Long-range intramolecular allostery and regulation in the dynein-like AAA protein Mdn1

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Mdn1 is an essential mechanoenzyme that uses the energy from ATP hydrolysis to physically reshape and remodel, and thus mature, the 60S subunit of the ribosome. This massive (>500 kDa) protein has an N-terminal AAA (ATPase associated with diverse cellular activities) ring, which, like dynein, has six ATPase sites. The AAA ring is followed by large (>2,000 aa) linking domains that include an ∼500-aa disordered (D/E-rich) region, and a C-terminal substrate-binding MIDAS domain. Recent models suggest that intramolecular docking of the MIDAS domain onto the AAA ring is required for Mdn1 to transmit force to its ribosomal substrates, but it is not currently understood what role the linking domains play, or why tethering the MIDAS domain to the AAA ring is required for protein function. Here, we use chemical probes, single-particle electron microscopy, and native mass spectrometry to study the AAA and MIDAS domains separately or in combination. We find that Mdn1 lacking the D/E-rich and MIDAS domains retains ATP and chemical probe binding activities. Free MIDAS domain can bind to the AAA ring of this construct in a stereo-specific bimolecular interaction, and, interestingly, this binding reduces ATPase activity. Whereas intramolecular MIDAS docking appears to require a treatment with a chemical inhibitor or preribosome binding, bimolecular MIDAS docking does not. Hence, tethering the MIDAS domain to the AAA ring serves to prevent, rather than promote, MIDAS docking in the absence of inducing signals.

midasin | dynein | AAA ATPase | ribosome biogenesis

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roteins in the AAA superfamily, which includes ~100 members in humans, are responsible for a diversity of mechanochemical activities in the cell, ranging from protein quality control to intracellular transport (1–5). Many AAA members self-assemble into homohexameric rings and use the energy from ATP hydrolysis to thread protein substrates through their central pores (1, 6). Two notable exceptions are the microtubule-based motor dynein and the ribosome biogenesis factor Mdn1 (also known as midasin, or Rea1 in Saccharomyces cerevisiae), each of which have six AAA domains, together with additional domains, expressed tandemly on the 60S precursor particles. It is not known how this unusually large (>500 kDa) mechanoenzyme coordinates the activities of its C-terminal MIDAS domain, which binds specific preribosomal proteins, with the ATPase cycle in its N-terminal AAA ring. Here, we show that the linking domains, which include a >500-amino acid disordered region, regulate an intramolecular binding interaction between the AAA and MIDAS domains. Surprisingly, the linking domains down-regulate MIDAS-AAA binding despite tethering these two domains together. This study provides insights into the regulation of Mdn1 function and into the design principles of large, multidomain mechanoenzymes.

Significance

The dynein-like AAA motor Mdn1 drives ribosome biogenesis by using ATP-fueled motions to dislodge assembly factor proteins from 60S precursor particles. It is not known how this unusually large (>500 kDa) mechanoenzyme coordinates the activities of its C-terminal MIDAS domain, which binds specific preribosomal proteins, with the ATPase cycle in its N-terminal AAA ring. Here, we show that the linking domains, which include a >500-amino acid disordered region, regulate an intramolecular binding interaction between the AAA and MIDAS domains. Surprisingly, the linking domains down-regulate MIDAS-AAA binding despite tethering these two domains together. This study provides insights into the regulation of Mdn1 function and into the design principles of large, multidomain mechanoenzymes.

Author contributions: K.J.M. and T.M.K. conceived and designed the project; K.J.M. and S.E.W. made plasmids and purified proteins; K.J.M. ran biochemical assays and analyses; P.D.B.O. performed native mass spectrometry experiments with supervision by B.T.C.; K.J.M. and Y.N. performed photo-crosslinking experiments and analyses; Y.S. synthesized Rbin-XL; and K.J.M. and T.M.K. wrote the manuscript with contributions from all other authors.

The authors declare no competing interest.

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Data deposition: Maps were deposited to Electron Microscopy Data Bank (https://www. ebi.ac.uk/pdbe/emdb/) in entries EMD-21911 and EMD-21912.

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inducing MIDAS docking had contrasting effects on ATPase activity: Deleting the helix 2 insert of AAA2 increased the ATPase rate of Mdn1 ∼10-fold, whereas addition of Rbin-1 inhibited the ATPase activity (9, 10). The interplay between MIDAS binding and ATPase activity in the AAA ring thus remains an open question.

The MIDAS-docked conformation induced by Rbin-1 structurally mimics the preribosome-bound form of Mdn1 (9). Mdn1 inhibited the ATPase activity (9, 10). The interplay between the AAA ring could be reconstituted as a two-protein interaction, asking if intramolecular docking of the MIDAS domain onto the Free MIDAS Protein Binds to Truncated Mdn1 Lacking a MIDAS Domain.

Methods

We predicted that tethering the MIDAS domains are little more than a passive tether, making it difficult to explain why they have remained relatively well-conserved (7)—and why the AAA ring and MIDAS domain have remained on the same polypeptide—throughout eukaryotic evolution. It may be expected that tethering the MIDAS domain serves to favor docking by increasing local concentration, but this hypothesis has not been tested.

