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Proteobacterial Origin of Protein Arginine Methylation and Regulation of Complex I Assembly by MidA

Graphical Abstract

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In Brief

The biological role of protein arginine methyltransferases (PRMTs) outside of the nucleus is poorly understood. Hameed et al. provide structural evidence for a proteobacterial origin of PRMTs. Their work suggests a methylation-controlled substrate release mechanism and reveals that arginine methylation controls complex I assembly, growth, and phototaxis in *Dictyostelium*.

Highlights

- We determined the 3D structure of a mitochondrial methyltransferase
- Structural similarity suggests that protein methylation exists in proteobacteria
- Binding studies suggest a methylation-controlled substrate release mechanism
- Methylation by MidA controls *Dictyostelium* complex I assembly, growth, and phototaxis

Data and Software Availability

5ZTZ
5ZZW
5ZU0

Proteobacterial Origin of Protein Arginine Methylation and Regulation of Complex I Assembly by MidA

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SUMMARY

The human protein arginine methyltransferase NDUFAF7 controls the assembly of the ~1-MDa mitochondrial complex I (CI; the NADH ubiquinone oxidoreductase) by methylating its subunit NDUFS2. We determined crystal structures of MidA, the *Dictyostelium* ortholog of NDUFAF7. The MidA catalytic core domain resembles other eukaryotic methyltransferases. However, three large core loops assemble into a regulatory domain that is likely to control ligand selection. Binding of MidA to NDUFS2 is weakened by methylation, suggesting a mechanism for methylation-controlled substrate release. Structural and bioinformatic analyses support that MidA and NDUFAF7 and their role in CI assembly are conserved from bacteria to humans, implying that protein methylation already existed in proteobacteria. *In vivo* studies confirmed the critical role of the MidA methyltransferase activity for CI assembly, growth, and phototaxis of *Dictyostelium*. Collectively, our data elucidate the origin of protein arginine methylation and its use by MidA/NDUFAF7 to regulate CI assembly.

INTRODUCTION

Protein methylation is a reversible signaling process (Chang et al., 2007; Wesche et al., 2017) in which protein methyltransferases (PMTs) covalently modify proteins by attaching one or several methyl groups onto arginines or lysines (PRMTs and PKMTs, respectively) (Copeland, 2013; Copeland et al., 2009; Eram et al., 2016). Both PRMTs and PKMTs use the cofactor S-adenosylmethionine (SAM) as a source of the methyl group and produce S-adenosylhomocysteine (SAH) and methylated arginines or lysines (Bauerle et al., 2015; Chiang et al., 1996). The mammalian PRMT family comprises 9 members (PRMT1–9) and shares a conserved catalytic core domain (Scha-

pira and Ferreira de Freitas, 2014). To our knowledge, no direct evidence for PRMTs in bacteria has been reported.

Currently, only nuclear PMTs and their actions on histone tails—and, hence, their contribution to transcriptional control—are well characterized (Bedford and Clarke, 2009). Structural and mechanistic data for PMTs outside the nucleus are sparse (Bedford and Clarke, 2009; Boriack-Sjodin and Swinger, 2016). In particular, structural data are lacking for mitochondrial PMTs (Rhein et al., 2017).

In humans, ~40% of mitochondrial oxidative phosphorylation disorders are linked to the dysfunctions of complex I (CI) of the respiratory chain (Janssen et al., 2006). CI is a multi-subunit NADH ubiquinone oxidoreductase that couples electron transfer from NADH to ubiquinone (Wirth et al., 2016; Zickermann et al., 2015). Its 14 central subunits are conserved from prokaryotes—from which the mitochondrion was most likely derived by endosymbiosis—to mammals (Gray, 2012). In eukaryotic cells, 30 or more additional subunits are included, producing a megadalton assembly that comprises a membrane-embedded arm and a peripheral arm (Vinothkumar et al., 2014; Wirth et al., 2016; Zickermann et al., 2015). A crucial step in the assembly of CI is the association of the integral membrane protein ND1 with components of the peripheral arm—in particular, NDUFS2—to form a 400-kDa precursor of CI (Mimaki et al., 2012).

