Figures and figure supplements

A dedicated diribonucleotidase resolves a key bottleneck for the terminal step of RNA degradation

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Figure 1. Orn has a stark preference for diribonucleotide cleavage in vitro. RNA nucleotides two to seven residues in length (1 μM, containing the corresponding \textsuperscript{32}P-labeled RNA tracer) were each subjected to cleavage over time with 5 nM (A, B) or 1000 nM OrnVc (C, D). Aliquots of each reaction were stopped at indicated times (min), and assessed by denaturing 20% PAGE (A, C). Quantification of the intensities of bands corresponding to the amount of uncleaved initial oligonucleotide over time are plotted as the average and SD of three independent experiments (B, D).

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Catalytic residues of OrnVc are required for degradation of $^{32}$P-AAAAAGG in vitro. Degradation of $^{32}$P-AAAAAGG by purified indicated His$_{10}$-MBP-OrnVc variants with 5 nM (A) or 1 µM (B). Samples were stopped at indicated time (min) and analyzed by denaturing 20% PAGE. Representative gel images of two independent assays are shown.

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Figure 2. Structures reveal Orn’s conserved substrate preference for diribonucleotides. Crystal structures of pGpG-bound *V. cholerae* Orn (A) and human REXO2 (B) are shown in comparison to *E. coli* RNase T bound to substrate (PDB 3nh1; *Hsiao et al.*, 2011) (C), another DnaQ-fold 3’-5’ exoribonuclease with a DEDD(h) active site motif. The top panels show ribbon representations of the dimeric enzymes. The insets are surface representations of the enzymes’ active sites shown in similar orientations. The bottom panel describes the active site residues involved in RNA binding and catalysis. Residue numbering for REXO2 refers to its cytosolic isoform lacking the mitochondria-targeting pre-sequence. The sequence logos in (D) were constructed based on multi-sequence alignments of Orn and RNase T orthologs. Overall sequence identity ranges from 43 to 70% for Orn and 46 to 69% for RNase T. Sequence identifiers are provided in Figure 2—source data 2. Sequence logos were plotted using WebLogo (*Crooks et al.*, 2004). Conserved residues of the active site’s DEDD motif (‘act.’; red), for ribonucleotide base binding (‘nuc.’; gray), and of the phosphate cap (‘P-cap’; purple) are highlighted.

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Figure 2—figure supplement 1. Structural comparison of Orn orthologs, RNase T and Exo I. (A) Superposition of Orn$_{Vc}$ with REXO2 (left) and the substrate-free state of Orn from Xanthomonas campestris (PDB 2gbz; Chin et al., 2006) (right). Rmsd values are reported for the alignment of a
monomer. (B) Surface properties of Orn enzymes. An alignment of Orn orthologs was used to map conservation scores onto the solvent accessible surface of Orn\textsubscript{Vc} (left). High degree of conservation overlaps with the acidic active site observed in the electrostatic potential map calculated with the APBS software (Baker et al., 2001) and based on Orn\textsubscript{Vc} (right). (C) Active-site view of \textit{E. coli} RNase T bound to dAAC (PDB 3v9z; Hsiao et al., 2012). (D) Active-site view of \textit{E. coli} Exo I bound to dT13 (PDB 4jrp; Korada et al., 2013). DOI: https://doi.org/10.7554/eLife.46313.007
Figure 2—figure supplement 2. Structural comparison of Orn_{Vc} and REXO2 bound to diverse diribonucleotides. (A) Crystal structures of REXO2 bound to pApA and pApG. The structural overview (left) shows a C-terminal helix that is unique to Orn orthologs in higher eukaryotes and in the current crystal forms is stabilized by crystal packing contacts. (B) Crystal structures of Orn_{Vc} bound to di-purine (pApA, pApG, pGpA), purine-pyrimidine (pGpC, pCpG), and di-pyrimidine (pCpU) substrates. Views are identical to those shown in Figure 2 for the REXO2:pGpG and Orn:pGpG complexes.
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Figure 3. Functional importance of active-site and phosphate-cap residues for Orn function. (A) In vitro enzyme activity. Degradation of $^{32}$P-pGpG (1 μM) by purified wild-type OrnVc or variants with alanine substitutions (5 nM) at the indicated sites was assessed. Samples were stopped at the indicated times (min) and analyzed by denaturing 20% PAGE. Representative gel images are shown with the indicated RNA size. Figure 3—figure supplement 4 shows the graphs of the means and SD of three independent experiments. (B) In vivo activity of alanine substituted ornVc alleles to complement P. aeruginosa Δorn. Overnight cultures of the indicated strains were allowed to stand for 10 min without agitation to allow bacterial aggregates to sediment. Representative images of the cultures of triplicated assays are shown.