In the current work, we investigate the role of the linking domains in the MIDAS docking cycle and the overall mechanism of Mdn1. Using recombinant proteins, we reconstitute MIDAS docking as a bimolecular interaction between separately expressed AAA and MIDAS domains. Whereas for full-length Mdn1 MIDAS docking (unimolecular) must be induced by the addition of Rbin-1, untethered (bimolecular) MIDAS docking is Rbin-1-independent with submicromolar affinity. Hence, quite the opposite of the expected effect of increasing local concentration, tethering the MIDAS domain negatively regulates docking. The separately expressed AAA ring can bind ATP and Rbin-1 similarly to the full-length protein, but Rbin-1 binding does not inhibit ATPase activity. Interestingly, inhibition can instead be induced by the addition of free MIDAS. Altogether, our results suggest that the linking domains are a regulatory element that prevents MIDAS docking in the absence of inducing signals, such as Rbin-1 treatment or preribosome binding, and that MIDAS docking negatively regulates ATPase activity in the AAA ring.

Results

Free MIDAS Protein Binds to Truncated Mdn1 Lacking a MIDAS Domain.

To investigate the role of the linking domains in Mdn1, we first asked if intramolecular docking of the MIDAS domain onto the AAA ring could be reconstituted as a two-protein interaction (Fig. 1L). We thus designed a streptavidin-Dynabead pull-down assay using S. pombe Mdn1 lacking the D/E-rich and MIDAS regions (hereafter Mdn1-ΔC, amino acids 1 to 3,911) as “prey” and MIDAS domain with a N-terminal biotinylated SNAP tag as “bait” (Fig. 1B). Both full-length Mdn1 (Mdn1-FL) and Mdn1-ΔC were expressed in insect cells with an N-terminal His-tag and purified using affinity, ion-exchange, and size-exclusion chromatography (Methods). Typical preparations yielded ∼0.05 mg of these very large proteins (∼540 and 450 kDa for Mdn1-FL and Mdn1-ΔC, respectively) per liter of insect cells. Purity and relative size of the proteins were assessed by SDS/PAGE and by size-exclusion chromatography, where each protein eluted as a single peak (SI Appendix, Fig. S1 A–C).

The MIDAS domain was expressed in bacteria as an N-terminal SNAP-tag fusion (hereafter WT-MIDAS), which we found aided in expression and ease of purification. The construct also included an N-terminal His-tag, which was removed by protease treatment during the purification protocol (affinity, ion-exchange, and size-exclusion chromatography; Methods). In addition to WT-MIDAS, we also made a SNAP-tagged construct lacking the conserved “MIDAS loop” (hereafter ΔLoop-MIDAS), which has been shown to be required for docking onto the Mdn1 AAA ring (Fig. 1B) (12). Typical yields were in excess of 2 mg of purified protein per liter of cultured bacteria. Purity of the MIDAS constructs were assessed by SDS/PAGE and size-exclusion chromatography (SI Appendix, Fig. S1 D and E). MIDAS constructs were biotinylated via the SNAP-tag (Methods) prior to size-exclusion chromatography (SI Appendix, Fig. S1F).

We ran the pull-down assays in the presence of the small-molecule inhibitor Rbin-1 (1 μM), as this compound is required to induce MIDAS docking in Mdn1-FL (9). We found that WT-MIDAS (1 μM) at a 15-fold molar excess pulled down 67 ± 7% (mean ± SD) of Mdn1-ΔC (65 nM input). The empty beads and ΔLoop-MIDAS (1 μM) each pulled down 10% or less (Fig. 1C and D), indicating no significant specific binding. Together, these data indicate that the MIDAS docking interaction can be reconstituted as a two-protein interaction, hereafter referred to as untethered docking.

Untethered MIDAS Docking Structurally Matches Tethered MIDAS Docking. We next asked if there was a stereotyped position on the Mdn1-ΔC AAA ring where free MIDAS would dock. To address this question, we performed negative-stain EM imaging of Mdn1-ΔC together with WT-MIDAS at a 1:10 molar ratio (25 and 250 nM, respectively) in the presence of Rbin-1 (1 μM). Raw images revealed monodisperse molecules of Mdn1-ΔC with a characteristic laddie-like appearance, as well as multiple small particles corresponding to the smaller MIDAS proteins (SI Appendix, Fig. S2 A and B).

We collected ∼13,000 particles and subjected them to 2D and 3D classifications followed by supervised 3D classification using as references the resolution-filtered cryo-EM structures of Mdn1-FL in AMP-PNP and ATP + Rbin-1 (PDB ID codes 6OR5 and 6ORB, respectively) (9). This method yielded two density maps, each at ∼25-Å resolution (Fig. 1 E–G and SI Appendix, Fig. S2C). Map 1 contained most of the particles (∼63%), and allowed for identification of the linker domain, AAA ring, and central pore. The top surface of the AAA ring, viewed with the linker facing upward, appeared relatively flat (Fig. 1E). Map 2, which corresponded to the remaining particles (∼37%), had additional density on the top surface of the AAA ring (Fig. 1F). Map 1 was aligned with map 2, and a difference map was generated. This analysis revealed that the additional density of map 2 appeared on the top surface of the AAA ring opposite the linker (Fig. 1G). No major changes in the linker domain were observed within the allowance of ∼25-Å resolution.

To check if the extra density corresponded to the MIDAS domain, we docked the previously determined models of Mdn1-ΔC together with WT-MIDAS at a 1:10 molar ratio (25 and 250 nM, respectively) in the presence of Rbin-1 (1 μM). Map 1 was aligned with the linker domain, and central pore. The top surface of the AAA ring, viewed with the linker facing upward, appeared relatively flat (Fig. 1E). Map 2, which corresponded to the remaining particles (∼37%), had additional density on the top surface of the AAA ring (Fig. 1F). Map 1 was aligned with map 2, and a difference map was generated. This analysis revealed that the additional density of map 2 appeared on the top surface of the AAA ring opposite the linker (Fig. 1G). No major changes in the linker domain were observed within the allowance of ∼25-Å resolution.

