play in the assembly and transfer of nascent clusters in the cytosol.

Herein we describe the use of steady state kinetic analysis and equilibrium binding studies to probe the NTPase domains of Nbp35 and Cfd1. The results reveal that homodimeric Nbp35 and the heterodimeric complex of Nbp35 and Cfd1 are slow ATPases, whereas the activity of the Cfd1 homodimer could not be detected. Because this result contradicts a recent report (8), we demonstrate that the ATP affinity and hydrolysis activity can be modulated by mutation of conserved residues in the ATPase site of Nbp35 and Cfd1. These results provide the first in vitro evidence that the CIA scaffolding proteins can bind and hydrolyze ATP. Our results thereby partly resolve this long-standing puzzle in FeS cluster biogenesis and enable further in vitro studies to probe the role that nucleotide binding and hydrolysis play in the assembly and transfer of FeS clusters from this large family of cluster scaffolds found across all domains of life.

**Experimental Procedures**

**Construction of Expression Vectors**—For construction of pHisCfd1, primers HisCfd1-f and HisCfd1-r were used to amplify Cfd1 from genomic DNA (Table 1). Purified PCR product was mixed with pRSF-Duet digested with BamHI and EcoRI (25 fmol each) and ligated via the one-step isothermal DNA assembly method of Gibson et al. (23), placing Cfd1 in multiple cloning site 1 in-frame with the N-terminal His tag in the cloning vector. For construction of pNC_complex, Nbp35 was amplified from genomic DNA using StrepNbp35-f and Nbp35-r, which adds the N-terminal StrepII tag (underlined) to Nbp35 (MADLWSHPQFEK-Nbp35) (Table 1). The plasmid harboring Cfd1, pHisCfd1, was digested with BglII and XhoI and then mixed with the Nbp35 PCR product (25 fmol each) and ligated via the one-step isothermal DNA assembly method. For construction of pHisNbp35, Nbp35 was amplified from genomic DNA using HisNbp35-f and Nbp35-r, adding an N-terminal His tag to Nbp35 (MADLWSHPQFEK-Nbp35) (Table 1). The PCR product was cloned into pRSF-Duet between the BglII and XhoI sites using the one-step isothermal DNA assembly method. For all constructs, successful ligations were confirmed via DNA sequencing.

**Protein Overexpression**—Plasmids containing the gene(s) of interest were transformed into chemically competent BL21(DE3) *Escherichia coli* cells and plated on LB agar plates containing 50 μg/ml kanamycin. A single colony was selected and used to inoculate LB medium (50 ml) containing 50 μg/ml kanamycin in a 250-ml Erlenmeyer flask and grown overnight at 37 °C with shaking at 250 rpm. The overnight culture was diluted 1:200 into LB medium (2 liters) containing 50 μg/ml kanamycin in a 4-liter baffled flask. Cultures were grown at 37 °C with shaking at 180 rpm to an $A_{600}$ of 0.7. Isopropyl 1-thio-β-D-galactopyranoside (1 mM) was added, and cells were harvested 3–4 h after isopropyl 1-thio-β-D-galactopyranoside addition.

**Cfd1-Nbp35 Complex Purification**—Protein purification was performed using buffers cooled to 4 °C. Cell paste (2 g) was resuspended in 20 ml of Buffer A (50 mM sodium phosphate (NaP), pH 8, 300 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol) supplemented with 5 mM imidazole and 1 mM PMSF, DNase (500 units of Pierce Universal Nuclease) and lysozyme (1 mg/ml) were added, and the mixture was stirred on ice for 30 min. Cell resuspension was sonicated and then clarified by centrifugation (40,000 × g for 45 min). Soluble lysate was combined with 2 ml of HisBind resin and rocked at 4 °C for 60 min.
The CIA Scaffold Is an ATPase

The resin was collected in a column and washed with Buffer A containing increasing concentrations of imidazole from 5 to 30 mM over 40–50 column volumes. Bound protein was eluted with Buffer A supplemented with 250 mM imidazole. Protein concentrations were pooled, and DTT was added to a final concentration of 5 mM. The solution was incubated with 2 ml of Strep-Tactin Superflow resin for 1 h, and then the resin was collected and washed with 5 column volumes of Buffer B (50 mM NaPi, pH 8, 300 mM NaCl, 10% glycerol, 5 mM DTT). The complex was eluted with 2.5 mM d-desethylbiotin in Buffer B. Fractions containing protein were pooled, exchanged into Buffer B, concentrated to 1–1.5 mg/ml using an Amicon Ultra centrifugal filter with Ultracel-30 membrane, stored at 80 °C. Protein concentration was determined via Bradford assay using BSA as a standard.

Nbp35 Purification—His-Nbp35 was purified using HisBind resin as described above for the complex except the column was washed with 80 column volumes of Buffer A supplemented with 5–20 mM imidazole because 30 mM imidazole resulted in slow elution of His-Nbp35. Eluted protein was immediately buffer-exchanged using a PD10 column into Buffer B. Protein was concentrated to 1–1.5 mg/ml using an Amicon Ultra centrifugal filter with Ultracel-30 membrane and stored at 80 °C. Protein concentration was determined via Bradford assay using BSA as a standard.

Cfd1 Purification—Cfd1 was purified using HisBind resin as described in the Nbp35-Cfd1 complex except Buffer A was replaced with Buffer C (25 mM Tris, pH 8, 500 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol), and the cells were lysed using a cell homogenizer at 20,000 p.s.i. Following elution from the HisBind column, Cfd1 was dialyzed against Buffer C (replacing 2-mercaptoethanol with 5 mM DTT) and then concentrated to 1–1.5 mg/ml using an Amicon Ultra centrifugal filter Ultracel-30 membrane and stored at 80 °C. Protein concentration was determined via Bradford assay using BSA as a standard.

Size Exclusion Chromatography—Size exclusion chromatography was carried out on Superdex 200 5/150 GL (GE Healthcare) at 4 °C. Samples were loaded in a volume of 10 μl at concentrations of 2 mg/ml. The column was equilibrated and eluted with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5% glycerol, 1 mM DTT. The molecular weight of the CIA scaffolding complexes was determined by comparison with standards of known molecular weight.

