Figures and figure supplements

ATPase activity of the DEAD-box protein Dhh1 controls processing body formation

Christopher Frederick Mugler et al
Figure 1. Loss of the ATPase activity of Dhh1 triggers bona fide processing body (PB) formation. (A) Known PB components localize to Dhh1\textsuperscript{DQAD} foci. Cells co-expressing the indicated PB component were grown to exponential growth phase, then shifted to glucose-rich or glucose starvation conditions.
Figure 1 continued

for 20 min and observed by confocal microscopy. Scale bar: 5 μm (B) Constitutive PB formation by Dhh1$^{DQAD}$ is rescued by the presence of wild-type Dhh1. Dhh1-GFP or Dhh1$^{DQAD}$-GFP was expressed from a CEN plasmid in DHH1 or dhh1Δ cells and were treated as in (A). Scale bar: 5 μm (C) Loss of ATPase activity mildly disrupts degradation of a Dhh1-tethered mRNA. Dhh1 or Dhh1$^{DQAD}$ was co-expressed as a PP7CP fusion protein in dhh1Δ cells expressing FBA1-PP7L. FBA1 mRNA levels were measured by qPCR and normalized to ACT1 mRNA. Graphs show mean mRNA levels from three independent experiments of biological triplicate samples. Error bars represent SD. A student’s t-test comparing Dhh1 and Dhh1$^{DQAD}$ is shown. Asterisks indicate p<0.005. (D) FBA1 mRNAs do not colocalize with PBs in Dhh1 or Dhh1$^{DQAD}$-expressing cells, suggesting functional mRNA decay, but enrich in PBs in xrn1Δ cells. The indicated strains were grown to exponential growth phase, shifted to either glucose-rich (2% glucose) or glucose starvation conditions for 20 min, fixed with paraformaldehyde and processed for smFISH. Depicted is a maximum projection of the central 10 planes of a 3D image. Insets show representative cells (1.67X magnification). The graph shows the quantification of a representative experiment (n = 2 biological replicates). Scale bar: 5 μm. (E) GFA1 mRNAs do not colocalize with PBs in Dhh1 or Dhh1$^{DQAD}$-expressing cells, suggesting functional mRNA decay, but enrich in PBs in xrn1Δ cells. The indicated strains were grown to exponential growth phase, shifted to either glucose-rich (2% glucose) or glucose starvation conditions for 20 min, fixed with paraformaldehyde and processed for smFISH as in (D). Insets show representative cells (1.67X magnification). The graph shows the quantification of a representative experiment (n = 2 biological replicates). Scale bar: 5 μm.

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Figure 1—figure supplement 1. Loss of ATPase activity of Dhh1 does not trigger stress granule formation. (A) Dhh1\(^{DQAD}\) does not trigger constitutive stress granule formation. Cells co-expressing Pab1-GFP were grown to exponential growth phase, then shifted to glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Scale bar: 5 \(\mu m\). (B) \(PAT1\) mRNAs do not colocalize with PBs in Dhh1 or Dhh1\(^{DQAD}\)-expressing cells, suggesting functional mRNA decay, but enrich in PBs in \(xrn1\Delta\) cells. The indicated strains were grown to exponential growth phase, shifted to either glucose-rich or glucose starvation conditions for 20 min, fixed with paraformaldehyde and processed for smFISH. Depicted is a maximum projection of the central 10 planes of a 3D image. Insets show representative cells (1.67X magnification). Scale bar: 5 \(\mu m\). (C) \(PGK1\) mRNAs do not colocalize with PBs in Dhh1 or Dhh1\(^{DQAD}\)-expressing cells, suggesting functional mRNA decay, but enrich in PBs in \(xrn1\Delta\) cells. The indicated strains were grown to exponential growth phase, shifted to either glucose-rich or glucose starvation conditions for 20 min, fixed with paraformaldehyde and processed for smFISH as in (A). Insets show representative cells (1.67X magnification). Scale bar: 5 \(\mu m\).

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Figure 2. ATP-bound, RNA-bound Dhh1 is required for robust PB formation. (A) Disruption of ATP-binding activity of Dhh1 interferes with PB formation. Wild-type Dhh1 or Dhh1Q-motif was co-expressed from a plasmid as a GFP fusion protein in dhh1Δ cells along with Dcp2-mCherry as a PB marker and grown to exponential growth phase, then shifted to either glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Images were also acquired using wide-field microscopy and PB formation was quantified using Diatrack 3.5 particle tracking software (see Materials and methods). Graphs represent average Dhh1-GFP or Dcp2-mCherry foci number per cell (n=3 biological replicates, >800 cells). Figure 2 continued on next page.
Figure 2 continued

(A) Disruption of RNA binding activity of Dhh1 interferes with PB formation. Wild-type or mutant Dhh1 was co-expressed from a plasmid as a GFP fusion protein in dhh1Δ cells along with Dcp2-mCherry as a PB marker and treated as in (A). Scale bar: 5 μm.

