Table S1. Identification of RG/RGG repeat sequences within the protein sequences of the DEAD-box family using phase separation databases, including PhaSepDB, PhaSePro, LLPSDB v2.0, and DrLLPS.

|  |  |  |
| --- | --- | --- |
| Gene | Species | The number of RG/RGG |
| DDX3X | Human | RG\*10 RGG\*1 |
| DDX4 | Human | RG\*5 RGG\*1 |
| DDX5 | Human | RG\*9 RGG\*2 |
| DDX6 | Human | RG\*2 |
| DDX17 | Human | RG\*4 RGG\*5 |
| DDX21 | Human | RG\*9 |
| DBP1 | Yeast | RG\*4 RGG\*3 |
| DBP2 | Yeast | RG\*3 RGG\*7 |
| DED1 | Yeast | RG\*4 RGG\*4 |
| DHH1 | Yeast | RG\*1 |
| LAF-1 | Caenorhabditis elegans | RG\*11 RGG\*8 |
| RhlE | Escherichia coli | RG\*4 RGG\*2 |
| DeaD | Escherichia coli | RG\*8 RGG\*1 |

Table S2. Plasmids used in this article

|  |  |
| --- | --- |
| Plasmid | Characterization of plasmids |
| pET23a-sfGFP | sfGFP(Superfold Green Fluorescent Protein) |
| pET23a-DED1NC | DED1N-sfGFP-DED1C |
| pET23a-DED1N | DED1N-sfGFP-DED1N |
| pET23a-DED1C | DED1C-sfGFP-DED1C |
| pET23a-DDX3XNC | DDX3XN-sfGFP-DDX3XC |
| pET23a-DDX3XN | DDX3XN-sfGFP-DDX3XN |
| pET23a-DDX3XC | DDX3XC-sfGFP-DDX3XC |
| pET23a-DBP1NC | DBP1N-sfGFP-DBP1C |
| pET23a-DBP1N | DBP1N-sfGFP-DBP1N |
| pET23a-DBP1C | DBP1C-sfGFP-DBP1C |
| pET23a-DED1N-RGG\*0 | DED1N(R51S/R62S)-sfGFP-DED1N(R51S/R62S) |
| pET23a-DED1N-RGG | DED1N(R51S)-sfGFP-DED1N(R51S) |
| pET23a-DED1N-GGRGGG | DED1N(R62S)-sfGFP-DED1N(R62S) |
| pET23a-DED1C-RGG\*0 | DED1C(R545S/R578S)-sfGFP-DED1C(R545S/R578S) |
| pET23a-DED1C-RGG\*3 | DED1C(A559R)-sfGFP-DED1C(A559R) |
| pET23a-DED1C-RGG\*4 | DED1C(A559R /S595R)-sfGFP-DED1C(A559R/S595R) |
| pET23a-DED1C-R2RG4 | DED1C(S581G)-sfGFP-DED1C(S581G) |
| pET23a-DED1C-R3RG4 | DED1C(A559R/S581G)-sfGFP-DED1C(A559R/S581G) |
| pET23a-DED1C-R4RG4 | DED1C(A559R/S581G/S595R)-sfGFP-DED1C(A559R/S581G/S595R) |
| pET23a-DED1C-R2GGRGG | DED1C(S576G/F577G)-sfGFP-DED1C(S576G/F577G) |
| pET23a-DED1C-R3GGRGG | DED1C(A559R/S576G/F577G)-sfGFP-DED1C(A559R/S576G/F577G) |
| pET23a-DED1C-R4GGRGG | DED1C(A559R/S576G/F577G/S595R)-sfGFP-DED1C(A559R/S576G/F577G/S595R) |
| pET23a-DED1C-R2GGRG4 | DED1C(S576G/F577G/S581G)-sfGFP-DED1C(S576G/F577G/S581G) |
| pET23a-DED1C-R3GGRG4 | DED1C(A559R/S576G/F577G/S581G)-sfGFP-DED1C(A559R/S576G/F577G/S581G) |
| pET23a-DED1C-R4GGRG4 | DED1C(A559R/S576G/F577G/S581G/S595R)-sfGFP-DED1C(A559R/S576G/F577G/S581G/S595R) |
| pET23a-DBP1N-RGG\*2 | DBP1N(N78R)-sfGFP-DBP1N(N78R) |
| pET23a-DBP1N-N18R | DBP1N(N18R)-sfGFP-DBP1N(N18R) |
| pET23a-DBP1N-N78R | DBP1N(N78R)-sfGFP-DBP1N(N78R) |
| pET23a-DBP1C-RGG\*0 | DBP1C(R550S/R555S)-sfGFP-DBP1C(R550S/R555S) |
| pET23a-DDX3XN-RGG\*2 | DDX3XN(S114G)-sfGFP-DDX3XN(S114G) |
| pET23a-DDX3XN-S28R | DDX3XN(S28R)-sfGFP-DDX3XN(S28R) |
| pET23a-DDX3XN-S114G | DDX3XN(S114G)-sfGFP-DDX3XN(S114G) |
| pET23a-DDX3XC-RGG\*2 | DDX3XC(S622G/G624SR)-sfGFP-DDX3XC(S622G/G624SR) |
| pET23a-DDX3XC-22 | DDX3XC(S622G/G624SR)-sfGFP-DDX3XC(S622G/G624SR) |
| pET23a-DDX3XC-25 | DDX3XC(S622G/Y638YR)-sfGFP-DDX3XC(S622G/Y638YR) |
| pET23a-DDX3XC-56 | DDX3XC(S622G/F633G)-sfGFP-DDX3XC(S622G/F633G) |
| pET23a-DDX3XC-59 | DDX3XC(S622G/F633G/Y638G)-sfGFP-DDX3XC(S622G/F633G/Y638G) |
| pET23a-DDX3XC-RGG | DDX3XC(S622G/G624SR)-sfGFP-DDX3XC(S622G/G624SR) |
| pET23a-DDX3XC-GGRGG | DDX3XC(S622G/G624R/Y638R)-sfGFP-DDX3XC(S622G/G624R/Y638R) |