Studies of human and *Dictyostelium discoideum* CI showed that the 400-kDa precursor assembly requires the catalytic activity of a PRMT called NADH dehydrogenase ubiquinone assembly factor 7 (NDUFAF7) in humans and mitochondrial dysfunction protein A (MidA) in *D. discoideum* (Carilla-Latorre et al., 2010; Rhein et al., 2013; Zurita Rendón et al., 2014). These data suggested that MidA and NDUFAF7 contribute to CI pre-assembly by dimethylating NDUFS2 on Arg85 (human numbering), which then interacts more strongly with the NDUFS7 subunit of CI. Together, NDUFS7 and dimethylated NDUFS2 form a combined surface enabling the stable interaction of the peripheral arm with the membrane-located protein ND1 (Carroll et al., 2013; Rhein et al., 2013; Zickermann et al., 2015).

Here, we present insights into the structural, mechanistic, and evolutionary bases of CI assembly control through protein arginine methylation by MidA.

Figure 1. The Architecture and Cofactor Binding of MidA Is Conserved in Proteobacteria

(A) Secondary structure representation of the MidA crystal structure bound to SAH (stick representation, with carbon atoms indicated in pale orange). The methyltransferase core domain is indicated in pale green. The additional domain is formed by three core domain loops (loop I, in red; loop II, in magenta; and loop III, in light pink).

(B) Zoom-in on the SAH binding pocket. SAH (stick representation, as in A) is indicated in its 2Fo - Fc electron density contoured at 0.5 σ . SAH-coordinating residues of MidA are indicated.

(C) ITC titrations for the interaction of MidA with SAM and SAH. Bottom panels indicate integrated values for ITC peaks of top panels. Representative of 3 replicates.

(D) Superimposition of the MidA catalytic core domain (green) onto PRMT5 (brown; PDB: 3uA3). Conserved catalytic residues of MidA and PRMT5 are indicated with magenta and blue sticks, respectively (RMSD = 3.32 Å) for about 61 residues.

(E) Superimposition of MidA (green) onto the putative protein Q6N1P6 (PDB: 1zkd) from *R. palustris* (orange) and ACR COG1565 (PDB: 4g67) from *B. thailandensis* (purple); RMSD = 1.68 Å. The eukaryotic loop missing in the bacterial orthologs is indicated by an asterisk.

(F) SAH binding residues conserved among MidA (green), Q6N1P6 (red), and ACR COG1565 (purple). SAH are indicated in the same color as MidA and ACR COG2565.

See also Figure S2.

RESULTS

The MidA Combines a Canonical PRMT Catalytic Domain with an Unusual Additional Domain

Using selenomethionine MAD phasing, we determined the crystal structure of *D. discoideum* MidA (residues 76–484, named MidA Δ 75) in its apo- and SAH-bound forms at resolutions of 2.6–2.8 Å (Figure 1A; Table S1). All structures were obtained in the space group P2₁2₁2₁, with three molecules in the asym-

metric unit. The apo- and cofactor-bound structures were highly similar in the crystal (root-mean-square deviation [RMSD] = 0.31 Å for C α atoms) and in solution (Figures S1A and S1B), implying that SAH binding is not associated with a large structural change.

Akin to other eukaryotic PRMTs, the catalytic domain of MidA Δ 75 forms an α - β sandwich and binds SAH across the tip of the central β sheet, in a pocket formed between loop regions (Figures 1A and 1B; Table S2). The ribose moiety is coordinated

by Glu200 and Asn205. The adenine is inserted between Phe295 and Ala201, with a water-mediated hydrogen bond to the backbone carbonyl of Gly241. The homocysteine amine group (NH₃⁺) is coordinated by the backbone carbonyls of Gly170 and Gly257, and the carboxy group is hydrogen bonded to Thr135.