HPLC Assays for NTP Hydrolysis—ATP and GTP hydrolysis was directly monitored by an HPLC assay monitoring for the production of nucleoside diphosphate. Typical assays (50 μl) contained 50 mM Tris, pH 8.0, 200 mM KCl, 10 mM MgCl2, 5 mM ATP or GTP, and 12 μM StrepNbp35-His-Cfd1 (24 μM polypeptide). Reactions were carried out at 37 °C for 1 (ATP) or 4 h (GTP), subsequently quenched by addition of an equal volume of ice-cold 1 N HCl, and incubated on ice for 5 min. The reaction was neutralized with an equal volume of 1 M Tris followed by centrifugation to remove precipitated protein. ATP and ADP were separated by HPLC using a Phenomenex Prodigy 5-μm ODS-2 150 × 4.60-mm 5-μm reverse phase column using the following mobile phase: Solvent A (100 mM K2HPO4, pH 6.0), Solvent B (10% methanol in A), and a gradient from 0 to 100% B over 12 min. ATP eluted at 4.4 min, and ADP eluted at 5.0 min. GTP and GDP were separated by HPLC using a Phenomenex Prodigy 5-μm ODS-2 150 × 4.60-mm 5-μm reverse phase column using the following mobile phase: Solvent A (50 mM NaH2PO4, pH 6.5, 2 mM tetrabutylammonium bromide, 3% acetonitrile), Solvent B (100% acetonitrile), and a gradient from 0 to 40% B over 15 min. GTP eluted at 7.4 min, and GDP eluted at 4.4.

Coupled Assay for ATP Hydrolysis—ATP hydrolysis was monitored via a continuous spectroscopic assay using pyruvate kinase and lactate dehydrogenase. Assays (500 μl) contained 50 mM Tris-HCl, pH 8, 200 mM KCl, 10 mM MgCl2, 4 mM phosphoenolpyruvate, 0.2 mM NADH, 1 mM DTT, 20 units of pyruvate kinase, 20 units of L-lactic dehydrogenase, 0.05–15 mM ATP, and an enzyme concentration of 1.5–3 μM CIA scaffold (monomeric polypeptide concentration). All assay components were incubated at 37 °C for 5 min, and reactions were initiated by the addition of CIA scaffold. Assays were monitored at 340 nm. Plots of initial velocity versus ATP concentration were fit to the Michaelis–Menten equation using the curve fitting software GraphPad Prism 6. All kcat values reported are per polypeptide (per ATPase site). All kinetic constants reported are the average of at least two independent determinations, and the error reported is the standard deviation calculated from the independent measurements.

Site-directed Mutagenesis—Site-directed mutants of Cfd1, Nbp35, and their complex were generated via a two-step mutagenesis protocol. PCR 1 was completed with the appropriate template (pHisCfd1, pNC_complex, or pHisNbp35), a mutagenic primer (Table 2), and either DuetDOWN1 or T7 Terminator (Novagen) as reverse primers for Cfd1 and Nbp35 mutagenesis, respectively. Following purification, the product from PCR 1 was used as a megaprimer for a second PCR with the appropriate template. Following PCR 2, the product was digested with DpnI, purified, and transformed into NEB 5-α
The CIA Scaffold Is an ATPase

TABLE 2
Primers for site-directed mutagenesis

| Primer   | Sequence (5′ → 3′)                                      |
|----------|--------------------------------------------------------|
| Cfd1K26A | GATCCCTTCCGAGAAGGGTGGCTGTCGGTAAAAG                   |
| Cfd1K31A | CTGTGGTACCTACAGCCACGGCCACACCC                        |
| Cfd1D53A | GTGGAATTTCGTTTGTTATTGATTCTTTAGACGAGCGCCACCT          |
| Cfd1D55A | GTGGAATTTCGTTTGTTATTGATTCTTTAGACGAGCGCCACCT          |
| Nbp3K81A | GAGTTTAATACGAGACGAGGCGGTGATGCCAGAAGCCGCGGG         |
| Nbp3K86A | GGCCTAAATGGGCTGAGGCAGGCGGTGCTGCTGGAATTTCC         |
| Nbp3D109N | GGTTGCACTAGGACCTTATTGAT                          |
| Nbp3D111A | GGTCCTCCTCCGAGAAGGGTGGCTGTCGGTAAAAG                   |

Chemically competent E. coli (New England Biolabs). Each mutation was confirmed by DNA sequencing.

Circular Dichroism Studies—Circular dichroism (CD) spectra were acquired with an Applied Photophysics CS/2 Chirascan CD spectrometer. Spectra of wild-type (WT) and mutant CIA scaffolding proteins were recorded from 200 to 260 nm at 25 °C using a 0.1-cm-pathlength cuvette. Proteins (0.1–0.3 mg/ml) were buffer-exchanged into 10 mM KPO4, pH 8.0, 100 mM KCl, 0.5 mM DTT prior to acquisition of spectra. All spectra are an average of at least three scans and were baseline-corrected to subtract the contribution from the buffer. The spectra were converted to mean residue ellipticity and smoothed using the software package included with the CD spectrometer. Spectra of wild-type (WT) and mutant CIA scaffolding proteins were converted to mean residue ellipticity and smoothed using the software package included with the CD spectrometer.

Fluorescence Anisotropy Assays for MantATP Binding—Equilibrium binding experiments were carried out at 25 °C using a SpectraMax5 plate reader. Using a black, round bottom 96-well plate, WT Nbp35 and D109NNbp35 (1–300 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.1 mg/ml BSA) containing 500 nM mantATP. Samples were covered in tin foil and allowed to equilibrate for 60 min at 25 °C. The anisotropy was measured by exciting at 355 nm and measuring the polarization of the emission at 448 nm using a 435-nm-cutoff filter. The anisotropy of each well was measured using three independent readings. The background for each well was recorded without mantATP and subtracted from the anisotropy reading in the presence of mantATP to determine the anisotropy (r) at each concentration of Nbp35.

