

Expanded View Figures

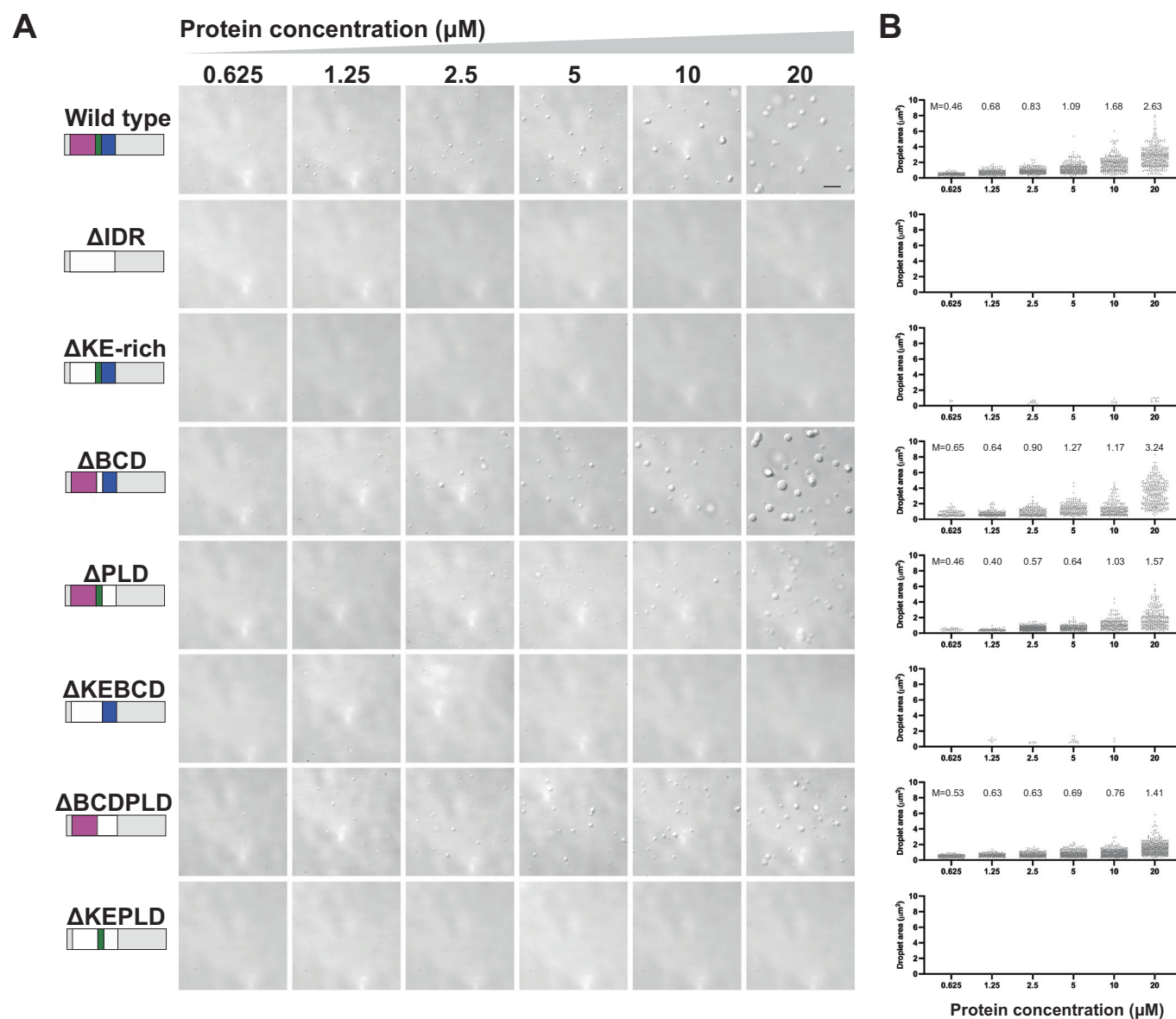


Figure EV1. *BtRho* variants harboring the KE-rich or PLD subdomains drive condensation.