To check if the extra density corresponded to the MIDAS domain, we docked the previously determined models of Mdn1-FL in the presence of AMP-PNP where the MIDAS domain was not seen to dock onto the Mdn1 ring and in presence of ATP + Rbin-1 (where the MIDAS domain was seen to dock onto the Mdn1 ring) (9) into Map 2 (Fig. 1H; additional views in SI Appendix, Fig. S2D). We found that the extra density was not accounted for by the AMP-PNP model, but was accounted for by the MIDAS domain (shown in red) in the ATP + Rbin-1 model. Hence, untethered docking occurs with 1:1 stoichiometry and at the position in Mdn1-FL expected from structural models for tethered docking. This result demonstrates that stereo-specific MIDAS docking does not require a tethered connection between the AAA and MIDAS domains.
Untethered MIDAS Docking Is Rbin-1–Independent. For wild-type Mdn1-FL in solution, significant docking of the MIDAS domain has not been seen unless Rbin-1 is present (9). Furthermore, tethering two domains should increase the effective concentration to promote binding. We therefore reasoned that the untethered docking reaction between Mdn1-ΔC and WT-MIDAS should be a low-affinity, Rbin-1–dependent interaction (Fig. 2A). To examine this, we first ran a pull-down assay in the absence of Rbin-1. We expected to see very little Mdn1-ΔC get pulled down. Surprisingly, we saw the opposite. Running the pull-down with just a 15-fold excess of WT-MIDAS bait to Mdn1-ΔC prey (1 μM to 65 nM), we were able to pull down ∼65% (Fig. 2B), similar to the amount pulled down in the presence of Rbin-1 (Fig. 1D).

To investigate the Rbin-1–dependence of untethered docking in more detail, we reran the pull-down assay at the same protein concentrations, but varied the Rbin-1 concentration. We found that the pelleted fraction was independent of the Rbin-1 concentration, with approximately equal fractions pulled down over a wide range (0 to 5 μM) of Rbin-1 concentrations (Fig. 2C and D and SI Appendix, Fig. S2E). Hence, in these reaction conditions, untethered docking is not sensitive to Rbin-1.

To estimate the strength of the untethered docking interaction, we next ran a titration pull-down using various concentrations of MIDAS-WT bait (0–1 μM to 65 nM Mdn1-ΔC prey) in the absence of Rbin-1. We observed an increase in the fraction of Mdn1-ΔC bound with increasing concentration of WT-MIDAS, although we were not able to achieve a complete (100%)
pull-down (Fig. 2 E and F and SI Appendix, Fig. S2F). We posit this incomplete pull-down is due to the large size or nucleotide state of Mdn1-ΔC. Nonetheless, we were able to analyze the dissociation constant by fitting a hyperbola to the fraction of Mdn1-ΔC depleted from the supernatant. We estimated a tight, submicromolar dissociation constant (K_D = 0.72 ± 0.58 μM, fit ± 95% CI). Hence, untethered docking is a surprisingly high-affinity bimolecular interaction and, contrasting the case of tethered docking, does not require Rbin-1. Together, the data in Fig. 2 do not support a model in which the linking domains serve to prevent MIDAS docking in the absence of Rbin-1.

Mdn1-FL and Mdn1-ΔC Have Similar ATPase Activities. Why is untethered docking Rbin-1–dependent while tethered docking is Rbin-1–independent? To address this question, we next sought to characterize the biochemical properties of Mdn1-ΔC relative to Mdn1-FL. We first ran steady-state ATPase assays on Mdn1-FL and Mdn1-ΔC as a function of ATP concentration to test for cooperativity between the six AAA domains and to assess affinities and turnover rates. We found that the ATPase rates for Mdn1-FL could be fit by a Michaelis-Menten model, with a k_cat of 2.4 ± 0.2 s^-1 and a K_M of 0.25 ± 0.05 μM (fit ± 95% CI; Fig. 3E and SI Appendix, Fig. S3). These values are close to those of other AAA proteins such as spastin (19). Expanding the fit to a Hill model returned a Hill coefficient within error of unity, indicating a lack of cooperativity in ATP binding and hydrolysis (SI Appendix, Fig. S3). These results contrast with other AAA proteins such as ClpXP (20, 21), which have Hill coefficients larger than unity. The ATPase activity of Mdn1-ΔC was similar to that of Mdn1-FL under our experimental conditions, with a k_cat of 2.6 ± 0.2 s^-1 and K_M of 0.53 ± 0.08 μM (Fig. 3F). Hence, the ATP hydrolysis properties of the Mdn1 AAA ring do not appear to be impacted by truncating off the MIDAS domain.

Mdn1-ΔC Can Bind ADP at All Sites and Exchange AMP-PNP at Multiple Sites. We next investigated the nucleotide binding in Mdn1-ΔC using native mass spectrometry (MS). We first characterized Mdn1-ΔC prepared in nucleotide-free buffer prior to native MS analysis (Methods). The mass spectrum revealed three distinct peaks corresponding to masses larger than the expected molecular weight of apo Mdn1-ΔC, and each subsequent peak ∼450 Da larger (Fig. 3G). The major peak had five Mg-ADP bound, but a fractional population had six Mg-ADP bound. Since Mg-ADP has a molecular weight of 451.5 Da, we assigned three peaks to be Mdn1-ΔC with five Mg-ADP bound, consistent with the six ATP-binding sites in Mdn1 (Fig. 3C). Further supporting this interpretation, removing the N-terminal His-tag of Mdn1-ΔC with a protease caused an equal and predictable shift in mass corresponding to the protease being cleaved from the highest mass peak to the next (Fig. 3H). Hence, the high molecular weight of apo Mdn1-ΔC (molecular weight of apo Mdn1-ΔC was estimated as 184,621 ± 1,132 Da, and each subsequent peak ∼450 Da larger (Fig. 3B). Since Mg-ADP has a molecular weight of 451.5 Da, we assigned three peaks to be Mdn1-ΔC with five Mg-ADP bound, consistent with the six ATP-binding sites in Mdn1 (Fig. 3C). Further supporting this interpretation, removing the N-terminal His-tag of Mdn1-ΔC with a protease caused an equal and predictable shift in mass for all three peaks (SI Appendix, Fig. S4).