Plots of anisotropy (r) versus the concentration of Nbp35 polypeptide were fit to Equation 1 using the curve fitting software GraphPad Prism6 where r_max is the maximum anisotropy, r_min is the minimum anisotropy, and K_D is the equilibrium binding dissociation constant.

\[ r = \left( r_{\text{max}} - r_{\text{min}} \right) \times \left( \frac{[\text{Nbp35}]}{[\text{Nbp35}] + K_D} \right) + r_{\text{min}} \]  

The stoichiometry of the mantATP binding was determined using the same anisotropy assay described above except the concentrations of mantATP and NaCl used were 30 μM and 300 mM, respectively. The molar equivalence point was determined by fitting the data points above and below the concentration of mantATP used in the assay to two linear regressions. The intersection point of the two lines indicated the stoichiometry of the interaction.

Results

The Quaternary Structure of the Nbp35-Cfd1 Complex Is Not Affected by Mutation of the NTPase Site—To understand the function of the nucleotide hydrolysis domain of Nbp35 and Cfd1, we began by characterizing their quaternary structures and determining whether mutation of their NTPase active sites affected their oligomerization. Previously it was reported that the Nbp35-Cfd1 complex is a heterotetramer (7). Because it was recently reported that a large fraction of Nbp35 and Cfd1 polypeptides form homocomplexes in addition to the heterocomplex in vivo (24), we determined the quaternary structure of these Nbp35-only and Cfd1-only homocomplexes, which have not been reported previously. We expressed and purified each different form of the CIA scaffold, a HisNbp35-only construct, a HisCfd1-only construct, and a StreptNbp35-HisCfd1 complex (Fig. 2).

In addition to co-expression of Nbp35 and Cfd1, we also investigated whether the Nbp35-Cfd1 complex could be reconstituted by mixing StreptNbp35 and HisCfd1 that were expressed and purified separately. Because Nbp35 and Cfd1 have different molecular weights (Fig. 2), formation of the Nbp35-Cfd1 complex could be easily assessed via SDS-PAGE analysis. Following mixing of separately purified StreptNbp35 and HisCfd1, the mixture was passed through a metal affinity chromatography column, and the eluted proteins were analyzed via SDS-PAGE. Under no conditions examined did we observe significant amounts of heterocomplex formation as little StreptNbp35 was found in the elution fraction (data not shown). Inclusion of EDTA to prevent chelation of zinc at the bridging cluster scaffolding site, tris(2-carboxyethyl)phosphine to prevent disulfide bond formation by the bridging cluster cysteines, or ATP in the buffer did not induce reorganization of the homocomplexes to form the heterocomplex. This observation suggests that the Nbp35-Cfd1 complex is not in rapid equilibrium with the Nbp35 and Cfd1 homocomplexes. This observation is in agreement with the recent report that differentially epitope-tagged Nbp35 and Cfd1 subunits separately expressed in yeast do not reorganize to form an Nbp35-Cfd1 complex in yeast cell-free extracts (24).

To determine the quaternary structure of Nbp35 and Cfd1, we utilized size exclusion chromatography. As shown in Fig. 3,
both the Nbp35-only construct and the Cfd1-only construct eluted predominantly as dimers. The Cfd1 elution profile was not monodisperse, suggesting that Cfd1 could have small amounts of tetrameric and monomeric species present. This result was somewhat surprising because the Nbp35-Cfd1 heterocomplex was reported previously to be a tetramer (7). Because we found that Nbp35 and Cfd1 homocomplexes are homodimers, we also analyzed the quaternary structure of our Nbp35-Cfd1 complex. Like Nbp35 and Cfd1, their heterocomplex eluted as a dimer (Fig. 3, dots). Therefore, all of our preparations of the CIA scaffolding complex, Nbp35, Cfd1, and the Nbp35-Cfd1 complex, were predominantly dimers that did not rapidly exchange subunits under our *in vitro* conditions.

Because some deviant Walker A enzymes undergo dynamic changes in their quaternary structure in response to nucleotide that are abolished by mutation of NTPase residues (22, 25, 26), we wanted to determine whether mutation of Nbp35 or Cfd1 would disrupt formation of the Nbp35-Cfd1 complex. It has been reported previously that mutation of the canonical lysines in the deviant Walker A signature, Lys-86 and Lys-31 in Nbp35 and Cfd1, respectively (Fig. 1C), inhibits cluster assembly at the scaffolding site (8). If mutation of these lysines prevents formation of the Nbp35-Cfd1 complex, then that could provide a molecular explanation for the defect in cluster scaffolding activity exhibited by these mutants *in vivo* because disruption of the Nbp35-Cfd1 interaction would also disrupt the cluster site poised at the dimer interface (Fig. 1).

To determine whether mutation of the NTPase site affects formation of the Nbp35-Cfd1 complex, we mutated both the canonical lysines as well as the deviant lysines (Lys-26 in Cfd1 and Lys-81 in Nbp35; Fig. 1C) of Nbp35 or Cfd1. In these experiments, WT or lysine mutants of Nbp35 and Cfd1 were co-expressed in *E. coli*, and the extent of heterocomplex formation was assessed via SDS-PAGE following our tandem affinity chromatography procedure. The lysine mutants K26A/Nbp35-Cfd1, Nbp35-K26A-Cfd1, and Nbp35-K31A-Cfd1 were expressed at similar levels as the wild-type Nbp35-Cfd1 complex, whereas little K31A/Nbp35 was present in crude extracts upon co-expression with wild-type Cfd1. For the remaining three mutants, intact complexes containing K86ANbp35-Cfd1, Nbp35-K26A-Cfd1, and Nbp35-K31A-Cfd1 could be obtained. Each lysine mutant complex was isolated with the Nbp35 and Cfd1 proteins in approximately a 1:1 ratio just as the wild-type Nbp35-Cfd1 complex (Fig. 2). We conclude from these experiments that mutation of lysines in the Walker A signature motif does not disrupt the Nbp35-Cfd1 interaction.