SAM and SAH bound to MidAΔ75, with comparable dissociation constants (K_D s) of 5.6 ± 0.5 and 3.1 ± 0.2 μ M, respectively (Figure 1C; Table S3A). Hence, post-catalytic exchange of SAH to SAM appears to be driven by the mitochondrial SAM:SAH ratio of 2:1 (Song et al., 2007) rather than by a decrease in cofactor affinity after methyl transfer. In agreement with our crystallographic model, substitution of the ribose-coordinating Glu200 by an alanine and the double mutation Gly170Val/Gly172Val (both located at the base of the ribose) abolished the binding of SAM and SAH (Figures 1C and S1C; Table S3A). The mutation Thr135Ala, designed to affect anchoring of the carboxy group, lowered the cofactor affinity below the limit of K_D determination in our isothermal titration calorimetry (ITC) assay (Figure S1C). The similar effect of mutations on both SAM and SAH, and their comparable binding thermodynamics (both driven by favorable entropy and enthalpy), suggested that SAM and SAH bind MidA in the same way. Assuming that SAM binds to MidA as does SAH, the methyl group would stick out into a cavity formed between the catalytic core and an additional domain. This additional domain is assembled from extended internal and N-terminal loops of the core domain and consists of a central four-stranded antiparallel β sheet, loosely flanked by three short α helices and connected by large loop regions (Figures 1A and S2). Although the mammalian PRMTs 1–9 have domains in addition to the catalytic domain, these are formed by either N- or C-terminal extensions to the catalytic core and, where known, adopt different structures (Schapira and Ferreira de Freitas, 2014).

Proteobacterial Origin of MidA and Complex I Assembly Control by Methylation

We only found close structural homologs for this additional domain in three bacterial proteins in the PDB: the putative protein Q6N1P6 from the β -proteobacterium *Rhodopseudomonas palustris* (PDB: 1zkd) and two proteins from the α -proteobacterium *Burkholderia thailandensis*, the “uncharacterized ACR COG1565” (PDB: 4f3n), and another COG1565 superfamily of proteins, labeled as “likely methyltransferase” (PDB: 4g67) (Table S2B). Q6N1P6 has not yet been described in a journal article, whereas the two *B. thailandensis* structures were mentioned without specific description in a publication regarding the “Essential Burkholderia Structome” (Baugh et al., 2013). All three bacterial structures also contain catalytic domains that closely matched the one of MidA, except for a 14-residue-long loop region present in MidA (214–227) and other eukaryotic homologs (Figures 1D and S3). Compared to MidA, the overall protein sequence homology and RMSD of the bacterial homologs are 52% and 1.7 Å, respectively. One of the bacterial homologs (PDB: 4g67) was crystallized with an SAH molecule, which is bound as in MidA. The SAH binding site is conserved in the other two proteins (Figures 1E, 1F, and S3). The conservation of the additional domain and of the cofactor binding site strongly suggested that MidA and these α and β proteobacterial proteins are evolutionarily related.

We also found close homologs of the eukaryotic NDUFS2 in *R. palustris* and *B. thailandensis* (WP_027277471.1 and WP_006025105.1, respectively, both with ~74% and 68% homology to *D. discoideum*) (Figure S4A). Importantly, NDUFS2 from these bacteria, *D. discoideum*, and human show the highest conservation in a 75-residue N-terminal fragment that contains the strictly conserved arginine, which is methylated by MidA/NDUFAF7 (Figure S4A). Conversely, although NDUFS2 and the overall architecture of CI are structurally conserved between *Thermus thermophilus* (a bacterium from the phylum Deinococcus-Thermus) and the fungus *Y. lipolytica* (Baradaran et al., 2013; Hunte et al., 2010), only *Y. lipolytica* NDUFS2 contains an arginine equivalent to the methylated arginine of the human or *Dictyostelium* NDUFS2 (Carroll et al., 2013; Rhein et al., 2013). *T. thermophilus* contains a threonine in this position, and, accordingly, there is no MidA/NDUFAF7 homolog in *T. thermophilus*. Consequently, this species appears to use a MidA/NDUFAF7-independent mechanism for CI assembly.

Considering that mitochondria derive from proteobacteria (Gray et al., 2001), these data suggest that the PRMT-regulated assembly of CI originated in this bacterial phylum and has later been transmitted through mitochondrial genes to eukaryotes, where the responsible PRMT gene later migrated into the nuclear genome. We, therefore, propose that the uncharacterized or tentatively annotated entries PDB: 1zkd, 4g67, and 4f3n are PRMTs that promote CI assembly by methylating NDUFS2 at an N-terminally located arginine.

