Electronic Supplementary Materials

G-quadruplex DNA inhibits unwinding activity but promotes liquid-liquid phase separation by the DEAD-box helicase Ded1p.

Experimental Methods

Oligonucleotides and recombinant protein purification

DNA and RNA oligonucleotides (Supplementary Table S1) were purchased from Integrated DNA Technologies, and Dharmacon, respectively. Oligonucleotides were purified as previously described. G4DNA and G4RNA were prepared as previously described. Recombinant Ded1p was purified as previously described.

RNA duplex unwinding

Unwinding reactions were performed and analyzed as previously described. Reaction conditions were as follows (concentrations listed are final). 50 nM radiolabeled duplex RNA (dsRNA) was incubated prior to reaction start with 1 nM Ded1p in helicase in reaction buffer [50 mM Tris–HCl (pH 8.0), 2.43 mM DTT, 0.21 mM EDTA, 0.02% (v/v) Triton X-100, 0.01% (v/v) IGEPAL, 0.5 mM MgCl₂, 50 mM NaCl, 40 mM KCl, 7.86% glycerol, 1 U/μl Rnasin (Roche)] for 5 min. Reactions were initiated at 19 °C by addition of 2 mM ATP, 2 mM MgCl₂, and 500 nM unlabeled 10 nt scavenger RNA to prevent reannealing of the unwound strand. Aliquots were removed at the indicated reaction times, and reactions were quenched with stop buffer containing 1% SDS, 50 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, and 20% glycerol. Samples were separated by 15% native PAGE. Gels were dried, visualized using a Molecular Dynamics PhosphorImager, and quantified using ImageQuant 5.2 software (Molecular Dynamics).

Fluorescence anisotropy binding assay

Experiments were conducted as previously described. 1 nM Fluorescein labelled tailless cMYC G4DNA (Supplementary Table S1), was incubated with varying concentrations of Ded1p (or Ded1p variants without either the N- or the C-terminal) at room temperature for 30 minutes in the binding buffer [40 mM Tris pH 8.0, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.05% NP-40]. Polarization was measured at 535 nm using a Perkin Elmer 1420 Victor³V Multi-label Counter with excitation at 485 nm. Data was converted to anisotropy and fit to the quadratic equation to determine the K_d value using KaleidaGraph software.
**Liquid-Liquid phase separation in vitro**

Ded1p (4 µM final) was incubated at room temperature for 1 h in helicase reaction buffer with 50 nM Cy5-labeled Ded1. DNA or RNA labeled with Cy3 was added as indicated. 10 µL of each reaction were spotted on a glass slide and a coverslip was applied. Samples were imaged within 30 minutes at RT. Granules were detected by monitoring Cy5-Ded1 or Cy3-Nucleic Acid on a Leica DM6000 microscope with a 20X objective. 20 images were taken for each condition at the same exposure settings for all conditions. Droplet number and diameter were quantified with ImageJ. Images were thresholded using the Otsu method and converted to a binary image. The diameter of each condensate was identified and quantified using ImageJ function “Analyze Particles”. The number of condensates of each size were counted in 0.5 µm bins and plotted for each condition using KaleidaGraph.

**Construction of the DED1-GFP strain**

The *DED1-GFP(S65T)-TRP1* yeast strain was constructed by “gene targeting” methods. In general, the 1901bp *GFP(S65T)-TRP1* cassette with 60 base pairs homology at each side to the C-terminally positioned of *DED1* chromosomal locus, was PCR cloned by using primer pair “DED1-GFP F2-1 and DED1-GFP R1-2” (Supplemental Table S1) and Pfu DNA polymerase from the pFA6a-GFP(S65T)-TRP1 plasmid. The PCR product was gel purified, and transformed into the *Saccharomyces cerevisiae* YPH499 strain by standard lithium acetate method, then plated on the SD-Trp+Ade agar plates for selection at 25°C 3-5 days. Candidate clones were individually picked up and patched onto new SD-Trp+Ade agar plate one round, then were individually inoculated into 10 mL SD-Trp+Ade liquid culture each at 25°C overnight. Cells were pelleted by centrifugation, and genomic DNA were extracted by “Smash and Grab” method. PCR and DNA sequencing confirmed the in-frame insertion of *GFP(S65T)-TRP1* cassette at the c-terminus of *DED1-ORF* in its genome locus.