The majority of the Mdn1-ΔC protein had five Mg-ADP bound, but a fractional population had six Mg-ADP bound. We hence wondered if we could shift weight from the four- and five-Mg-ADP–bound peaks into the six-Mg-ADP–bound peak by active pulling (Fig. 2 E and F and SI Appendix, Fig. S2F). We posit this incomplete pull-down is due to the large size or nucleotide state of Mdn1-ΔC. Nonetheless, we were able to analyze the dissociation constant by fitting a hyperbola to the fraction of Mdn1-ΔC depleted from the supernatant. We estimated a tight, submicromolar dissociation constant (K_D = 0.72 ± 0.58 μM, fit ± 95% CI). Hence, untethered docking is a surprisingly high-affinity bimolecular interaction and, contrasting the case of tethered docking, does not require Rbin-1. Together, the data in Fig. 2 do not support a model in which the linking domains serve to prevent MIDAS docking in the absence of Rbin-1.

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![Diagram](image-url)
adding Mg-ADP to the MS analysis buffer. Indeed, we saw that addition of low micromolar amounts of Mg-ADP substantially shifted the amounts of Mdn1-ΔC to higher masses with up to six bound Mg-ADP (Fig. 3D). At the highest Mg-ADP condition (5 μM), the majority of Mdn1-ΔC had six Mg-ADP bound. Hence, it is possible to fill all six nucleotide pockets in Mdn1-ΔC with ADP.

We next prepared Mdn1-ΔC with 1 mM Mg-AMP-PNP prior to exchange into MS analysis buffer. In this sample, we saw only two peaks, each shifted toward slightly heavier mass than the nucleotide-free sample (Fig. 3E). The difference in mass between these two peaks again corresponded to the molecular weight of Mg-ADP. The mass of the lower peak could correspond to two AMP-PNP plus two ADP or three AMP-PNP plus one ADP, depending on the number of magnesium ions bound (SI Appendix, Table S1). Hence, the major peak likely corresponds to Mdn1-ΔC with five nucleotides bound. While attaining mass spectra for Mdn1-FL proved difficult, likely due to the disorderly and highly charged D/E-rich region, we note that we previously observed five nucleotides bound to Mdn1-FL in the cryo-EM structure determined in the presence of AMP-PNP (9). Based on these native mass spectrometry data for Mdn1-ΔC and EM data for Mdn1-FL, we speculate that these constructs have similar nucleotide-binding characteristics. Only three or fewer subunits in Mdn1-ΔC exchanged from ADP into AMP-PNP under our experimental conditions. As a point of comparison, cytoplasmic dynein exchanges AMP-PNP at three, or possibly all four, of its nucleotide-binding–competent AAA modules (22, 23). Together, these data indicate that truncating off the D/E-rich and MIDAS domain does not substantially alter the biochemical activity of Mdn1, and that Rbin-1–independent docking of free WT-MIDAS on Mdn1-ΔC is therefore not likely due to differences between Mdn1-FL and Mdn1-ΔC nucleotide binding or hydrolysis. We note that additional experiments are going to be needed to more firmly establish this.

**Mdn1-ΔC Can Bind Rbin-XL.** Treatment of Mdn1-FL with the small molecule inhibitor Rbin-1 structurally mimics the pre-60S ribosomal subunit-bound conformation (9). While Rbin-1 treatment is required for tethered docking, it is not required for untethered docking. We hence asked if Rbin-1 can bind to or inhibit Mdn1-ΔC. To test for inhibitor binding, we used the available structure–activity relationship data (11) to design a Rbin-1 analog that incorporates both a diazirine group for photo–cross-linking and an alkyne group for click chemistry (Fig. 4A). We synthesized this analog (SI Appendix, Supplemental Text), which we named Rbin-XL, and found that it inhibited Mdn1-FL in an ATPase assay (EC₅₀ of 0.23 ± 0.33 μM, fit ± 95% CI), validating its use (Fig. 4B and SI Appendix, Fig. S5A). We reasoned that, if the Rbin-1–independence of untethered MIDAS docking stems
from differences in the Mdn1-ΔC AAA ring, Rbin-XL binding might be abrogated.