To confirm via a second independent method that our mutations did not significantly affect the structure of the heterocomplex, we analyzed the secondary structure content of the two canonical lysine mutants, K26ANbp35-Cfd1 and Nbp35-K31A-Cfd1, via CD. In both cases, the CD spectrum of the mutant was similar to that of the wild-type complex. All spectra had a similar shape with a minimum at 205 nm and a shoulder at 222 nm, and the amplitude of the spectra did not differ by more than 5% between the wild type and the mutant protein. These results corroborate our conclusion that the lysine mutants do not significantly alter the protein structure. We conclude from these CD and affinity purification experiments that the defect in cluster biogenesis observed upon mutation of the canonical lysines cannot be attributed to a defect in heterocomplex formation.

### The CIA Scaffold Is an ATPase

To test whether the NTPase homology domains in Nbp35 and Cfd1 confer functional NTPase activity on the proteins, we first used a discontinuous assay method to measure ATP or GTP hydrolysis in which evolution of the ADP or GDP product was detected by reverse phase HPLC. Incubation of the purified Nbp35-Cfd1 complex with 5 mM ATP revealed the time-dependent and protein-dependent production of ADP (Fig. 4). Little if any GDP production was observed under similar reaction conditions (not shown). Comparison of the size of the ADP peak with known amounts of an ADP standard indicated that the Nbp35-Cfd1 complex hydrolyzed ATP with a specific activity of approximately 13 nmol min⁻¹ mg⁻¹. This activity is similar to the rate reported for the bacterial ortholog ApbC using a discontinuous

---

**Figure 3**. Size exclusion chromatography analysis of Cfd1 (thick solid line), Nbp35 (thick solid line) and the Nbp35-Cfd1 complex (dots). The expected elution volume of monomer, Nbp35-Cfd1 dimer, and Nbp35-Cfd1 tetramer are indicated with gray dashed lines.

**Figure 4**. The Nbp35-Cfd1 complex hydrolyzes ATP. ATP (5 mM) and the Nbp35-Cfd1 complex (12 μM dimer) were incubated for 1 h at 37°C. The reaction was quenched by acid, incubated on ice for 5 min, and neutralized with 1 M Tris; precipitated protein was removed by centrifugation; and the supernatant was analyzed by reverse phase HPLC. ATP (4.4 min) and ADP (5.0 min) retention times were identified by comparison with standards. mAU, milliabsorbance units.
assay measuring phosphate production (17 nmol min\(^{-1}\) mg\(^{-1}\)) (8, 14). These results suggested that the Nbp35-Cfd1 complex is indeed an active NTPase and that ATP is the physiologically relevant substrate. This conclusion is also in agreement with the co-crystallized ADP ligand observed with the archaeal homolog from *Archaeoglobus fulgidus* (Fig. 1, A and B) (Protein Data Bank code 3KB1).

**Steady State Kinetics of ATP Hydrolysis**—To further characterize the nucleotide hydrolysis activity of the CIA scaffold, we used a continuous coupled enzymatic assay to measure the specific activities of the Nbp35-Cfd1 complex as well as the Nbp35 and Cfd1 homodimers. The coupling assay measures the evolution of the ADP product from the ATPase reaction by including pyruvate kinase and lactate dehydrogenase as coupling enzymes plus phosphoenolpyruvate and NADH so that ADP formation results in the equimolar consumption of NADH, loss of which can be monitored spectrophotometrically at 340 nm.

In the presence of 5 mM ATP, the Nbp35-Cfd1 complex catalyzed the hydrolysis of ATP with a specific activity of 60 nmol min\(^{-1}\) mg\(^{-1}\). This is consistent with the observation of ATPase activity for this complex in the discontinuous assay described above. The Nbp35 homodimer also possessed ATPase activity with a specific activity of 115 nmol min\(^{-1}\) mg\(^{-1}\). Chemical reconstitution of the iron-sulfur clusters in Nbp35 homodimer or the Nbp35-Cfd1 complex did not significantly affect the ATPase specific activity. These activity levels lie in the same range as the activity reported for both apo and chemically reconstituted ApbC (45.7 nmol min\(^{-1}\) mg\(^{-1}\)) measured using a similar coupled assay (14). We also note that the specific activity of the Nbp35-Cfd1 complex was 4.5-fold higher using the coupled assay as compared with the discontinuous assay. A similar observation has been made with ApbC, which is inhibited by ADP and therefore has a 3-fold lower specific activity in the discontinuous assay that allows for a buildup of ADP versus the continuous assay where ADP is continually turned over by the coupling enzymes.

In contrast to Nbp35 and Nbp35-Cfd1, Cfd1 alone exhibited minimal nucleotide hydrolysis activity (<15 nmol min\(^{-1}\) mg\(^{-1}\)). Because mutation of residues in the active site of Cfd1 did not affect the observed ATP hydrolysis rate, we concluded that the low activity observed is the background in our ATP hydrolysis assay and that on its own Cfd1 has no or very little ATP hydrolysis activity *in vitro*.

To enable a more detailed comparison of the kinetic properties of Nbp35 and the Nbp35-Cfd1 complex, we determined the steady state kinetic constants for ATP hydrolysis (Fig. 5 and Table 3). Nbp35 and the Nbp35-Cfd1 complex showed similar values for \(k_{cat}\) of 4.6 ± 0.1 and 3.9 ± 0.6 min\(^{-1}\), respectively. The \(K_m\) for the complex, 5.0 ± 1.0 mM, was 10-fold higher than that of the Nbp35 homodimer, giving the heterodimeric complex a ~15-fold lower catalytic efficiency. Compared with the bacterial homolog ApbC, which is a negatively cooperative ATPase with a \(k_{cat}\) of 1.92 min\(^{-1}\) and \(K_m\) values of 1.6 ± 1.1 and 300 ± 43 μM for the low and high \(K_m\) respectively, the steady state constants for the yeast enzymes are similar with the only outlier being the larger \(K_m\) of the Nbp35-Cfd1 complex (14).