Methylation Controls Substrate Binding Affinity

We next investigated the catalytic mechanism of MidA. Recombinant MidAΔ75 methylated NDUFS2-derived peptides *in vitro*, as confirmed by mass spectrometry (Table S3B). With increasing incubation times, we initially detected monomethylation and then increasingly dimethylation *in vitro*, revealing a slow and stepwise catalysis under the conditions used.

We failed to obtain crystal structures of MidA:substrate peptide complexes. However, all three MidAΔ75 molecules of the asymmetric unit positioned their eukaryote-specific loop so that its Lys219 points directly into the active site of an adjacent molecule (Figure 2A). This crystallographic positioning of Lys219 appears to mimic a substrate arginine, barring access for added substrate peptides in this crystal lattice. In support, the substrate peptide for CARM1, the only PRMT for which a complex structure is available (PDB: 5dx0) (Boriack-Sjodin et al., 2016), presents the substrate arginine in a position equivalent to that of Lys219. We did not observe these crystallographic substrate-mimic complexes for MidAΔ75 *in vitro* (Figures S1A and S1B; Experimental Procedures) but cannot rule out that the first 75 residues or additional factors can stabilize such autoinhibitory complexes *in vivo*.

An *in silico* substitution of MidA Lys219 by an arginine suggested that the aliphatic arginine side chain carbons are channelled by Phe133 and Tyr352 from both sides (Figures 2B and 2C). The charged arginine head group would be attracted into the pocket, toward the cofactor, by Glu258, Asp261, and Asp381. In this position, the arginine guanidino nitrogen atoms would be at a distance of about 3 Å from the methyl group of SAM. Given the charge surrounding the arginine head group,

Figure 2. Crystallographic Mimicry of the Catalytic Mechanism of MidA and MidA Interaction with Its Substrate

(A) In the crystal lattice, each MidA molecule inserts its eukaryotic loop into the active site of another MidA molecule. Global view of two MidA molecules (indicated in green and violet), where the eukaryotic loop of one molecule (violet) inserts lysine 219 (magenta stick) into the active site between the substrate selection domain and catalytic core of the other molecule (green) bound to SAH (pink sticks).

(B) Zoom-in on the catalytic region of MidA, where substrate mimic K219 (magenta stick) and surrounding residues forming the binding pocket and SAH are indicated.

(C) The substrate mimic K219 is replaced *in silico* with R219 (magenta stick) in the catalytic pocket, and SAH is replaced by SAM (pink stick).

(D) ITC titrations for the interaction between MidA (wild-type and mutants) and NDUFS2 peptides or MBP-NDUFS2. Bottom panels show integrated values for ITC peaks of top panels. Representative of 3 replicates.

single or double methylation is predicted to lower the affinity of the substrate. In agreement, MidA Δ 75 bound *in vitro* to unmethylated, monomethylated, and dimethylated NDUFS2-derived peptides encompassing the substrate arginine Arg55 (*D. discoideum* numbering), with K_D s of about 2, 7, and 20 μ M, respectively (Figures 2D and S4B; Table S3A). Whereas the unmethylated peptide bound with a highly favorable enthalpy (offset by a large negative entropy), the associations of the mono- and dimethylated peptides were characterized by an increasing loss of favorable enthalpy and concomitant increase in entropy. This loss of strong enthalpy contribution would be in agreement with a loss of polar and charged interactions, whereas the increase in favorable entropy changes would be in line with an increase in hydrophobic interactions, all of which are features fitting the stepwise dimethylation of the substrate arginine.

MidA binds to maltose-binding protein (MBP)-fused (unmethylated) recombinant NDUFS2, with an affinity comparable to the one of the unmethylated peptide (Figures 2D and S4B; Table S3A). However, the thermodynamics of binding differed markedly, showing a reduced entropic penalty and an associated loss in favorable enthalpy changes for NDUFS2, as compared to the peptide mimic (Table S3A). This difference suggested that the full-length NDUFS2 forms additional interactions and/or pre-structures the methylation site.

Possible Role of the Additional Domain in Substrate Selection

Residues 11–51 of *D. discoideum* NDUFS2 are minimally required to bind to MidA (Carilla-Latorre et al., 2010). These residues are conserved across bacteria and eukaryotes

Figure 3. MidA Protein-Protein Interactions

(A) MidA residues are colored based on residue conservation from blue (highly conserved) to red (not conserved). Left and right panels show 90° views.