(A) DIC microscopy of in vitro condensation assays of wild-type *BtRho* and variants at the indicated protein concentrations. The results corresponding to protein concentrations of 5, 10, and 20 μM are also shown in Fig. 2A. Three independent experiments were performed, and a representative experiment is shown. Scale bar: 5 μm . (B) Quantification of droplet size formed in (A) for each *BtRho* variant. For each condition, the counted droplets were from three different fields of view of the same sample. The median (M) value is also indicated for each condition.

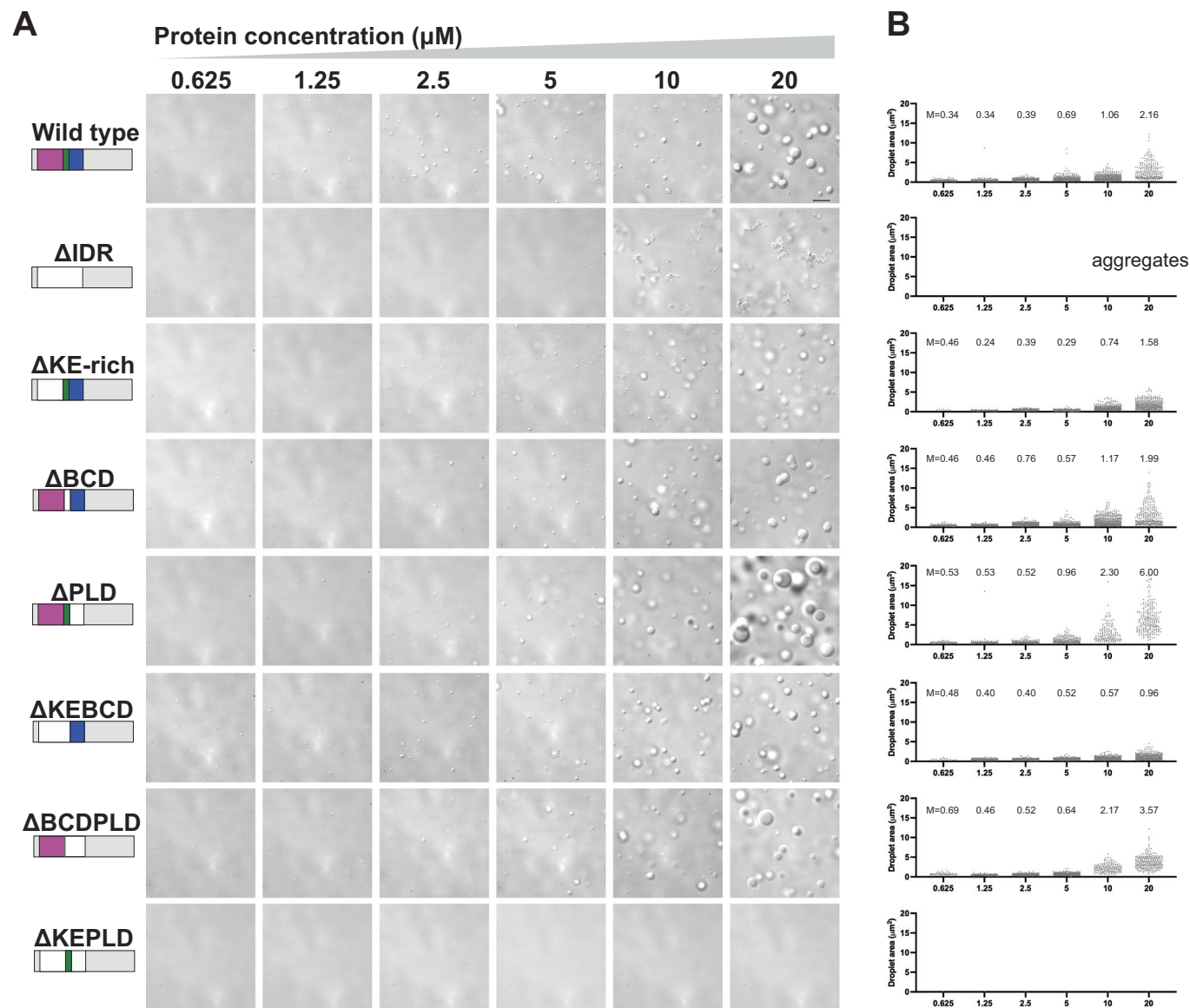


Figure EV2. BtRho variants harboring the KE-rich or PLD subdomains form large condensates in the presence of the crowding factor dextran.