**Phen-DC3 treatment and high-resolution microscopy analysis**

The *S. cerevisiae* yeast strain G0144 [YPH499, *DED1-GFP(S65T)-TRP1*] was streaked out from -80°C stock onto a SD-Trp+Ade agar plate, grown at 28°C for 3-4 days. Individual clones were picked from the plate, and incubated into 10 mL SD-Trp+Ade each, grown at 28°C in a dark incubator to an OD$_{600}$ of approximately 1. Then, Phen-DC3 was added into the culture to final concentration 10 µM, and cells continued to grow at 28°C in a dark incubator. At each indicated time-point, 1 mL cell culture was transferred from the culture tube into a new tube, mixed with 156 µL 37% Formaldehyde (to final 5% Formaldehyde), and incubated on a rotator at 25°C for 1 hr. Fixed cell samples were harvested by centrifugation at 15,800xg for 1 minute, then washed by
900 µL PBS twice, and re-suspended in 100 µL PBS, then stored in 4°C dark box. Cell samples were stained with DAPI, and subjected to high-resolution microscopy analysis on the ZEISS LSM-880 microscope with Airyscan (using PlanApo 63x/1.4 oil DicII objective) in the UAMS Digital Microscopy Core facility.

**G4RNA unfolding**

Experiments were conducted as previously described. All concentrations listed are final. 32P-labelled G4RNA was folded by heating to 95°C for 10 minutes in 100 mM KCl and slowly cooling to room temperature. G4RNA (2 nM) was pre-incubated with 5 mM ATP and 10 mM MgCl₂, in the reaction buffer [40 mM Tris pH8.0, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.01% NP-40] at 25°C. Reactions were initiated by addition of Ded1p (400 nM) and DNA trap (Q-trap) complementary to the G4-forming region (15 nM) for trapping the unfolded RNA. Aliquots were removed and quenched at various time-points with 50 mM EDTA, 0.5% SDS, and 0.9 µM C-trap (unlabeled G4DNA). Samples were separated by 20% native PAGE, visualized using a Typhoon Trio PhosphorImager, and quantitated using ImageQuant software.

**RNA:DNA hybrid duplex unwinding**

Experiments were conducted as previously described. All concentrations listed are final. RNA:DNA hybrid duplex was formed by mixing the 32P-labelled RNA strand with complementary DNA strand at 1:1.2 ratio, heating to 95°C for 5 minutes, and slowly cooling to room temperature. The hybrid duplex (2 nM) was pre-incubated with 5 mM ATP and 10 mM MgCl₂, in the reaction buffer [40 mM Tris pH8.0, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.01% NP-40] at 25°C. Reactions were initiated by addition of Ded1p (400 nM) and DNA trap (60 nM) complementary to the displaced DNA strand. Aliquots were removed and quenched at various time-points with 50 mM EDTA, 0.5% SDS. Samples were separated by 20% native PAGE, visualized using a Typhoon Trio PhosphorImager, and quantitated using ImageQuant software.
References for Supplementary Materials

1. P. D. Morris, A. J. Tackett, K. Babb, B. Nanduri, C. Chick, J. Scott and K. D. Raney, *J. Biol. Chem.*, 2001, **276**, 19691–19698.

2. A. K. Byrd and K. D. Raney, *J. Biol. Chem.*, 2015, **290**, 6482–6494.

3. J. Gao, A. K. Byrd, B. L. Zybalov, J. C. Marecki, M. J. Guderyon, A. D. Edwards, S. Chib, K. L. West, Z. J. Waldrip, S. G. Mackintosh, Z. Gao, A. A. Putnam, E. Jankowsky and K. D. Raney, *Chem. Commun.*, 2019, **55**, 4467–4470.

4. Q. Yang and E. Jankowsky, *Biochemistry*, 2005, **44**, 13591–13601.

5. E. Jankowsky and A. Putnam, *Methods Mol. Biol.*, 2010, **587**, 245–264.

6. W. K. Huh, J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman and E. K. O'Shea, *Nature*, 2003, **425**, 686–691.

7. M. S. Longtine, A. McKenzie, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippson and J. R. Pringle, *Yeast*, 1998, **14**, 953–961.