To test for Rbin-XL binding, we performed a competition binding assay employing Rbin-XL and Rbin-1 (Fig. 4C). Mdn1 constructs were mixed with either Rbin-XL alone (2 μM) or with Rbin-XL and a 25-fold molar excess of Rbin-1 and incubated on ice for 30 min. We then initiated Rbin-XL cross-linking by exposure to UV light, followed by click-chemistry-mediated attachment of rhodamine to the Rbin-XL alkyn. Finally, we ran SDS/PAGE and measured the ratio of rhodamine signal to Coomassie signal. A decrease in this ratio in the Rbin-1-added lane relative to the Rbin-XL-alone lane indicates binding of Rbin-XL prior to the photo-cross-linking process that can be competed off by Rbin-1. For Mdn1-FL, we indeed saw a robust decrease in signal in the Rbin-1-added lane relative to the Rbin-XL-alone lane (Fig. 4D and E and SI Appendix, Fig. S5B). We saw a similar signal decrease for Mdn1-ΔC, providing evidence for specific binding of Rbin compounds. We also note cross-linking to BSA, visible as rhodamine signal. As incubation with Rbin-1, prior to UV exposure, does not suppress the signal, we interpret this as nonspecific cross-linking of Rbin-XL with BSA (blocking agent). Nonspecific binding can also account for the partial suppression of signal in the Mdn1-ΔC ATPase activity shown no response to Rbin-1, even at very high (100 μM) concentrations. Similar to Rbin-1, Rbin-XL did not inhibit Mdn1-ΔC in our assay conditions (SI Appendix, Fig. S5A). Hence, Rbin compounds can bind to Mdn1-ΔC without inhibiting it, indicating that the C-terminal domains are required for coupling compound binding to functional inhibition.

The decoupling of Rbin-1 binding and inhibition in Mdn1-ΔC suggests that the chemical inhibitor does not directly interfere with ATP binding, but rather works through an allosteric mechanism that requires the MIDAS domain. To test for an allosteric mechanism, we performed ATPase competition assays with Rbin-1 using Mdn1-FL. We found that the addition of 1 μM Rbin-1 reduces the $k_{cat}$ but not the $K_{M}$ (2.1 ± 0.2 s$^{-1}$ vs. 1.1 ± 0.2 s$^{-1}$ and 0.20 ± 0.04 μM vs. 0.24 ± 0.09 μM for DMSO control and 1 μM Rbin-1 conditions, respectively) of ATP-dependent Mdn1-FL ATPase activity (Fig. 5B and C). Hence, Rbin-1 inhibition of Mdn1-FL is noncompetitive, consistent with an allosteric mechanism. Together with the data in Fig. 4, these data suggest that the chemical inhibitor does not directly interfere with ATP binding.
Results show that the MIDAS domain is required for inhibition by Rbin-1. While Rbin-1 did not inhibit Mdn1-ΔC alone, it did lead to further inhibition when Mdn1-ΔC was mixed with WT-MIDAS (Fig. 5D). No decrease was seen when WT-MIDAS was denatured before use or when ΔLoop-MIDAS was used. These results show that the MIDAS domain is required for inhibition by Rbin-1.

We next measured the Mdn1-ΔC ATPase rate as we titrated the WT-MIDAS concentration with no Rbin-1 present. We observed dose–response inhibitory behavior that plateaued at high WT-MIDAS concentrations (Fig. 5E). The EC50 measured here (0.09 ± 0.13 μM) serves as an upper-limit estimate of the K1 for the MIDAS docking interaction: the exact mechanism of inhibition for MIDAS-driven ATPase inhibition is unknown, but for common models (i.e., noncompetitive, mixed inhibition, etc.), K1 ≤ EC50 (24). This estimate supports the submicromolar dissociation constant estimated from pull-down assays (Fig. 2F). The ATPase plateauing at a nonzero value (0.65 ± 0.21 s⁻¹ and 0.32 ± 0.26 s⁻¹ for DMSO and Rbin-1, respectively) but reduced the lower plateau value (0.65 ± 0.21 s⁻¹ and 0.32 ± 0.26 s⁻¹ for DMSO and Rbin-1, respectively).

suggest that Mdn1-ΔC can bind, but cannot be inhibited by, Rbin-1.

**Binding of Free MIDAS Protein Reduces the ATPase Rate of Mdn1-ΔC.** Given that chemical inhibitor binding is insufficient for ATPase inhibition of Mdn1-ΔC (Figs. 4 and 5A–C), we next asked if MIDAS docking, in the absence of Rbin-1, could lead to inhibition. We thus ran steady-state ATPase assays with Mdn1-ΔC and free WT-MIDAS. We found that the addition of WT-MIDAS to Mdn1-ΔC (250 nM to 25 nM, matching the stoichiometry used in the negative-stain EM experiments; Fig. 1E–H) reduced the ATPase rate nearly twofold (Fig. 5D). Addition of heat-denatured WT-MIDAS or ΔLoop-MIDAS (similarly at 250 nM) did not significantly decrease the ATPase rate of Mdn1-ΔC. Hence, the ability of a given MIDAS construct to influence the ATPase rate of Mdn1-ΔC correlated with its ability to bind to it (Figs. 1D and 5D).

We next ran the same experiment in the presence of Rbin-1. While Rbin-1 did not inhibit Mdn1-ΔC alone, it did lead to further inhibition when Mdn1-ΔC was mixed with WT-MIDAS (Fig. 5D). No decrease was seen when WT-MIDAS was denatured before use or when ΔLoop-MIDAS was used. These results show that the MIDAS domain is required for inhibition by Rbin-1.

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We next measured the Mdn1-ΔC ATPase rate as we titrated the WT-MIDAS concentration with no Rbin-1 present. We observed dose–response inhibitory behavior that plateaued at high WT-MIDAS concentrations (Fig. 5E). The EC50 measured here (0.09 ± 0.13 μM) serves as an upper-limit estimate of the K1 for the MIDAS docking interaction: the exact mechanism of inhibition for MIDAS-driven ATPase inhibition is unknown, but for common models (i.e., noncompetitive, mixed inhibition, etc.), K1 ≤ EC50 (24). This estimate supports the submicromolar dissociation constant estimated from pull-down assays (Fig. 2F). The ATPase plateauing at a nonzero value (0.65 ± 0.21 s⁻¹ and 0.32 ± 0.26 s⁻¹ for DMSO and Rbin-1, respectively) but reduced the lower plateau value (0.65 ± 0.21 s⁻¹ and 0.32 ± 0.26 s⁻¹ for DMSO and Rbin-1, respectively).
Discussion

In this study, we assay the roles of the linking domains in the AAA protein Mdn1, a critical component of the ribosome biogenesis machinery. We find that the linking domains serve to prevent MIDAS docking onto the AAA ring in the absence of Rbin-1 treatment or preribosome binding. Moreover, MIDAS docking negatively regulates ATPase activity in the AAA ring. These results provide insights into the design principles of Mdn1 and of large, multidomain AAA proteins in general.