**Mutation of ATPase Site Attenuates Hydrolysis Activity**—To rule out the possibility that the ATPase activity seen for the yeast scaffold proteins might be due to the presence of a contaminating ATPase, we mutated several active site residues expected to be important for enzymatic activity and measured the ATP hydrolysis of the mutant proteins using the coupled assay. Two regions were selected for mutation: the two lysines of the Walker A signature motif already described and a DXD motif located about 20 residues downstream from the Walker A motif (Fig. 1, B and C). This motif, also known as the Walker A’ or switch I region, is absolutely conserved in the MRP/Nbp35 family of cluster scaffolding NTPases as well as in a subset of other deviant Walker A enzymes, such as the bacterial cell division regulator MinD (9). The DXD motif likely plays a role in magnesium ion binding and/or water activation (Fig. 1B), and mutation of this region in MinD resulted in an ATP hydrolysis defect (21, 27). Because this region was not mutated in the ApbC study or in previous genetic studies of Nbp35 and Cfd1 (4, 5, 8, 14), it is currently unknown how mutations affect the ATP hydrolysis activity of the MRP/Nbp35 scaffolds or their cluster biogenesis activity *in vivo*.
For the Nbp35 homodimer, mutation of either of the two aspartates in the DXD motif reduced ATPase activity, lowering it close to or below background levels (Fig. 5A and Table 3). This result confirms that the ATPase activity seen with the wild-type protein is not due to contaminating ATPase as mutation of the Nbp35 active site affects the ATP hydrolysis kinetics. Interestingly, mutation of the canonical lysine, Lys-86, in the Walker A signature motif produced only modest changes in the kinetic constants (Table 3 and Fig. 5A, triangles). Although mutation of the canonical lysine in some deviant Walker A enzymes reduces nucleotide hydrolysis activity, mutation of the canonical lysine, Lys-121, in ApbC did not knock out nucleotide hydrolysis activity, similar to the results we report here (14, 25, 26). In contrast, mutation of the deviant Walker A lysine, K81A, altered the steady state kinetic properties by increasing the $K_m$ for ATP such that the enzyme could not be saturated with substrate even at 15 mM ATP (data not shown). Unfortunately, the negative cooperativity exhibited by this mutant prevented us from estimating the $k_{cat}/K_m$ from the slope of the curve at low concentrations of ATP. Because three of the four mutations to Nbp35 significantly altered its ATP hydrolysis kinetics, our mutagenesis studies suggest that a majority of the ATPase activity observed is due to the Nbp35 polypeptide.

We additionally tested how these same mutations to Nbp35 affected ATPase kinetics of the Nbp35-Cfd1 complex. K86A-Nbp35-Cfd1, D109NNbp35-Cfd1, and D111A-Nbp35-Cfd1 all displayed significantly reduced ATP hydrolysis to near or below background levels (Fig. 5B). This change in ATP hydrolysis activity cannot be attributed to disruption of the Nbp35-Cfd1 complex as each mutant construct, K86A-Nbp35-Cfd1, D109NNbp35-Cfd1, and D111A-Nbp35-Cfd1, could be obtained with a 1:1 ratio of Nbp35 and Cfd1 (Fig. 2 for lysine mutant; data not shown for aspartate mutants). Furthermore, comparison of the CD spectra of K86A-Nbp35-Cfd1, D109NNbp35-Cfd1, and D111A-Nbp35-Cfd1 with WT heterodimer did not reveal any significant change in secondary structural content. These observations are consistent with the Nbp35-Cfd1 complex being responsible for a majority of the ATP hydrolysis activity observed.

We additionally tested the effects of mutating the Walker A lysines and switch I aspartates of Cfd1 on the ATPase activity of the Nbp35-Cfd1 complex. Although the wild-type Cfd1 homodimer did not have ATPase activity above the background, we wanted to determine whether any mutations to Cfd1 could affect the activity of the Nbp35-Cfd1 complex. We found that mutation of either lysine in the Walker A motif, K26A or K31A, resulted in decreased ATP hydrolysis activity of the Nbp35-Cfd1 complex (Fig. 5C, triangles). As already discussed, we know these mutations do not disrupt formation of the Nbp35-Cfd1 complex (Fig. 2). These results demonstrate that amino acids within the Cfd1 polypeptide play an important role in the ATP hydrolysis activity of the Nbp35-Cfd1 complex. Mutation to the DXD motif of Cfd1 resulted in more subtle changes to the ATPase activity. Mutation of Asp-55 resulted in a minor 2-fold reduction in $k_{cat}/K_m$ of the Nbp35-D55ACfd1 complex (Fig. 5B, open circles, and Table 3). The Nbp35-D55ACfd1 construct displayed a long and variable lag phase in its ATP hydrolysis kinetics, preventing determination of the initial rate and the kinetic constants. SDS-PAGE analysis of Nbp35-D55ACfd1 and Nbp35-D55ACfd1 demonstrated that each aspartate mutant was competent to form the 1:1 complex with Nbp35 (not shown). Together, these results corroborate our conclusion that the Nbp35-Cfd1 complex is an ATPase and that Cfd1 plays an important role in the ATP hydrolysis activity of the Nbp35-Cfd1 complex as a whole.

MantATP Binding Studies with Nbp35—Because our finding that Nbp35 and the Nbp35-Cfd1 complex are ATPases is in variance with a recent publication (8), we sought to further probe the interaction of these proteins with nucleotide using a second, orthogonal method. Specifically, we performed direct binding experiments to test whether Nbp35 could bind nucleotide and if so whether the observed binding affinity was consistent with the hypothesis that the ATPase activity we observed is in fact due to Nbp35. We decided to focus on Nbp35 for these binding experiments because it has the highest specific activity at 5 mM ATP as compared with the Nbp35-Cfd1 complex. We reasoned that if the observed ATPase activity were due to a contaminant then the Nbp35 homodimer would have the largest amount, thus making it easier to detect.