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Figure 3—figure supplement 1. Contribution of catalytic residues and His10-MBP tag to binding interaction between OrnVc and pGpG. The dissociation constants ($K_d$) of indicated purified (A) His$_{10}$-MBP-Orn$_{Vc}$ variants and (B) untagged Orn$_{Vc}$ to pGpG were measured by DRaCALA. Fraction bound from data was plotted and determined by nonlinear regression. All data shown represent average and SD of three independent experiments. DOI: https://doi.org/10.7554/eLife.46313.016
**Figure 3—figure supplement 2.** Immunoblot analysis of Orn in *P. aeruginosa* ∆orn expressing indicated orn alleles. Proteins in whole cell lysates of strains expressing the indicated HA-tagged orn allele were separated by 12% SDS-PAGE and probed with anti-HA antibodies. All images were performed in duplicate independently. A representative image of two independent experiments is shown.

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Figure 3—figure supplement 3. Molecular weight determination indicates that Orn\textsubscript{Vc} variants with point mutations at the phosphate cap and active site remain dimeric. (A) Absolute molecular weights (black data points across elution peaks are plotted on the right axis; theoretical monomer and dimer molecular weights, horizontal dashed lines) of wild-type Orn\textsubscript{Vc} and point mutants were determined using SEC-MALS (90°-light scattering: colored, solid lines; refractive index signal: black, dashed lines; plotted on the left axis).

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Figure 3—figure supplement 4. pGpG degradation by purified wild-type Orn<sub>Vc</sub> or variants with indicated alanine substitutions. 1 μM pGpG was treated with 5 nM of the indicated Orn protein and sampled at indicated time points. The graphs show quantification of three independent data sets, of which representative radiographs are shown in Figure 3A.
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Figure 4. Orn, cleaves all diribonucleotides. (A) Orn (5 nM) was incubated with di-purine (pRpR), purine-pyrimidine (pRpY or pYpR), or di-pyrimidine (pYpY) diribonucleotides (1 μM) containing the corresponding 32P-labeled RNA tracer. Aliquots of each reaction were stopped at the indicated times (min) and assessed by denaturing 20% PAGE. (B) Quantification of the intensities of bands corresponding to the amount of diribonucleotide cleaved at the 10 min time point. Results are the average and SD of duplicate independent experiments. Orn cleaves all diribonucleotides to nucleoside monophosphates, albeit to varying extents. Diribonucleotides consisting of two purines (pRpR) were hydrolyzed most efficiently, with over 90% of starting RNAs processed by 10 min. A majority (>75%) of diribonucleotides with a 5' purine (pRpY) were also processed by the 10 min endpoint. However, diribonucleotides with a 5' pyrimidines exhibited moderately reduced levels of cleavage. Di-pyrimidine (pYpY) substrates, and in particular pUpC and pCpC, showed the slowest turnover from the substrates tested. These results demonstrate that while all diribonucleotides are acceptable substrates for Orn, the enzyme is likely to exhibit moderate preferences for diribonucleotides that contain a 5' purine.

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Figure 4—figure supplement 1. Effect of GpG and pAp on Orn activity. (A) Cleavage of 130 nM $^{32}$P-pGpG by 5 nM Orn in the absence or presence of unlabeled pAp, GpG or GMP (100 μM). (B) Fraction bound of $^{32}$P-pGpG to 200 nM Orn in presence of no competitor, 100 μM pGpG or 100 μM GpG. (C) Fraction bound of $^{32}$P-pAp to different concentration of Orn$_{Vc}$ was determined by DRaCALA. $K_d$ could not be measured as $^{32}$P-pAp binding to Orn was not saturated. (B) (C) All data shown represent duplicate-independent experiments.