(B) Residue conservation of MidA mapped onto the protein surface. Left and right panels show the same 90° views as in (A). The red circle outlines a prominent conserved hydrophobic pocket on the substrate selection domain. The SAH is indicated by an orange stick model. The eukaryotic loop is indicated by an asterisk.

(C) Top panel: modeled structure of the tripartite complex of *D. discoideum* MidA (green) with NDUF2 (cyan) and ND1 indicated with gray spheres. The orientation of MidA is upside down, compared with that presented in (A) and (B), to align with the usual viewing orientation of CI. The minimal NDUF2 residues for MidA binding (residues 11–51) are indicated in orange. Bottom panel: modeled structure of the tripartite complex formed by *D. discoideum* NDUF2 (coloring is as in A), ND1 (gray spheres), and NDUF7 (dark green). The respective orientations of NDUF7, ND1, and NDUF2 were obtained from the experimental structures of mammalian CI. MidA has been docked *in silico* onto NDUF2.

(Figure S4A). *In silico* docking with a homology model of *D. discoideum* NDUF2 produced a high-ranked docking pose that placed NDUF2 Arg55 (corresponding to the human Arg85) in a position optimal for methylation. In this position, NDUF2 residues 11–51 contacted a conserved surface cavity on the additional MidA domain (Figures 3A and 3B). This cavity has typical ligand-binding features; namely, a hydrophobic core (Ile105, Val267, Val280, Phe296, Leu362, Ile373, and Leu374) surrounded by polar (Gly291, Gln298, Gln322, and Ser360) and charged (Arg269, Asp372, and Glu324) residues (Figure 3C). These observations suggested a role of the additional domain in ligand binding and selection, in agreement with our biophysical binding data. Therefore, we suggest naming it the “regulatory domain.”

This MidA:NDUF2 complex is incompatible with the association of NDUF7 with NDUF2 in sheep and human CI (Figure 3C), in agreement with a model that foresees that MidA/NDUF7 is replaced by NDUF7 after NDUF2 methylation. However, the docked MidA:NDUF2 complex is compatible with the association of NDUF2 with ND1 in CI (Figure 3C) (Fiedorczuk et al., 2016; Guo et al., 2017). The additional regulatory domain of MidA could stabilize this tripartite complex by interacting with ND1. Complexes containing NDUF7, NDUF2, and ND1 are, indeed, found in cells (Rhein et al., 2013; Sánchez-Caballero et al., 2016; Zurita Rendón et al., 2014), suggesting that MidA/NDUF7:NDUF2:ND1 complexes can be stable under certain conditions.

Even with NDUF2 bound, the opening between the catalytic and regulatory domains on the opposite side is sufficient to allow substitution of SAH by SAM without substrate dissociation. Thus, the same NDUF2 arginine could be dimethylated without intermittent dissociation of the monomethylated substrate. If the local SAM concentration drops markedly below

the SAM:MidA K_D of $\sim 5 \mu\text{M}$, then unmethylated or monomethylated MidA:NDUF2 complexes could persist. Consequently, the MidA:NDUF2 association, as well as their tripartite complex with ND1, might be stable under low-SAM conditions.

Biological Relevance of MidA Methylation

Previous *in vitro* biochemical analysis of the activities and levels of *Dictyostelium* oxidative phosphorylation (OXPHOS) complexes showed that MidA knockout in *Dictyostelium* causes a CI-specific defect (Carilla-Latorre et al., 2010). To investigate the effects of blocking SAM and SAH binding to MidA in living organisms, we used Seahorse respirometry on MidA knockout *D. discoideum* (Lay et al., 2016). We created transformants of the MidA knockout mutant that ectopically expressed wild-type (WT) MidA or point mutants, where we mutated residues on both sides of the SAH-binding cleft (single mutants: Glu200Ala, Asn205Ala, and Thr135Ala; double mutants: Gly170Val/Gly172Val and Gln257Ala/Phe259Ala; Figure 1B).