Table S3. Primers associated with targeted mutations

|  |  |
| --- | --- |
| Primers | Primer Sequences (5’-3’) |
| DED1-R51S-F | GCGGCTACAACGGTGGCAGTGGCGGTGGCAGCTTCTTTAG |
| DED1-R51S-R | GAAGCTGCCACCGCCACTGCCACCGTTGTAGCCGCCGTTG |
| DED1-R62S-F | CTTTAGCAACAACCGTAGTGGTGGTTACGGCAACGGTGG |
| DED1-R62S-R | CGTTGCCGTAACCACCACTACGGTTGTTGCTAAAGAAGC |
| DED1-R545S-F | CAGAAGCAACAGCCGTAGTGGCGGTTTCGGTCGCAACAAC |
| DED1-R545S-R | TTGCGACCGAAACCGCCACTACGGCTGTTGCTTCTGCTAC |
| DED1-R578S-F | GCAGAGATAACTCTTTCAGTGGCGGTAGTGGCTGGGGTAG |
| DED1-R578S-R | CCCCAGCCACTACCGCCACTGAAAGAGTTATCTCTGCTTC |
| DED1-A559R-F | ACAGAGATTACCGTAAGAGAGGAGGCGCTAGCGCAGGCGG |
| DED1-A559R-R | TGCGCTAGCGCCTCCTCTCTTACGGTAATCTCTGTTGTTG |
| DED1-S595R-F | CTTCTGGCTGGGGTAACAGAGGTGGTTCAAACAACTCTTC |
| DED1-S595R-R | GTTGTTTGAACCACCTCTGTTACCCCAGCCAGAAGACTTG |
| DED1-S581G-F | TCTTTCAGAGGCGGTGGTGGCTGGGGTAGCGATTCCAAG |
| DED1-S581G-R | TCGCTACCCCAGCCACCACCGCCTCTGAAAGAGTTATC |
| DED1-S576G/F577G-F | GAAGCAGAGATAACGGTGGCAGAGGCGGTAGTGGCTGGGG |
| DED1-S576G/F577G-R | CCACTACCGCCTCTGCCACCGTTATCTCTGCTTCTTGAAG |
| DBP1-N18R-F | CATCAACAACAAAGAGAGGGGTGGTGGTGGCGGCAAATC |
| DBP1-N18R-R | GCCGCCACCACCACCCCTCTCTTTGTTGTTGATGCTTAAATTAG |
| DBP1-N78R-F | TTTTCTAAGGAAAGAGGTGGAGGGACGAGTGCGAACTATAAC |
| DBP1-N78R-R | ATAGTTCGCACTCGTCCCTCCACCTCTTTCCTTAGAAAAGCC |
| DBP1-R550S-F | GTCAAGACAGAATTCAAGTGGTGGAAGAACTAGGGGAGG |
| DBP1-R550S-R | CCCCTAGTTCTTCCACCACTTGAATTCTGTCTTGACAGG |
| DBP1-R555S-F | TCAAGAGGTGGAAGAACTAGTGGAGGTCAAGAGGTGGAAGAACTAG |
| DBP1-R555S-R | GAAAAAACCTCCTCCTCCACTAGTTCTTCCACCTCTTGAATTC |
| DDX3X-S28R-F | CTTCAGATAATCAGCGTGGAGGAAGTACAGCCAGCAAAG |
| DDX3X-S28R-R | TGGCTGTACTTCCTCCACGCTGATTATCTGAAGAGTTCAG |
| DDX3X-S114G-F | CAGCCGTGGTGACAGAGGTGGCTTTGGCAAATTTGAACG |
| DDX3X-S114G-R | AATTTGCCAAAGCCACCTCTGTCACCACGGCTGCCAATG |
| DDX3X-S622G-F | CGCAAGCAGCAGCCGCGGTGGCGGAGGTGGCCACGGTAG |
| DDX3X-S622G-R | CGTGGCCACCTCCGCCACCGCGGCTGCTGCTTGCGCGGC |
| DDX3X-G624SR-F | CAGCAGCCGCAGTGGCAGTCGAGGTGGCCACGGTAGCAGCAG |
| DDX3X-G624SR-R | TGCTACCGTGGCCACCTCGACTGCCACTGCGGCTGCTGCTTG |
| DDX3X-G624R-F | CAGCAGCCGCAGTGGCCGAGGTGGCCACGGTAGCAGCAG |
| DDX3X-G624R-R | TGCTACCGTGGCCACCTCGGCCACTGCGGCTGCTGCTTG |
| DDX3X-F633G-F | CGGTAGCAGCAGAGGAGGTGGTGGAGGTGGCTATGGAGG |
| DDX3X-F633G-R | CATAGCCACCTCCACCACCTCCTCTGCTGCTACCGTGGC |
| DDX3X-Y638YR-F | ATTTGGTGGAGGTGGCTATCGTGGAGGCTTTTACAACAGTG |
| DDX3X-Y638YR-R | TGTTGTAAAAGCCTCCACGATAGCCACCTCCACCAAATCCTC |
| DDX3X-Y638R-F | ATTTGGTGGAGGTGGCCGTGGAGGCTTTTACAACAGTG |
| DDX3X-Y638R-R | TGTTGTAAAAGCCTCCACGGCCACCTCCACCAAATCCTC |
| DDX3X-Y638G-F | ATTTGGTGGAGGTGGCGGTGGAGGCTTTTACAACAGTG |
| DDX3X-Y638G-R | TGTTGTAAAAGCCTCCACCGCCACCTCCACCAAATCCTC |