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Figure 2—figure supplement 1. Loss of ATP binding and RNA binding by Dhh1 disrupts PB localization of other PB factors. Xrn1-GFP (A), Dcp1-GFP (B), or Edc3-GFP (C) was co-expressed in dhh1Δ cells along with pDHH1-TAP, pDHH1-Q-motif-TAP, or pDHH1-3X-RNA-TAP and Dcp2-mCherry as a PB marker and treated as in Figure 2A. A student’s t-test comparing DHH1 and either dhh1Δ-Q-motif or dhh1Δ-3X-RNA is shown. Asterisks indicate p<0.005 (**), or p<0.05 (*). Scale bar: 5 µm.

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Disruption of RNA-binding, but not ATP-binding, affects the ability of tethered Dhh1 to promote mRNA decay. (A) Dhh1Q-motif is functional in mRNA decay when tethered to an mRNA. Dhh1 or Dhh1Q-motif was co-expressed as a PP7CP fusion protein in dhh1Δ cells expressing FBA1-PP7L. FBA1 mRNA levels were measured by qPCR and normalized to ACT1 mRNA. Graphs show mean mRNA levels from three independent experiments of biological triplicate samples. Error bars represent SD. A student’s t-test comparing Dhh1 and Dhh1Q-motif is shown. (B) Loss of RNA-binding activity disrupts degradation of a Dhh1-tethered mRNA. Dhh1 or Dhh13X-RNA was co-expressed as a PP7CP fusion protein in dhh1Δ cells expressing FBA1-PP7L. FBA1 mRNA levels were measured by qPCR and normalized to ACT1 mRNA. Graphs show mean mRNA levels from three independent experiments of biological triplicate samples. Error bars represent SD. A student’s t-test comparing Dhh1 and Dhh13X-RNA is shown. Asterisks indicate p<0.005. (C) Wild-type and Dhh1 mutant proteins are expressed to similar levels. Western blot of Dhh1 and Dhh1 mutant protein expression from cells in exponential growth phase from Figure 2A and B. Dhh1 was detected using an anti-Dhh1 antibody. Hxk1 was used as a loading control.

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Figure 3. Loss of the ATPase activity of Dhh1 disrupts PB dynamics. (A) Loss of the ATPase activity of Dhh1 does not alter the dynamics of known PB components. Fluorescence recovery after photobleaching experiments (FRAP) were performed on cells expressing the indicated GFP-tagged PB components.

Figure 3 continued on next page...
component. Cells were glucose starved for 20 min to allow PBs to form, then PBs were bleached and recovery of GFP fluorescence to the PB was followed over time. Recovery of PB components is presented as an averaged data plot of FRAP recovery curves from three independent experiments (n > 8 PBs per experiment, typically ~12 PBs per experiment). Error bars represent SD. (B) The ATPase activity of Dhh1 is required for proper PB disassembly. dhhlΔ cells expressing Dhh1-GFP or Dhh1DQAD-GFP were glucose starved for 30 min to allow PBs to form and then treated with either 50 μg/mL cycloheximide or solvent only (DMSO) for 2 hr and disappearance of Dhh1-GFP or Dhh1DQAD-GFP foci per cell was monitored for 2 hr. Each time point image is a maximum-projection of 8 z-stacks at a distance of 0.4 μm. The graph shows foci number per cell measurements for Dhh1 and Dhh1DQAD normalized to 1 to account for differences in PB formation between Dhh1 and Dhh1DQAD (n = 3 biological replicates, >100 cells). Error bars represent SEM. Scale bar: 5 μm.

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**A. PB disassembly**

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<tr>
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<td>Untreated</td>
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<tr>
<td>DMSO</td>
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**B.**

30' glucose starvation, then 2 hr drug treatment

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**Figure 3—figure supplement 1.** Loss of ATPase activity of Dhh1 disrupts the PB dynamics of other PB components. (A) Loss of ATPase activity of Dhh1 affects disassembly of Dcp2 foci. Dcp2-mCherry was expressed in dhh1∆ cells co-expressing Dhh1-GFP or Dhh1^DQAD^-GFP from a plasmid, and cells were glucose starved for 30 min.