(A) DIC microscopy of in vitro condensation assays of wild-type BtRho and variants at the indicated protein concentration and in the presence of 10% w/v dextran. The results corresponding to protein concentration 20 μM are also shown in Fig. 2A. Three independent experiments were performed, and a representative experiment is shown. Scale bar: 5 μm . (B) Size quantification of droplets formed in (A) for each protein variant. For each condition the droplets counted were from 3 different fields of view of the same sample. The median (M) value is also indicated for each condition.

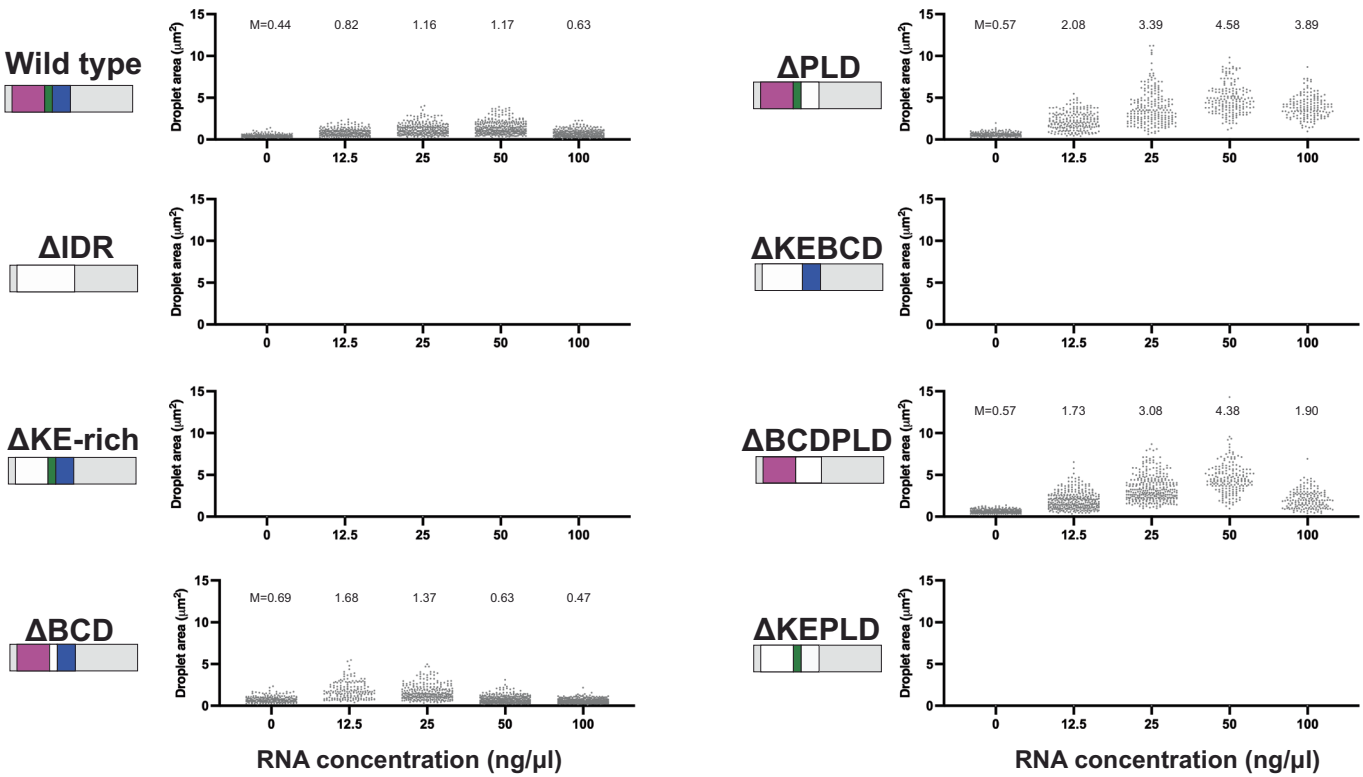


Figure EV3. The KE-rich subdomain is required for RNA-dependent *Btrho* condensation in vitro.