In Seahorse respirometry, the fraction of O_2 consumption attributable to CI (rotenone sensitive) in carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP)-uncoupled mitochondria was reduced markedly in the MidA null mutant, compared to the parental AX4 strain (Figure 4A). Ectopic expression of WT MidA in the null mutant background reversed a large part (but not all) of this reduction in respiratory activity. The point mutants fell into two groups (Figure 4A): (1) the class E mutants (the mutated residues are essential for MidA function), which could not rescue the CI defect in the null mutant (Glu200Ala, Gly170Val/Gly172Val, and Asn205Ala); and (2) the class N mutants (the mutated residues are not essential for function), which rescued the defect (Gln257Ala/Phe259Ala and Thr135Ala). The differences in CI activity among the CI-defective strains (class E and null mutant) and among “rescued” strain groups

Figure 4. Phenotypic Analysis of Parental and MidA Mutant Strains of *Dictyostelium*

For all measurements, means and SEMs (red bars) were calculated for multiple independent experiments with multiple strains. For all analyses, significance probabilities are for two-sample t-tests comparing the rescued strains (class N mutants and WT) with either the CI-deficient strains (class E mutants and null) or the parental control strain (AX4). Significance probabilities within each of the groups (red boxes in A) are for ANOVA among the strain groups in that box. All data are represented as mean $\pm$ SEM.

(A) Fraction of uncoupled respiration attributable to CI. O₂ consumption rates by CI were measured as the rotenone-sensitive fraction of CCCP-uncoupled respiration in living cells.

(B) Accuracy of phototaxis by multicellular slugs of the parental and MidA mutant strains of *Dictyostelium*. The error bars represent 90% confidence intervals.

(C–H) O₂ consumption rates (OCRs) attributable to various components of respiration in the parental and MidA mutant strains of *Dictyostelium*. (C) O₂ consumption by CI in uncoupled mitochondria. (D) O₂ consumption by complex II in uncoupled mitochondria. (E) Basal O₂ consumption rate. (F) O₂ consumption by ATP synthesis. (G) O₂ consumption by mitochondrial "proton leak." (H) "Nonmitochondrial" O₂ consumption.

(I and J) Growth rates by the parental and MidA mutant strains of *Dictyostelium*. Generation times during growth in liquid medium (I) were measured for the parental AX4 strain, the null mutant, and 22 strains expressing either the WT or one of the point mutant forms of MidA. (J) Plaque expansion rate. Error bars indicate 95% confidence intervals.

See also [Supplemental Information](#).

(class E) were statistically insignificant, whereas the difference between these two groups was highly significant.

Phototaxis of the multicellular “slug” stage of *Dictyostelium* is known to be impaired in the MidA null mutant; this defect is rescued completely by ectopic expression of the WT MidA protein (Carilla-Latorre et al., 2010). The phototaxis defect of the null mutant (compared to the parental strain AX4) persisted in strains expressing MidA class E mutants but was completely rescued by WT MidA and by the class N point mutants (Figure 4B).

Previously, we reported biochemical assays indicating that the mitochondrial respiratory defect in the MidA null mutant is specific to CI (Carilla-Latorre et al., 2010). The Seahorse respirometry verified this in direct measurements of the physiological activity of various components of respiration in intact cells (Figures 4C–4H). Measuring the absolute rate of O₂ consumption (picomoles per minute per 10⁵ cells) by different components of respiration confirmed that the defect in CI (Figure 4C) is specific and does not extend to complex II (Figure 4D), the ATP synthase (Figure 4F), or the “proton leak” (the use of respiratory energy to support mitochondrial functions other than ATP synthesis) (Figure 4G). We found no significant differences in ATP steady-state levels among the strains, consistent with the rate of synthesis of ATP being maintained homeostatically at similar rates in the CI-deficient and rescued cells (Figure S5).

By contrast, basal respiration (Figure 4E) and, in particular, its “non-mitochondrial” component (direct O₂ consumption by other cellular enzymes, such as oxygenases) were elevated in the CI-deficient mutants and only partially restored to WT levels in the “rescued” strains (Figure 4H). This confirms the presence of an additional non-mitochondrial defect in the “rescued” strains that was not attributable to the reduced CI activity. In fact, the absolute CI activities in the rescued strains were not significantly different from that of the original parental strain AX4 (Figure 4C), showing that the CI deficiency was restored to normal in the “rescued” strains. Its proportional contribution to total CCCP-uncoupled O₂ consumption remained below AX4 levels (Figure 4A), primarily because of the elevated contribution by non-mitochondrial O₂ consumption. For the same reason, the elevated rate of basal O₂ consumption in the CI-deficient strains was only partially restored to parental AX4 levels by the class N mutant or WT proteins (Figure 4E).