How might the Mdn1 linker and D/E-rich region serve to regulate the docking of the MIDAS domain onto the AAA ring? One possibility is that the lengths of these domains are finely tuned such that the MIDAS domain can only reach the AAA ring when the protein is in a specific conformation or state of flexibility. Both the linker and D/E-rich domains are large, and tend to scale in size with one another in different organisms (7, 9). For S. pombe, the D/E-rich region is 517 amino acids long, and the distance from the end of the linker to the docking position is ~24 nm (9). To gain insight into whether the lengths and mechanics of the linking regions may play a role in limiting tethered MIDAS docking, we ran a simple Brownian dynamics simulation. We modeled the D/E-rich region as a worm-like chain and used a Monte Carlo approach to simulate tethered diffusion of the MIDAS domain (Fig. 6A). We find that extending the D/E-rich region such that the MIDAS dock would introduce ~1.3 pN of intramolecular tension (Fig. 6A and B). Furthermore, we find that the MIDAS domain only spends ~0.02% of its time within 2 nm of the docking site (Fig. 6C). Although this model is very simple, as it ignores volume exclusions and attractive potentials, it raises the possibility that the relative sizes of the linker domain and D/E-rich tethers may underlie the inefficiencies of tethered MIDAS docking. It furthermore emphasizes how changes to the structure or overall flexibility of the linker domain, particularly at the hinge point (a point of proposed flexibility in the linker; ref. 9; Fig. 6C), may substantially alter MIDAS docking dynamics. A second possibility, which we do not favor, is that the negatively charged (pI = 3.7) D/E-rich region physically sequesters the MIDAS domain while in specific conformations. Further functional and structural studies of Mdn1 will be needed to test these and other potential models.

Several multimeric AAA proteins such as Yme1 (25), katanin (26, 27), and numerous others (28) are thought to hydrolyze ATP in a sequential rotary fashion. Mdn1 is unlikely to function in such a way because (i) only four of the six AAA modules in Mdn1 can hydrolyze ATP (11); (ii) Rbin-1 can bind to, but cannot inhibit, Mdn1-ΔC (Figs. 4 and 5A); and (iii) inhibition by Rbin-1 or MIDAS is partial (Fig. 5A and E). Mdn1 further differs from canonical AAA proteins in that it does not show positive cooperativity in its ATPase activity (Fig. 3A), and in that its binding partner (the MIDAS domain) inhibits, rather than activates, ATPase activity (5). Mdn1 may instead divide labor among its AAA domains, similar to dynein (8, 22, 29–31).

Our results also provide insights into the mechanisms by which Rbin compounds inhibit Mdn1. Partial inhibition of Mdn1 ATPase has been seen previously for Rbin compounds (~40%) (11). Even Mdn1 with a Walker B mutation in AAAs, which has an ATPase rate nearly 10-fold lower than wild-type, is partially inhibited (~50%) by Rbin-1 (9). Rbin-1 treatment also induces tethered MIDAS docking in Mdn1-FL (9). Here we find that both of these effects occur independent of Rbin-1 when separately expressed WT-MIDAS protein is mixed with Mdn1-ΔC (Figs. 2 and 5D and E). We also find by Rbin-XL binding experiments (Fig. 4) and by ATPase assays with both WT-MIDAS and Rbin-1 present (Fig. 5D and E) that Rbin-1 can bind to Mdn1-ΔC. However, without MIDAS present, Rbin-1 cannot inhibit Mdn1-ΔC (Fig. 5A). Taken altogether, these results argue against the possibility that Rbin-1 works exclusively on the AAA ring. Instead, we propose that Rbin-1 works by relieving the regulatory effects of the linking domains. Rbin-1 may enable tethered MIDAS docking, which in turn inhibits Mdn1-FL ATPase. In support of this allosteric mechanism, we find that Rbin-1 is a noncompetitive inhibitor of Mdn1-FL (Fig. 5B and C). The two-step inhibition of Mdn1-ΔC by MIDAS and Rbin-1 may indicate a mixed mechanism for the separated proteins, or potentially separate effects on two or more of the active ATPase sites.

Mdn1 may not follow the canonical AAA mechanism of threading substrates through the central pore of its hexameric AAA ring (2). Here, we extend existing models (9, 10, 12, 15) to suggest that Mdn1 instead uses its MIDAS domain to (i) simultaneously bind to both an assembly factor and its own AAA ring, (ii) transmit information about substrate binding (mimicked by Rbin-1) to the AAA ring (Figs. 4 and 5), and (iii) transmit force produced in the AAA ring to the assembly factor. This MIDAS-mediated mechanism may be a more general alternative strategy utilized by a subclass of AAA proteins. One example is the MoxR-group AAA protein CbbQ, a Rubisco activase that requires a MIDAS-containing cofactor protein to function (32–34). Recent structural work has shown that the MIDAS-containing von Willebrand factor A (VWA) domain of this cofactor docks onto the hexameric AAA ring of CbbQ in a bimolecular interaction, and furthermore that the VWA domain
communicates information about substrate binding to the AAA ring (32). Other AAA proteins such as RavA, chelatase, and VWA8 either work with a MIDAS-containing cofactor or have a fused VWA domain (2, 35–39). We propose that the MIDAS-mediated mechanism is a general paradigm for some AAA proteins, and furthermore propose that keeping the MIDAS domain on the same polypeptide as the AAA domains, as opposed to in a separate protein, enables an additional level of regulation in Mdn1.