8. R. D. Gietz and R. A. Woods, *Methods Enzymol.*, 2002, **350**, 87–96.

9. C. S. Hoffman, *Curr. Protoc. Mol. Biol.*, 1997, **39**, 13.11.1-13.11.4.
**Supplementary Table S1. Sequences of oligonucleotides.**

| Name                                                                 | Sequence                                                                 |
|----------------------------------------------------------------------|-------------------------------------------------------------------------|
| **PCR for *DED1-GFP(S65T)-TRP1* yeast strain construction**          |                                                                          |
| *DED1-GFP F2-1*                                                      | 5′-TAGCGATTCCAAGTCTTCTGGCTGGGTAACACGCGGTTTCAAACACCTCTTCGAGCTCGTTTTAAACCTCCTTA-3′ |
| *DED1-GFP R1-2*                                                      | 5′-GAAAGAAAATATAAGACATGCTAGACGAGAAAACGAAGAATCCCTCACCCTAGTTTGTCTGAAAGAATTCGAGCTCGTTTTAAACCTCCTTA-3′ |
| **G4DNA Binding**                                                   |                                                                          |
| Tailless cMYC                                                       | 5′-TGGGTTGGGTAGGGTGGTTT-6FAM-3′                                        |
| **RNA:RNA duplex unwinding with G4DNA or ssDNA competitor**         |                                                                          |
| RNA Strand 1                                                        | 5′-UUACCGGUCUUAAAACAAAAACAAAAAACAACAAAA-3′                              |
| RNA Strand 2                                                        | 3′-AAUGCCACGA-5′                                                        |
| Annealing Trap                                                      | 5′-AGCACCGUAA-3′                                                        |
| G4DNA competitor                                                    | 5′-TGGGTTGGGTAGGGTGGTTT-3′                                              |
| ssDNA competitor                                                    | 5′-TCCCTCCCTACCCCTCCT-3′                                               |
| **Ded1 granules *in vitro***                                         |                                                                          |
| G4DNA                                                              | 5′-T(15)GAGGGTGGGTTAGGGTGGGTAA-Cy3                                    |
| ssDNA                                                              | 5′-TGTTGTTGTTGTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTAAGTACAGAAG-Cy3      |
| ssRNA                                                              | 3′-GCUUUACGGUGCUAAAACAAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAA-3′ |
| **G4RNA unfolding**                                                 |                                                                          |
| Tailless G4RNA                                                      | 5′-GUUGGUGGUGGUGUGUGUGGU-3′                                            |
| Qtrap DNA-1                                                         | 5′-ACACCAACCACCACCACC-3′                                               |
| Ctrap DNA                                                          | 5′-GTTGGTGGTGGTGGTGTGT-3′                                              |
| **RNA:DNA hybrid duplex unwinding**                                 |                                                                          |
| RNA                                                                 | 5′-GUUGGUGGUGGUGGUGGU13-3′                                              |
| Complementary DNA                                                   | 3′-CAACCCACCACCCACACCACA-5′                                             |
| DNA trap                                                           | 5′-GTTGGTGGTGGTGGTGTGT-3′                                              |
| **16bp RNA duplex unwinding sequence**                              |                                                                          |
| RNA Strand 1                                                        | 5′-GCUGCUCUUACCGGUCUAAAACAAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAAACAAA-3′ |
| RNA Strand 2                                                        | 3′-CGCAGAAAUUGCACACCA-5′                                               |

Guanines involved in tetrad formation are underlined.

Duplex forming regions are italicized.
Supplementary Video 1. Three-dimensional scan of *DED1*-GFP cell after 19 hours of Phen-DC3 treatment. (a) 360 degree spinning around the x-axis. (b) 360 degree spinning around the y-axis.

Supplementary Figure S1. LLPS with unlabeled nucleic acid. Representative microscopy images with Cy5 labeled Ded1p and unlabeled ssRNA, under conditions identical to those shown in Figure 3. Sensitivity of the Cy3 channel in the image on the lower right is increased by a factor of 5, indicating no bleed-through of the Cy5 fluorescence.

Supplementary Figure S2. Phen-DC3 inhibits G4RNA destabilization by Ded1. (a) Diagram illustrating the experiments. 32P-labelled tailless G4RNA was pre-incubated with Mg2+, in the presence of ATP. Ded1 was added to initiate the reaction, along with a DNA trap (Qtrap). The destabilized G4RNA will be single-stranded and rapidly complemented with Qtrap to form a stable RNA:DNA duplex (blue and black duplex). At increasing times, the reactions were quenched by adding excess Ctrap with EDTA and SDS. The Ctrap will form a duplex with the leftover Qtrap (blue and green duplex). (b) Gel images of tailless G4RNA unfolded by Ded1 with indicated experimental conditions. (c) The reaction progress curves. Data were fit to a single exponential. Experiments were repeated twice independently.