For these binding experiments, we used the fluorescent ATP analog mantATP and measured its binding to Nbp35 by fluorescence anisotropy. Titration of mantATP (0.5 μM) with increasing concentrations of Nbp35 resulted in a saturable change in the observed anisotropy that was magnesium-dependent (Fig. 6A). Using both a direct HPLC assay and the coupled enzymatic assay, we observed that little if any mantATP was hydrolyzed during the course of the experiment (not shown). This observation confirms that the anisotropy assay is measuring the binding of mantATP and not mantADP. Fitting the hyperbolic binding curve to Equation 1 allowed us to calculate a $K_D$ of 15.6 ± 1.9 μM for the Nbp35-mantATP interaction.

To determine that the change in anisotropy observed is due to binding of mantATP to Nbp35 and not to a low abundance contaminant, we also examined the stoichiometry of the interaction. To do this, we repeated the fluorescence anisotropy assay but this time held the concentration of mantATP at 30 μM (just above the $K_D$) to titrate the number of binding sites present. Under these conditions, the binding curve followed the expected quadratic behavior with an equivalence point of 24 μM mantATP (Fig. 6B). Because the calculated number of binding sites is comparable with the concentration of mantATP in the assay,
The CIA Scaffold Is an ATPase

we demonstrate that each Nbp35 polypeptide can bind one mantATP. This 1:1 stoichiometry establishes that the fluorescence anisotropy binding experiment is directly reporting on Nbp35 binding, not the binding of mantATP to a low abundance contaminant.

Having demonstrated that mantATP binds to Nbp35 stoichiometrically and with reasonable affinity, we also wanted to determine how the mutation of a residue demonstrated to be important for the ATPase activity of Nbp35 would affect the affinity for mantATP. For this experiment, we examined the affinity of D109N Nbp35 for mantATP as the D109N mutation reduced the ATPase activity of Nbp35 below the background rate. Mutation of the first aspartate in the switch I region of Nbp35 resulted in an increased $K_D$ for nucleotide. Because we could not saturate the binding, we can determine that the binding affinity was decreased at least 13-fold to a $K_D \approx 200 \mu M$. Thus, the drop in ATP hydrolysis activity observed for this mutant (Fig. 5A, filled circles) can be attributed to a defect in substrate binding.

Discussion

Here we utilized both nucleotide binding and hydrolysis assays and mutagenesis studies to establish that Nbp35 and the Nbp35-Cfd1 complex are ATPases, whereas Cfd1 does not possess detectable hydrolysis activity. For both the Nbp35 homodimer and the Nbp35-Cfd1 heterodimer, we identified several mutations of the ATPase site that affect the rate of nucleotide hydrolysis. We also utilized fluorescence anisotropy assays of mantATP binding to Nbp35 as an orthogonal method to probe the ATPase site. We demonstrate that Nbp35 binds mantATP stoichiometrically and that the affinity of the Nbp35-mantATP interaction can be modulated by mutagenesis of the active site of Nbp35. All together, this work demonstrates that the CIA scaffold is an ATPase.

At this point, we do not understand the discrepancies between our data and the report from Lill and co-workers (8) that none of the CIA scaffolding proteins alone or in combination display a measurable affinity for nucleotide or detectable nucleotide hydrolysis activity. However, we note that the specific activity of Nbp35-Cfd1 measured with our discontinuous assay, 13 nmol min$^{-1}$ mg$^{-1}$, is close to the lower limit of detection in the previous report. Thus, small differences in how the protein was purified or assayed could explain the observed variances. For example, we observed that the Nbp35-Cfd1 complex hydrolyzed nucleotide with a specific activity that was 4.5-fold higher in the coupled assay versus the discontinuous assay. A similar observation was made with the ApbC bacterial homolog of Nbp35 and Cfd1, which hydrolyzed nucleotide 3-fold slower in the discontinuous assay versus the coupled assay; this difference was attributed to product inhibition from ADP (14). Not only could the assay format influence the observed ATPase activity, but we also observed that the purification strategy could affect the resulting hydrolysis kinetics. For example, we discovered that a tandem affinity purification of Nbp35-Cfd1 was absolutely required to obtain reproducible hydrolysis kinetics. Use of just the nickel-nitrilotriacetic acid column resulted in isolation of a mixture of $^{15}$N Cfd1 and $^{35}$N Nbp35-$^{15}$N Cfd1. Because Cfd1 and Nbp35-Cfd1 have differing ATPase activities, changes in their relative ratios in the one-step purification procedure resulted in changes to the $V_{\text{max}}$ with different preparations of the protein. The relatively slow ATP hydrolysis combined with preparation to preparation variance in the $V_{\text{max}}$ mimicked the results one would expect if the observed ATPase activity was due to a contaminant. Use of tandem affinity chromatography to isolate the heterodimer away from the Cfd1 homodimers resulted in significantly improved reproducibility in the hydrolysis kinetics of the heterodimer. Finally, we noticed during development of the fluorescence anisotropy binding assays that the $K_D$ for mantATP was strongly influenced by the buffer composition. The ionic strength of the buffer as well as the salt components of the buffer affected the measured nucleotide affinity. Therefore, differences in the assay format, buffer composition, and/or purification procedure likely contributed to the discrepancies between our study and the previous report.

Our results also provide insights into the quaternary structure of the CIA scaffold and its influence on ATPase activity. Nbp35 and Cfd1 are both required for the biosynthesis of FeS clusters in the cytosol. Although it is well known that these proteins can form a heterocomplex in vitro and in vivo (7, 8, 12), recent work utilizing overexpressed, epitope-tagged constructs of Nbp35 and Cfd1 demonstrated that up to 60% of the Nbp35 and Cfd1 polypeptides form homocomplexes in yeast (24). It remains to be established what if any differing functional roles the Nbp35 and Cfd1 homocomplexes and the heterocomplex play in vivo. However, any differences that can be discerned via in vitro assays could provide insight into this question. We observed that the ATPase activity of Nbp35 and Cfd1 homocomplexes and the Nbp35-Cfd1 heterocomplex possess distinct ATP hydrolysis kinetics. Although Nbp35 homodimers and the Nbp35-Cfd1 complex both hydrolyzed nucleotide, Cfd1 on its own did not. Steady state kinetic analysis revealed further differences. Although both Nbp35 homodimer and the Nbp35-Cfd1 heterodimer displayed similar $k_{\text{cat}}$ values, the heterodimer had a $K_m$ of 5 mM, which was 10-fold higher than the $K_m$ of Nbp35. Therefore, Nbp35 homodimer, Cfd1 homodimer, and the Nbp35-Cfd1 heterocomplex all display differences in their ATP hydrolysis kinetics.