The *Dictyostelium* MidA null mutant was defective in growth, both on bacterial lawns and in liquid medium (Torija et al., 2006). We found that neither phenotype could be rescued by ectopic expression of the class E mutant proteins, while both phenotypes were rescued, at least partially, by the expression of WT or class N mutant proteins (Figures 4I and 4J).

Collectively, this *in vivo* analysis revealed that SAH and SAM binding of MidA—and, hence, its methyltransferase activity—was crucial for normal growth, phototaxis, and O₂ consumption attributable to CI.

DISCUSSION

(De)methylation of proteins is increasingly recognized as a way to transduce signals in eukaryotes, akin to protein phosphorylation. While methylation of histones has been well described, methylation of mitochondrial proteins is still poorly understood.

Herein, we describe the crystal structure of a PRMT from mitochondria. The structure of the *D. discoideum* MidA protein, a close homolog of the mammalian NDUFAF7, is unusual, because it combines a canonical PRMT catalytic fold with a domain not found in other eukaryotic protein families (herein named the “regulatory domain”). However, we found the catalytic and regulatory domains of MidA to be structurally preserved in functionally uncharacterized homologs from α and β proteobacteria. This structural similarity of catalytic and regulatory domains (including positioning of the SAH cofactor) implies that the eukaryotic *midA/NDUFAF7* was originally a mitochondrial gene that was later integrated into nuclear DNA and, hence, that proteobacteria use protein arginine methylation. Moreover, the conservation of the methylation site of NDUFS2 in proteobacterial NDUFS2 homologs, but not in the *T. thermophilis* structure, suggests that protein-methylation-controlled assembly of CI has originated in proteobacteria.

Our analysis supports that the substrate NDUFS2 is recognized through interactions with the regulatory domain of MidA. However, full-length NDUFS2 does not bind more strongly to MidA than a short peptide mimic of the region surrounding the substrate arginine, suggesting that the regulatory domain controls substrate selection, whereas the binding affinity is dominated by the interaction between the substrate arginine and the catalytic core domain. We observed that monomethylation lowered the affinity of substrate peptides 3-fold, while dimethylation lowered the affinity 10-fold, suggesting that methylation controls the binding affinity. Our structural analysis supports that the SAH-to-SAM exchange is possible while the substrate remains bound to MidA. Hence, NDUFS2 could stay bound to MidA until dimethylation weakens the interaction sufficiently to release the substrate. Consequently, the dissociation of MidA and NDUFS2 would be regulated by the local concentrations of the cofactors, which can vary considerably. Thus, our data propose a mechanism in which local SAM and SAH levels control CI assembly.

Our investigations also characterized the importance of the SAM binding site (and, hence, of the methyltransferase activity of MidA) for CI assembly *in vivo*, with marked repercussions on growth and phototaxis of *Dictyostelium*. Interestingly, not all MidA null mutant abnormalities were completely rescued by ectopic expression of the WT or class N mutants of the protein. While these “rescued” strains exhibited normal phototaxis and absolute CI activity, they still had growth defects and elevated “nonmitochondrial” O₂ consumption. One explanation is that the original null mutant may have contained an additional undetected genetic change that is unconnected to MidA function. While this is possible, the probability that a random, spontaneous mutation affects any given phenotype is very small—even for multigenic phenotypes—and, in this case, because it involves impaired growth, would be selected against during strain isolation and growth in culture (Wilczynska et al., 1997). The simplest explanation is that the WT and mutant forms of MidA are expressed at levels that are higher than normal in the rescue transformants, because the vectors used have strong promoters and are integrated into the genome in multiple copies (Barth et al., 1998). The overexpression of functional MidA could exalt off-target methylations or other interactions and thus produce additional, abnormal phenotypes.