Together, our data shed light on the role of the linking domains in regulating the docking of the MIDAS domain onto the AAA ring. In contrast to the expectation that tethering the MIDAS domain might favor docking by increasing local concentration, we find that untethering the MIDAS domain leads to tight (submicromolar) binding that no longer requires Rbin-1. Regulation by these large linking domains provides insight into why Mdn1 has remained a single polypeptide, and one of the largest enzymes in the genome, throughout eukaryotic evolution. Long-range regulation between N- and C-terminal domains is also reminiscent of other ATPase enzymes such as kinesin (40, 41), dynein (42), and receptor tyrosine kinase (43).

Materials and Methods

Protein Expression and Purification. S. pombe Mdn1-FL and Mdn1-ΔC (amino acids 1 to 3,911) were cloned into pFastbac HTC (Thermo Fisher 10584027) and expressed in insect cells as previously reported (9, 11). Recombinant baculoviruses were generated using the Bac-to-Bac system (Thermo Fisher). High Five cells (Thermo Fisher BB8520) were grown to ∼3 million cells per milliliter in Express Five SFM (Thermo Fisher 10486025) supplemented with antibiotic-antimycotic (Life Technologies 15240-062) and 16 mM L-glutamine (Life Technologies 25030-081) prior to infection with P2 virus at a stock of 1:50 virus:media ratio. Cells were cultured in suspension (27 °C, shaking at 115 rpm) for 48 h prior to harvesting.

Mdn1-FL and Mdn1-ΔC were purified using a modified version of a previously reported protocol (9, 11). All purification steps were carried out at 4 °C. Cells were lysed using a Dounce homogenizer (Thomas Scientific) in −25 °L of lysis buffer (50 mM Tris [pH 7.5], 400 mM NaCl, 1 mM MgCl2, 10% wt/vol glycerol, 20 mM imidazole, 5 mM 2-mercaptoethanol, 20 μM SNAP-Biotin (NEB S9110S) following the manufacturer-suggested protocol prior to sizing as above. Biotinylation was verified using the colorimetric HABA assay (Pierce 28005), and protein concentration was determined using the colorimetric Bradford assay (Bio-Rad 5000006).

NADH-Coupled Steady-State ATPase Assay. ATPase assays were carried out using time-course fluorescence measurements in a Synergy Neo Microplate reader (340 nm excitation, 440 nm emission) (9). All assays were carried out in Mdn1 assay buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 1 mM DTT) supplemented with 500 μM NADH (Sigma N7410), 1 mM phosphoenolpyruvatic acid (Sigma P7127), 30 U/ml D-lactic dehydrogenase, and 30 U/ml pyruvate kinase (Sigma P1506). Unless otherwise stated, 1 mM of Mg-ATP (Sigma A2383) was present. In experiments with Rbin-1, Rbin-ΔC, or additional MIDAS protein present, 2% DMSO and 2 mM sodium sulfate were added to the assay buffer. Reaction velocities were determined by linear fitting to fluorescence time course data and dividing by the total Mdn1 concentration (25 nM). ATPase rates (V) as a function of ATP concentration (S) were fitted to the Michaelis–Menten equation:

\[
V = \frac{k_{\text{cat}} [S]}{K_m + [S]}
\]

where h reports the Hill coefficient. For Rbin and WT-MIDAS titrations, ATPase rates (V) were fitted to a dose–response equation:

\[
V = \frac{A - b}{1 + \frac{h}{[S]}} + b
\]

where A reports the rate with no Rbin present and b reports the maximal inhibition (11). Fits were weighted by the inverse SEM. All data analysis and fitting were performed in MATLAB.

Rbin-XL Binding Assay. For Rbin-XL binding experiments, input protein (325 nM Mdn1-FL or Mdn1-ΔC or 1.5 μM WT-MIDAS) was mixed with 0 μM Rbin-XL (control lane), 2 μM Rbin-XL (binding lane), or 2 μM Rbin-XL + 50 μM Rbin-1 (competition lane) in binding buffer (20 mM Hepes [pH 7.5], 150 mM NaCl, 1 mM MgCl2, 3% wt/vol glycerol, 1 mM EDTA, 50 μM ATP). For all click chemistry, a Hepes-based substrate buffer was used (20 mM Hepes [pH 7.5], 150 mM NaCl, 1 mM MgCl2, 3% wt/vol glycerol, 1 mM EDTA). The eluent was concentrated to below 0.2 mg/ml using 100-Kd-cutoff Amicon filters as above. Final protein concentration was determined using the colorimetric Bradford assay (Bio-Rad 5000006).

The SNAP-MIDAS construct was generated by subcloning Mdn1 aa 3,911 to 4,381 into pFastbac HTC (Thermo Fisher 10584027) and expressed in insect cells as previously reported (9, 11). Recombinant baculoviruses were generated using the Bac-to-Bac system (Thermo Fisher). High Five cells (Thermo Fisher BB8520) were grown to ∼3 million cells per milliliter in Express Five SFM (Thermo Fisher 10486025) supplemented with antibiotic-antimycotic (Life Technologies 15240-062) and 16 mM L-glutamine (Life Technologies 25030-081) prior to infection with P2 virus at a stock of 1:50 virus:media ratio. Cells were cultured in suspension (27 °C, shaking at 115 rpm) for 48 h prior to harvesting.