**Supplemental Figure S1.** A) Expression of DDX3XNC/ DDX3XN/ DDX3XC in a cell-free system (4 h at 30℃) followed by 30 min incubation at different temperatures (30, 37, and 42℃). Phase separation and aggregation were assessed by fluorescence microscopy. B) Solubility analysis using Western blot analysis of the supernatant (S) and pellet (P) fractions at 37℃.



**Supplemental Figure S2.** Comparison of self-aggregation in living cells, cell-free protein synthesis (CFPS) systems, and purified proteins at different temperatures (25, 30, 37 ℃). Left panel: Fluorescence microscopy images with a Scale bar of 20 μm. Right panel: Enlarged fluorescence images highlighting the morphology of the aggregates (Scale bar, 2 μm).

**Phase-separated protein expression purification**

The pET28a-DED1NC plasmid was constructed and transformed into E. coli BL21 (DE3) for protein expression. The monoclonal strains were harvested and cultured in LB medium at 37°C with an oscillation frequency of 220 rpm until OD600 = 0.6-0.8. They were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Cat. I1020, Solarbio) and cultured overnight at 18°C and 220 rpm for protein expression. Bacteria were resuspended in high salt lysis buffer (500 mM NaCl, 20 mM Tris, 20 mM imidazole, 1 mM PMSF, pH 7.5) and sonicated. It was then purified on a nickel column (Cat. P2011, Solarbio) and eluted in 8ULB buffer (200 mM NaCl, 20 mM Tris, 200 mM imidazole, 8 M urea, pH 7.5). Finally, 10 kDa ultrafiltration tubes (Cat. UFC801096, Solarbio) were used and the solvent was changed to high salt buffer (500 mM NaCl, 20 mM Tris, pH 7.5) to prevent phase separation. Quick freeze in liquid nitrogen and store at -80°C.