Protein arginine methylation can alter protein structure and function and, hence, has to be strictly regulated. In this regard, the self-association observed in all three independent MidA Δ 75 molecules in our crystal lattice might represent a mechanism for autoinhibition. Although MidA and NDUFAF7 dimers had been reported (Carilla-Latorre et al., 2010), our small angle X-ray scattering (SAXS) experiments with MidA Δ 75 did not show stable multimeric associations *in vitro*. Future work is, therefore, needed to assess whether (autoinhibitory) MidA and NDUFAF7 multimers can be stabilized *in vivo* through their flexible N-terminal residues or other interactions.

EXPERIMENTAL PROCEDURES

Protein Cloning, Expression, and Purification

Glutathione S-transferase (GST)-fused MidA Δ 75 (residues 76–484) was cloned into the pGEX 6P1 vector and expressed and purified from BL21 (DE3) cells (Rosano and Ceccarelli, 2014).

Crystallization and Structure Determination

Crystals of MidA Δ 75 alone and mixed with SAM or SAH at a 1:10 molar ratio were grown with the hanging drop vapor diffusion method. The structures were solved, and initial models were built using Phenix from X-ray data.

ITC

MidA Δ 75 was extensively dialyzed into degassed HEPES buffer and placed into the measurement cell. Ligands were dissolved into the last dialyzate and placed into the syringe. Titrations were carried out using a MicroCal VP-ITC calorimeter (Malvern) and an MicroCal ITC200 (Malvern) at 25°C.

Mass Spectrometric Analysis

The molecular weight difference for mono and di-methylation (+14.01565 and +28.0313 Da) on arginine was considered as a variable modification. All the peptides below 3,000 Da were analyzed, and changes were observed manually using mass spectrometry.

Microscale Thermophoresis

MidA Δ 75 was N-terminally labeled with RED dye (NT-647). Serial dilutions of unlabeled ligands were mixed with 20 nM labeled MidA and loaded into standard Monolith NT capillaries for measurements in a NanoTemper Monolith NT.015T.

SAXS Analysis

On the SWING beamline, proteins were either injected directly into the measurement quartz capillary or injected at 10 mg/mL through a high-performance liquid chromatography (HPLC)-piloted size exclusion column. Individual SAXS frames of 1.5 s with a 1-s gap time were collected. Data were scaled, merged, and analyzed using ATSAS software.

Dictyostelium Strains and Culture Conditions

All the strains used in this study were derived from the MidA null mutant (originally isolated from the parental WT strain AX4). The cultures were purified and stored as frozen stocks.

Phenotypic Analysis

Phototaxis

The phototaxis assay was performed using the methods developed by Fisher et al. (1981) and by Fisher and Annesley (2006).

Plaque Expansion Rates

D. discoideum strains were inoculated in the center of a normal agar plate containing a lawn of *E. coli* B2 with 4 replicates for each strain. The plates were incubated at 21°C, and the diameter of the plaque was measured twice a day at approximate 8-hr intervals for 100 hr.

Growth in HL-5 Broth

The axenic growth of *D. discoideum* strains was conducted, and the growth rate was measured as previously described (Bokko et al., 2007).

Seahorse Respirometry

The method was described in detail Lay et al. (2016).

Bioinformatic Analysis

SWISS-MODEL was used for homology modeling (Arnold et al., 2006). ClusPro2.0 was used for protein docking (Kozakov et al., 2017). Structural homologs were identified using DALI (Holm and Rosenström, 2010), and the ConSurf server (Landau et al., 2005) was used for projecting sequence conservation onto the 3D structure.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for crystallographic data and models for MidA reported in this paper are PDB: 5TZT, 5ZZW, and 5ZUO.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.07.075>.

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AUTHOR CONTRIBUTIONS

S.T.A. and K.S. conceived the idea for the study. U.F.S.H. performed protein crystallization. U.F.S.H. and S.T.A. performed crystallographic data recording, analysis, and structure solution. S.T.A. performed SAXS data analysis. U.F.S.H. performed biophysical experiments. U.F.S.H. and S.T.A. performed data analysis. O.S., S.T.L., S.J.A., and P.R.F. performed *in vivo* analysis. C.J. helped in protein studies. S.T.A., U.F.S.H., P.R.F., and K.S. performed manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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