*Figure 3—figure supplement 1 continued on next page*
Figure 3—figure supplement 1 continued

to allow PBs to form, followed by treatment with either 50 μg/mL cycloheximide or solvent only (DMSO) for 2 hr. Disappearance of Dcp2-mCherry foci over time is monitored (n = 3 biological replicates). Each time point image is a maximum-projection of 8 z-stacks at a distance of 0.4 μm. Scale bar: 5 μm (B) Two-hour cycloheximide treatment does not disrupt cell viability. The indicated cells were glucose starved for 30 min, and then treated with or without 50 μg/mL cycloheximide and 5-fold serial dilutions were plated on SD (-URA) + 2% dextrose media. DOI: 10.7554/eLife.18746.009
Figure 4. The ATPase activity of Dhh1 is stimulated in vitro and in vivo by Not1. (A) ATPase activity of Dhh1 is stimulated by Not1. The ATPase activity of full-length Dhh1 or Dhh1\textsuperscript{DQAD} was measured with increasing concentrations of Not1\textsuperscript{MIF4G}. Graphs represent average ATPase activity (n=3). Error bars

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represent SD. (B) Disruption of Dhh1 interaction with the MIF4G region of Not1 by mutation of conserved residues in Dhh1. TAP-tagged Dhh1, Dhh1\(^{5X-Not}\), or untagged Dhh1 were purified from cells in exponential growth phase using IgG-coupled magnetic beads and co-purifying Not1-3HA was detected by Western blot. Quantification of Not1 to Dhh1 ratio is plotted with SEM (n=5 biological replicates). A representative Western blot is shown. A student’s t-test comparing Dhh1 and Dhh1\(^{5X-Not}\) is shown. Asterisks indicate p<0.01. (C) Mutations in the Not1-binding surface of Dhh1 trigger constitutive PB assembly. Wild-type or mutant Dhh1 was co-expressed from a plasmid as a GFP fusion protein in dhh1\(^{-}\) cells along with Dcp2-mCherry as a PB marker and grown to exponential growth phase, then shifted to either glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Images were also acquired using wide-field microscopy and PB formation was quantified using Diatrack 3.5 particle tracking software. Graphs represent the average Dhh1-GFP foci or Dcp2-mCherry foci number per cell (n=3 biological replicates, >800 cells per experiment). Error bars represent SD. Scale bar: 5 \(\mu m\).

DOI: 10.7554/eLife.18746.014
**Figure 4—figure supplement 1.** Not1 is a specific activator of the ATPase activity of Dhh1. (A) ATPase activity of Dhh1 is weakly stimulated by RNA. ATPase activity of full-length Dhh1 was measured with increasing concentrations of polyU RNA. Graphs represent average ATPase activity (n=3). **Figure 4—figure supplement 1 continued on next page**
biological replicates). Error bars represent SD. (B) Not1 is a specific activator of Dhh1. ATPase activity of full-length Dhh1 or Dbp5 was measured in the presence of the indicated protein. Graphs represent average ATPase activity (n=3 biological replicates). Error bars represent SD. (C) Not1 binding is diminished in a ATP-binding mutant of Dhh1. TAP-tagged Dhh1 or Dhh1\textsuperscript{Q-motif} were purified from cells in exponential growth phase using IgG-coupled magnetic beads and co-purifying Not1-3HA was detected by Western blot. Quantification of Not1 to Dhh1 ratio is plotted with SEM (n=4 biological replicates). A representative Western blot is shown. A student’s t-test comparing Dhh1 and Dhh1\textsuperscript{Q-motif} is shown. Asterisks indicate p<0.01. (D–F) Known PB components localize to Dhh1\textsuperscript{5X-Not} foci. Xrn1-GFP (D), Dcp1-GFP (E), or Edc3-GFP (F) was co-expressed in DHH1 or dhh1\textsuperscript{5X-Not} cells, along with Dcp2-mCherry as a PB marker. Cells were grown to exponential growth phase, then shifted to glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Images were also acquired using wide-field microscopy and PB formation was quantified using Diatrack 3.5 particle tracking software. Graphs represent the average foci number per cell (n = 3 biological replicates, >800 cells per experiment). Error bars represent SD. Scale bar: 5 μm.

DOI: 10.7554/eLife.18746.015
Figure 4—figure supplement 2. Tethered Dhh1 does not require ATPase activation by Not1 to promote mRNA decay. (A) Dhh1<sup>5X-Not</sup> does not show a significant defect in mRNA decay when tethered to an mRNA. Dhh1 or Dhh1<sup>5X-Not</sup> was co-expressed as a PP7CP fusion protein in <i>dhh1Δ</i> cells expressing FBA1-PP7<sub>L</sub>. FBA1 levels were measured by qPCR and normalized to ACT1 mRNA. Graphs show mean mRNA levels from three independent experiments of biological triplicate samples. Error bars represent SD. (B) Wild-type and Dhh1 mutant proteins are expressed to similar levels. Western blot of Dhh1 and Dhh1 mutant protein expression from cells in exponential growth phase. Dhh1 was detected using an anti-GFP antibody. Hxk1 was used as a loading control.