Besides differences in the ATPase activity of the wild-type proteins, our mutagenesis studies of Nbp35 revealed more distinctions between the homodimer and the heterodimer. For example, the K81ANbp35 homodimer could be purified with a yield and purity similar to those of the wild-type Nbp35, whereas K81ANbp35-Cfd1 could not be purified due to very low expression of K81ANbp35 upon its co-expression with Cfd1. We noted a similar behavior with the D109ANbp35 that was overcome with the more conservative D109N mutation. Some Nbp35 mutations also had different effects on ATPase activity in the homodimer versus the heterodimer. Mutation of the canonical lysine of Nbp35, K86A, in the homodimer resulted in minor changes to the hydrolysis kinetics, whereas the ATPase activity of K86ANbp35-Cfd1 was below background. These results demonstrate the existence of allosteric communication across the dimer interface because mutation of Nbp35 resulted in differing functional consequences depending on its dimeric partner.
Although the Cfd1 homodimer did not possess detectable ATPase activity, our results demonstrate that Cfd1 is not a passive bystander in the Nbp35-Cfd1 complex. Mutation of either lysine of the Walker A signature of Cfd1 resulted in decreased ATPase activity of Nbp35-Cfd1 (Fig. 5C). This observation again demonstrates that the Nbp35 and Cfd1 ATP binding sites are in allosteric communication with one another. This is perhaps not surprising given what is known about the structure of other deviant Walker A NTPase subfamilies that bind nucleotide at the dimer interface (Protein Data Bank code 3KB1 and Refs. 19, 21, 22, and 28). The structure of the A. fulgidus homolog confirms that the MRP/Nbp35 subfamily forms the expected nucleotide sandwich structure (Fig. 1) (Protein Data Bank code 3KB1). This structural arrangement predicts that the Nbp35 ATPase site and the Cfd1 ATPase site will be located close to one another at the heterodimer interface. Our mutagenesis studies demonstrate that this predicted arrangement must allow Cfd1 to allosterically affect ATP binding or hydrolysis activity at the Nbp35 active site because mutation of Cfd1 affected the ATP hydrolysis kinetics of the complex. Further investigations will be required to determine whether Nbp35 can allosterically influence Cfd1, allowing Cfd1 to become competent for nucleotide hydrolysis upon formation of the heterodimer.

Although our work provides the first in vitro biochemical evidence that the CIA scaffold is an ATPase and reveals how the quaternary structure of the scaffold affects its ATP hydrolysis activity, an important question remains: what is the role of nucleotide hydrolysis in cluster biogenesis? Because Nbp35, Cfd1, and their homologs in the MRP/Nbp35 family of cluster scaffolds are deviant Walker A ATPases, we looked to what is known about the function of nucleotide binding and hydrolysis in other subfamilies of this large and functionally diverse family. Generally, NTPases in this family utilize nucleotide to orchestrate dynamic protein-protein interactions, and they do this via two main mechanisms. Some deviant Walker A NTPases, including the MinD and ParA subfamilies, utilize nucleotide binding to drive dimerization, creating a protein-protein interaction site unique to the dimer (18). Other deviant Walker A enzymes, including the NifH and Get3 subfamilies, are constitutive dimers that undergo a large conformational change upon nucleotide binding (17, 19). Our work suggests that Nbp35 and Cfd1 are more like the NifH and Get3 subfamilies, which are dimers that undergo a conformational change upon binding ATP. First, we observed that Nbp35, Cfd1, and the Nbp35-Cfd1 complex are constitutive dimers (Fig. 3). Second, we observed that mutation of the deviant Walker A motif did not result in disruption of the heterocomplex between Nbp35 and Cfd1 (Fig. 2). Our work predicts that ATP binding to Nbp35 and Cfd1 drives a conformational change that could gate dynamic interactions with other proteins in the CIA pathway.

It is also interesting that we observed an Nbp35-Cfd1 heterodimer when these proteins have been reported previously to form a heterotetramer (7). Other subfamilies of deviant Walker A ATPases, like the Get3 subfamily, are known to form higher order oligomers that are functionally important (29). We speculate that differences in how the Nbp35-Cfd1 complex is purified could affect its quaternary structure. Indeed, the bacterial homolog ApbC is a mixture of monomers and dimers in its isolated state and a mixture of tetramers and dimers following chemical reconstitution of an FeS cluster in its scaffolding site (15). It would be interesting to determine what conditions induce tetramerization of Nbp35-Cfd1 heterodimer and if so whether the dimer to tetramer oligomerization has a functional role in cluster biogenesis in vivo. Clearly, more biochemical and biophysical studies will be required to characterize the structure of the CIA scaffold, its ATPase kinetic mechanism, and the functional implications of ATP binding and hydrolysis for cluster biogenesis by these proteins. The in vitro assays for nucleotide binding and hydrolysis reported here represent the first step toward understanding why the MRP/Nbp35 family of FeS cluster scaffolds requires an ATPase site to assemble FeS clusters and transfer these nascent clusters to downstream cluster biogenesis proteins or apoprotein recipients in vivo.

Author Contributions—E. J. C. and J. D. G. designed experiments, collected data, and analyzed results. G. J. T. and N. M. F. collected data and analyzed results. D. L. P. designed the study, analyzed data, and wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—We thank Boston University for funding this work in the form of startup funds. We also thank Jia Gao, Mihayl Petkov, Tudor Gradinariu, Isaac Banda, and Harry Griffin for construction of clones and mutants and Xin Sun for development of the discontinuous ATPase assay and critical reading of the manuscript. The CD spectrometer was purchased under National Science Foundation Grant CHE1126545.