Supplementary Figure S3. Phen-DC3 inhibits RNA:DNA duplex unwinding by Ded1. (a) Diagram illustrating the experiments. 32P-labelled 3'-tailed RNA:DNA hybrid duplexes (2nM) were pre-incubated with Mg2+, in the presence or absence of ATP. Helicase Ded1 (400 nM) was added to initiate the reaction, along with a DNA trap (60 nM). At increasing times, the reactions were quenched with 50 mM EDTA and 0.5% SDS. (b) Gel images of 3'-tailed RNA:DNA hybrid duplex unwound by Ded1 with indicated experimental conditions. (c) The reaction progress curves. Data were fit to a single exponential. Experiments were repeated twice independently.

Supplementary Figure S4. Phen-DC3 inhibits RNA:RNA duplex unwinding by Ded1. (a) Diagram illustrating the experiments. 32P-labelled 3'-tailed RNA:RNA duplexes (0.5 nM) were pre-incubated with helicases Ded1 (200 nM) in helicase reaction buffer (40 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5 mM MgCl2, 2 mM DTT, 1 unit/µL RNasin, 0.01% NP40, 8% glycerol) at 19°C for 5 minutes. ATP/Mg2+ (2 mM) was added to initiate the reaction. At increasing times, the reactions were quenched by
adding stop buffer (50 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, 20% glycerol). All Phen-DC3 reactions had 1% final DMSO concentration. (b) Gel images of 3’-tailed RNA:RNA duplex unwound by Ded1 with indicated experimental conditions. (c) The graph of the unwinding rate constants. Data were fit to a single exponential.
Supplementary Figure S2

**a**

![Diagram](image)

**b**

| Time, min | RNA:DNA | ssRNA | sG4RNA |
|-----------|---------|-------|--------|
| 0         |         |       |        |
| 25        |         |       |        |
| 5         |         |       |        |
| 10        |         |       |        |

**c**

| Time (min) | Ded1+ATP | Trap only | Ded1+ATP +10uM PhenDC3 | Ded1+ATP +0.5uM PhenDC3 |
|------------|-----------|-----------|------------------------|-------------------------|
| 0          | 0.2       | 0.2       | 0.2                    | 0.2                     |
| 2          | 0.4       | 0.4       | 0.4                    | 0.4                     |
| 4          | 0.6       | 0.6       | 0.6                    | 0.6                     |
| 6          | 0.8       | 0.8       | 0.8                    | 0.8                     |
| 8          | 1.0       | 1.0       | 1.0                    | 1.0                     |
| 10         | 1.0       | 1.0       | 1.0                    | 1.0                     |

**Ded1+ATP**  $k = 1.02 \pm 0.03$ min$^{-1}$

**Trap only**  $k = 0.41 \pm 0.01$ min$^{-1}$

**Ded1+ATP+10uM PhenDC3**

**Ded1+ATP+0.5uM PhenDC3**
Supplementary Figure S3

a 3'-tailed RNA/DNA hybrid duplex unwound by Ded1

b 3'-tailed RNA/DNA hybrid duplex unwound by Ded1

Ded1+ATP  Ded1, w/o ATP  Ded1+ATP +10uM PhenDC3  Ded1+ATP +0.5uM PhenDC3

Time, min 0 2.5 5 10 15 20 0 2.5 5 10 15 20 0 2.5 5 10 15 20 0 2.5 5 10 20

RNA:DNA

ssRNA

k = 2.06 ± 0.15 min⁻¹
k = 2.34 ± 0.14 min⁻¹

Ded1+ATP +10uM PhenDC3

Ded1, w/o ATP

Ded1+ATP +0.5uM PhenDC3

Ded1+ATP

Fraction RNA/DNA unwound

Time (min)
Supplementary Figure S4

(a) ATP, Mg^{2+}\quad \text{Helicase}\quad \text{RNA:RNA} + \rightarrow \text{RNA:RNA} +

(b) 3'-tailed 16bp RNA:RNA duplex unwound by Ded1

| Time (min) | No DMSO | 1% DMSO | 0.1 uM DC3 | 1 uM DC3 | 10 uM DC3 |
|-----------|---------|---------|------------|----------|-----------|
| 0 10 30 1' 3' 15' |         |         |            |          |           |
| 0 10 30 1' 3' 15' |         |         |            |          |           |
| 0 10 30 1' 3' 15' |         |         |            |          |           |
| 0 10 30 1' 3' 15' |         |         |            |          |           |

(c) PhenDC3 Effect on $k_{unw}$

PhenDC3 Effect on $k_{unw}$

2 mM ATP

- No DMSO
- 1% DMSO
- 0.1 uM DC3
- 1 uM DC3
- 10 uM DC3

$k_{unw}$ (min$^{-1}$)

0 0.5 1 1.5 2 2.5 3