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Figure 5. Mutations in Dhh1-binding surface of Not1 trigger constitutive PB assembly. Not1 or Not1<sup>9X-Dhh1</sup> was co-expressed with Dhh1-GFP and Dcp2-mCherry and grown to exponential growth phase, then shifted to either glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Images were also acquired using wide-field microscopy and PB formation was quantified using Diatrack 3.5 particle tracking software (see Materials and methods). Graphs represent average Dhh1-GFP foci or Dcp2-mCherry foci number per cell (n=3 biological replicates, >800 cells per experiment). Error bars represent SD. Scale bar: 5 μm.

DOI: 10.7554/eLife.18746.017
Figure 5—figure supplement 1. Not19X-Dhh1 triggers PB assembly. (A–C) Known PB components localize to Not19X-Dhh1 foci. Xm11-GFP (A), Dcp1-GFP (B), or Edc3-GFP (C) was co-expressed in NOT1, or not19X-Dhh1 cells, along with Dcp2-mCherry as a PB marker. Cells were grown to exponential growth phase, then shifted to glucose-rich or glucose starvation conditions for 20 min and observed by confocal microscopy. Images were also acquired using wide-field microscopy and PB formation was quantified using Diatrack 3.5 particle tracking software. Graphs represent average foci number per cell (n=3 biological replicates, >800 cells per experiment). Error bars represent SD. A student’s t-test comparing localization between Not1 and Not19X-Dhh1 is shown. Asterisks indicate p<0.005 (**), or p<0.05 (*). Scale bar: 5 μm (D) Not19X-Dhh1 mutant is expressed to wild-type Not1 levels. Western blot of Not1 or Not19X-Dhh1 mutant protein expression from cells in exponential growth phase. Not1 was detected using Rabbit IgG, and Dhh1 was detected using an anti-Dhh1 antibody. Hxk1 was used as a loading control.

DOI: 10.7554/eLife.18746.018
Figure 6. Dhh1 PB dynamics can be recapitulated in vitro. (A) Formation of liquid Dhh1-droplets depends on the presence of the RNA analog polyuridylic acid (polyU) and increases with increasing protein concentration. Recombinant mCherry-tagged Dhh1 was diluted into a low salt buffer and incubated at 4°C for 1 hr. Formation of liquid droplets was observed by fluorescence microscopy. Scale bar: 10 μm. (B) Dhh1 liquid droplet formation requires ATP. Dhh1 liquid droplets were formed as in (A), in the presence or absence of ATP and the creatine kinase ATP regeneration system. Scale bar: 20 μm. (C) Addition of Not1MIF4G or RNase A, but not buffer alone, dissolves pre-formed Dhh1 liquid droplets. Dhh1 liquid droplets were pre-formed for 20 min at 4°C, followed by the addition of 5 μM Not1MIF4G or RNase A. Scale bar: 10 μm. (D, E) Pre-incubation with Not1MIF4G prevents formation of Dhh1, but not Dhh1DQAD liquid droplets. Reactions were imaged after 1h incubation at 4°C. Scale bar: 10 μm.
DOI: 10.7554/eLife.18746.019
Figure 6—figure supplement 1. Single point mutants in the ATP binding site of Dhh1 affect PB assembly and liquid droplet formation. (A) Single mutations in the Q-motif of Dhh1 show minor defects in PB formation. Wild-type Dhh1, Dhh1^{F66R} or Dhh1^{Q73A} were expressed from a plasmid as a GFP fusion protein in dhh1^D cells along with Dcp2-mCherry as a PB marker and grown to exponential growth phase, then shifted to either glucose-rich or glucose starvation conditions for 20 min and observed by wide-field microscopy. PB formation was quantified using Diatrack 3.5 particle tracking software. Graphs represent average Dhh1-GFP or Dcp2-mCherry foci number per cell (n=3 biological replicates, >300 cells per experiment). Error bars represent SD. A student’s t-test comparing Dhh1^{F66R} or Dhh1^{Q73A} with Dhh1 is shown. Scale bar: 5 μm (B) Dhh1 liquid droplet formation requires ATP binding. Recombinant mCherry-tagged Dhh1, Dhh1^{QAD} or Dhh1^{F66R} were diluted into a low salt buffer and incubated at 4°C for 1 hr in the presence of ATP and 0.075 mg/mL polyU. Formation of liquid droplets was observed by fluorescence microscopy. Scale bar: 10 μm DOI: 10.7554/eLife.18746.020
Figure 7. Model: The ATPase cycle of Dhh1 controls PB assembly and disassembly. An ATP- and RNA-bound conformation of Dhh1 nucleates PB formation, while stimulation of Dhh1’s ATPase activity by Not1 promotes granule disassembly.

DOI: 10.7554/eLife.18746.025