Mdn1-FL and Mdn1-ΔC were purified using a modified version of a previously reported protocol (9, 11). All purification steps were carried out at 4 °C. Cells were lysed using a Dounce homogenizer (Thomas Scientific) in −25 °L of lysis buffer (50 mM Tris [pH 7.5], 400 mM NaCl, 1 mM MgCl2, 10% wt/vol glycerol, 20 mM imidazole, 5 mM 2-mercaptoethanol, 20 μM SNAP-Biotin (NEB S9110S) following the manufacturer-suggested protocol prior to sizing as above. Biotinylation was verified using the colorimetric HABA assay (Pierce 28005), and protein concentration was determined using the colorimetric Bradford assay (Bio-Rad 5000006).
on a Novex 4 to 20% Tris-Glycine gel (Thermo Fisher XP04205BOX). Rhodamine imaging was done using a Biorad ChemiDoc system, and Coomassie imaging was done using a LI-COR Odyssey system. Image analysis was done in ImageJ (National Institutes of Health; imagej.nih.gov). Details of Rbin-XL synthesis are in SI Appendix, Supplemental Text.

Dynabead Pull-Down Assay. Mdn1-ΔC (65 nM) and biotinylated MIDAS protein (either WT-MIDAS or ΔLoo-MIDAS, 1 μM unless otherwise stated) were incubated on ice (20 to 40 μL initial reaction volume) for 30 min in binding buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM MgCl₂, 0.5 mM EGTA, 1 mM DTT, 1 mM Mg-ATP, 2 mM NaSO₄, 0.05 mg/mL BSA, 0.002 to 0.02% Triton X-100, and 2% DMSO). MIDAS protein was left out for empty bead control. Rbin-1 was added at 1 μM for example pull-downs and 0 to 50 μM for the titration experiment. The reaction was then mixed with buffer-equilibrated Dynabeads M-280 streptavidin (Invitrogen ND2050) and incubated on ice for 30 min with mixing. The Dynabeads were separated with a magnet, and the supernatant was collected. The pellet was resuspended in SDS/PAGE loading buffer (50 mM Tris-HCl, 0.01% bromophenol blue, 6% glycerol, 100 mM DTT, 2% SDS) and boiled for 3 min for elution. Fractions were analyzed by SDS-PAGE on precast Novex 4 to 20% Tris-Glycine gels (Thermo Fisher XP04205BOX). Imaging was done using a LI-COR Odyssey system. All image analysis was done in ImageJ using the Gel tool to measure band intensities. For the WT-MIDAS titration experiment, data were fitted to a hyperbola:

\[ f = \frac{[\text{MIDAS}] + [\text{Mdn1-ΔC}] + K_\text{D}}{[\text{MIDAS}] + [\text{Mdn1-ΔC}] + K_\text{D}^{-1} - 4 \cdot [\text{Mdn1-ΔC}] \cdot [\text{MIDAS}]} \]

The fit was weighted by the inverse SEM at each data point.

Electron Microscopy Sample Preparation and Data Collection. A 3.5-μL aliquot of purified Mdn1-ΔC + MIDAS sample at total 0.025 mg/mL (250 nM of nonbiotinylated SNAP-MIDAS and 25 nM of Mdn1-ΔC) was adsorbed for 1 min on a glow-discharged copper grid covered with a thin carbon film. The grid was blotted and washed two times with water before being stained with a 0.75% (wt/vol) uranyl formate solution as described (44). Specimens were imaged with a Phillips CM10 electron microscope equipped with a tungsten filament and operated at an acceleration voltage of 100 kV. Micrographs were collected at a calibrated magnification of 41,513 (set equal to the viscosity of water), \( 21911 \) and \( 21912 \).

Brownian Dynamics Modeling of Tethered MIDAS Domain. For modeling motion of the tethered MIDAS domain, the MIDAS domain was treated as a 4-nm-diameter sphere undergoing Brownian motion while tethered by D/E-rich region. The D/E-rich region was modeled as a worm-like chain (entropic spring) (52, 53). Motion of the tethered MIDAS domain was modeled using the overdamped Langevin equation:

\[ \frac{d\mathbf{x}}{dt} = \gamma B \mathbf{x} + \mathbf{F}_{\text{therm}} + \mathbf{F}_{\text{tether}} \]

where \( \gamma = k_B T / \zeta \) is the drag coefficient of a sphere with radius \( r \) in fluid with viscosity \( \eta \) (set equal to the viscosity of water), \( D \) is the diffusion constant, and \( F_{\text{tether}} \) is a Gaussian white noise process with mean zero (55). This equation was integrated numerically using modified Euler’s method, such that \( x(t) \) is updated every 1-ns time step \( \Delta t \) by:

\[ x_{n+1} = x_n + \sqrt{2 D} \Delta t \text{R} + \frac{1}{\gamma B} \mathbf{F}_{\text{therm}} + \frac{1}{\gamma B} \mathbf{F}_{\text{tether}} \Delta t \]

The simulation was run for a total duration of 10 ms. The MIDAS docking site was approximated to be \( (y_0, z_0) = (6.28, 0.22, 9.6) \) nm away from the start of the D/E-rich tether based on the cryo-EM structure of Mdn1-FL (9). Time spent within a 2-nm region was calculated by counting the number of 1-ns time steps spent in the region. All modeling was done in MATLAB.

Data Availability. Data for EM studies were deposited to the EMDB (EMD-21911 and EMD-21912).

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