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Figure 5. Orn acts as a dinucleotidase in cell lysates. Degradation of $^{32}$P-GG (A) and $^{32}$P-AAAAAGG (B) by whole cell lysates of wild-type, orn mutant, orn mutant complemented with ornVc, or ornVc, D12A. For Δorn and Δorn complemented with ornVc, D12A, 100 nM of purified OrnVc was added at 30 min time point and incubated for an additional 10 min. Samples were stopped at the indicated time and analyzed by 20% denaturing PAGE. Representative gel images of triplicated assays are shown with the indicated RNA size. Graphs show quantitation of triplicate data for indicated RNA species over time. DOI: https://doi.org/10.7554/eLife.46313.021
Catalytic residues of Orn\textsubscript{Vc} are required for degradation of \textsuperscript{32}P-AAAAAGG in whole-cell lysates. Degradation of \textsuperscript{32}P-AAAAAGG by whole-cell lysates of the indicated strain (PA14, \textDelta orn mutant complemented with pMMB-orn\textsubscript{Vc} \textL{18}A or pMMB-orn\textsubscript{Vc} \textH{158}A). For \textDelta orn expressing orn\textsubscript{Vc} \textL{18}A or orn\textsubscript{Vc} \textH{158}A, 100 nM of purified, wild-type Orn\textsubscript{Vc} protein was added at the 30 min time point and reactions were stopped 10 min thereafter. Samples were stopped at indicated time and analyzed by denaturing 20% PAGE. Representative gel images of two independent experiments are shown.

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Figure 6. Small-colony phenotype of Δorn is independent of c-di-GMP signaling. Bacterial cultures were diluted and dripped on LB agar plates with the indicated concentration of IPTG. After overnight incubation, representative images of the plates were taken and shown for (A) PA14 and PA14 ΔpelA harboring pMMB vector with the indicated diguanylate cyclase gene and (B) PA14 Δorn and PA14 Δorn ΔpelA-G with pMMB and pMMB-orn. Experiments were performed in triplicate.

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Small-colony phenotype of \(\Delta\text{orn}\) is complemented by functional \(\text{orn}_{\text{Vc}}\) alleles. Bacterial cultures were diluted, 10 \(\mu\text{L}\) was spotted on LB agar plates with the indicated concentration of IPTG and drip to form parallel lines of bacteria. After overnight incubation,

Figure 6—figure supplement 1 continued on next page
representative images of the plates were taken of PA14 Δorn and PA14 Δorn ΔpelA-G with pMMB alone or containing the indicated allele of ornVc. Experiments were performed in triplicate and one representative image of the plates is shown.
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**Figure 6—figure supplement 2.** *P. aeruginosa* PAK Δorn mutants show a small-colony phenotype. Indicated bacterial strains were diluted and dripped on (A) LB agar plates or (B) LB agar plates containing 50 μg/mL carbenicillin with indicated concentration of IPTG and incubated overnight at 37°C. All experiments were performed in triplicate and one representative image of the plates is shown.

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Figure 7. Model for Orn’s cellular function as a diribonucleotidase. RNA degradation is initiated by fragmentation via endoribonucleases (e.g. RNase E and RNase III) that cleave unstructured or structured RNA sequences. RNA fragments are processed further at their 3’ termini by 3’→5’ exoribonucleases (e.g. PNPase, RNase R, and RNase II). Their combined activity produces mononucleotides and various terminal diribonucleotides from the original RNA fragments. The pGpG (GG) linear diribonucleotide is also produced by linearization of c-di-GMP by specific phosphodiesterases, EAL- and HD-GYP-domain-containing enzymes, which terminate c-di-GMP signaling. In Pseudomonas aeruginosa and likely other organisms that rely on Orn for growth, Orn is the only diribonucleotidase that cleaves diribonucleotides to mononucleotides. In the absence of Orn, diribonucleotides accumulate with a drastic impact on cellular physiology, ranging from transcriptional changes, small-colony phenotypes and growth arrest, depending on the organism. Orn is also unique because it acts as the second phosphodiesterase in the degradation of c-di-GMP by clearing the pGpG intermediate. In an orn mutant, c-di-GMP levels are elevated through feedback inhibition of the c-di-GMP-degrading phosphodiesterases by pGpG, leading to the associated biofilm phenotypes.

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