References
1. Fuss, J. O., Tsai, C. L., Ishida, J. P., and Tainer, J. A. (2015) Emerging critical roles of Fe-S clusters in DNA replication and repair. Biochem. Biophys. Acta 1853, 1253–1271
2. Yeatch, J. R., McMurray, M. A., Nelson, Z. W., and Gotschling, D. E. (2009) Mitochondrial dysfunction leads to nuclear genome instability via an iron-sulfur cluster defect. Cell 137, 1247–1258
3. Paul, V. D., and Lill, R. (2015) Biogenesis of cytosolic and nuclear iron-sulfur proteins and their role in genome stability. Biochim. Biophys. Acta 1853, 1528–1539
4. Vitale, G., Fabre, E., and Hurt, E. C. (1996) NBP35 encodes an essential and evolutionary conserved protein in Saccharomyces cerevisiae with homology to a superfamily of bacterial ATPases. Gene 178, 97–106
5. Roy, A., Solodovnikova, N., Nicholson, T., Antholine, W., and Walden, W. E. (2003) A novel eukaryotic factor for cytosolic Fe-S cluster assembly. EMBO J. 22, 4826–4835
6. Hausmann, A., Aguilar Netz, D. J., Balk, J., Pierik, A. J., Mühlenhoff, U., and Lill, R. (2005) The eukaryotic P loop NTPase Nbp35: an essential component of the cytosolic and nuclear iron-sulfur protein assembly machinery. Proc. Natl. Acad. Sci. U.S.A. 102, 3266–3271
7. Netz, D. J., Pierik, A. J., Stümpfig, M., Mühlenhoff, U., and Lill, R. (2007) The Cfd1–Nbp35 complex acts as a scaffold for iron-sulfur protein assembly in the yeast cytosol. Nat. Chem. Biol. 3, 278–286
8. Netz, D. J., Pierik, A. J., Stümpfig, M., Bill, E., Sharma, A. K., Pallesen, L. J., Walden, W. E., and Lill, R. (2012) A bridging [4Fe–4S] cluster and nucleotide binding are essential for function of the Cfd1–Nbp35 complex as a scaffold in iron-sulfur protein maturation. J. Biol. Chem. 287, 12365–12378
9. Leipe, D. D., Wolf, Y. I., Koonin, E. V., and Aravind, L. (2002) Classification and evolution of P-loop GTPases and related ATPases. J. Mol. Biol. 317, 41–72
10. Boyd, J. M., Dreveland, R. M., Downs, D. M., and Graham, D. E. (2009)
Archaeal ApbC/Nbp35 homologs function as iron-sulfur cluster carrier proteins. *J. Bacteriol.* **191**, 1490–1497

11. Schwenkert, S., Netz, D. J., Frazzon, J., Pierik, A. J., Bill, E., Gross, J., Lill, R., and Meurer, J. (2010) Chloroplast HCF101 is a scaffold protein for [4Fe-4S] cluster assembly. *Biochem. J.* **425**, 207–214

12. Stehling, O., Netz, D. J., Niggemeyer, B., Rösser, R., Eisenstein, R. S., Puccio, H., Pierik, A. J., and Lill, R. (2008) Human Nbp35 is essential for both cytosolic iron-sulfur protein assembly and iron homeostasis. *Mol. Cell. Biol.* **28**, 5517–5528

13. Schmid, B., Einsle, O., Chiu, H. J., Willing, A., Yoshida, M., Howard, J. B., and Rees, D. C. (2002) Biochemical and structural characterization of the cross-linked complex of nitrogenase: comparison to the ADP-AIF4-stabilized structure. *Biochemistry* **41**, 15557–15565

14. Bange, G., and Sinning, I. (2013) SIMIBI twins in protein targeting and localization. *Nat. Struct. Mol. Biol.* **20**, 776–780

15. Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., and Rees, D. C. (1997) Structure of ADP-AIF4-stabilized nitrogenase complex and its implications for signal transduction. *Nature* **387**, 370–376

16. Leonard, T. A., Butler, P. J., and Löwe, J. (2005) Bacterial chromosome segregation: structure and DNA binding of the Soj dimer—a conserved biological switch. *EMBO J.* **24**, 270–282

17. Wu, W., Park, K. T., Holyoak, T., and Lutkenhaus, J. (2011) Determination of the structure of the MinD-ATP complex reveals the orientation of MinD on the membrane and the relative location of the binding sites for MinE and MinC. *Mol. Microbiol.* **79**, 1515–1528

18. Mateja, A., Szlachcic, A., Downing, M. E., Dobosz, M., Mariappan, M., Hegde, R. S., and Keenan, R. J. (2009) The structural basis of tail-anchored membrane protein recognition by Get3. *Nature* **461**, 361–366

19. Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345

20. Pallesen, L. J., Solodovnikova, N., Sharma, A. K., and Walden, W. E. (2013) Analysis of MinD mutations reveals residues required for MinE stimulation of the MinD ATPase and residues required for MinC interaction. *J. Bacteriol.* **187**, 629–638

21. Fu, H. L., Ajees, A. A., Rosen, B. P., and Bhattacharjee, H. (2010) Role of signature lysines in the deviant walker a motifs of the ArsA ATPase. *Biochemistry* **49**, 356–364

22. Zhou, H., Schulze, R., Cox, S., Saez, C., Hu, Z., and Lutkenhaus, J. (2005) Analysis of MinD mutations reveals residues required for MinE stimulation of the MinD ATPase and residues required for MinC interaction. *J. Bacteriol.* **187**, 629–638

23. Zhou, T., Radaev, S., Rosen, B. P., and Gatti, D. L. (2001) Conformational changes in four regions of the *Escherichia coli* ArsA ATPase link ATP hydrolysis to ion translocation. *J. Biol. Chem.* **276**, 30414–30422

24. Rome, M. E., Rao, M., Clemons, W. M., and Shan, S. O. (2013) Precise timing of ATPase activation drives targeting of tail-anchored proteins. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 7666–7671