Size quantification of droplets formed in Fig. 2B for each investigated protein. For each condition the droplets counted were from three different fields of view of the same sample. The median (M) value is also indicated for each condition.

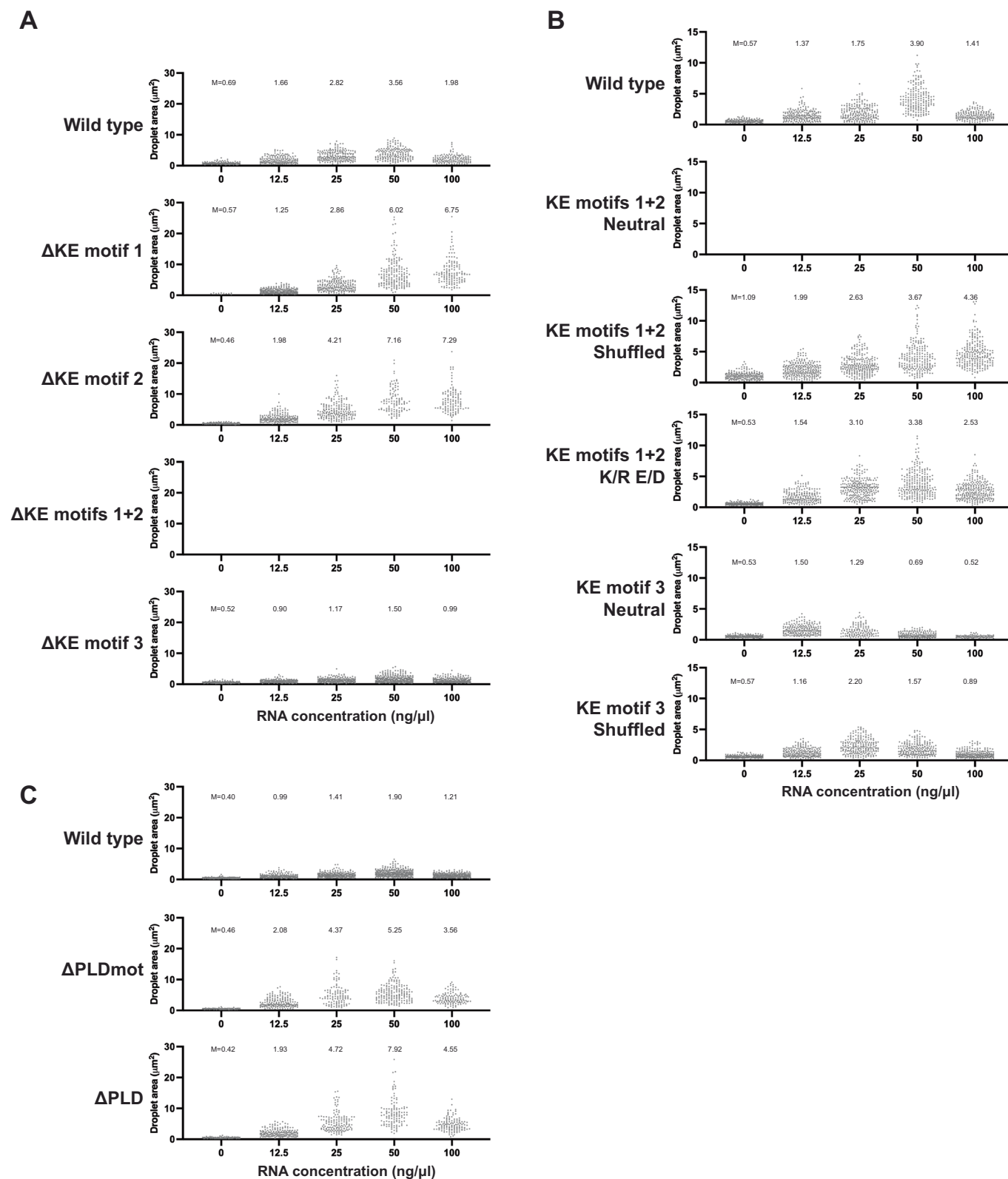
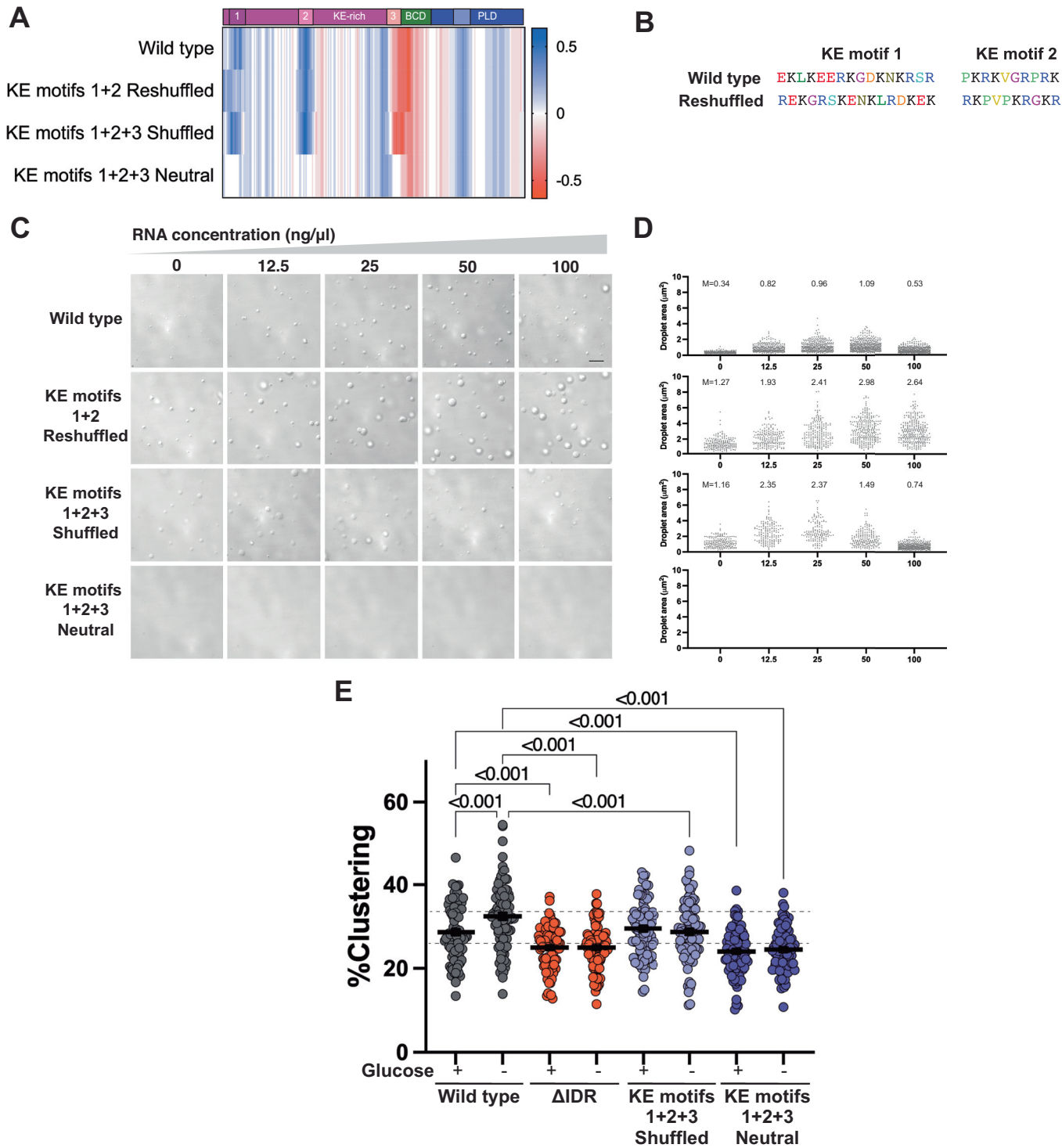


Figure EV4. Sequence motifs within the KE-rich and PLD subdomains control RNA-dependent BtRho condensation in vitro.

Size quantification of droplets formed in Fig. 3D (A), Fig. 3F (B) and Fig. 4C (C) for the indicated variants. For each condition, the droplets counted were from three different fields of view of the same sample. The median (M) value is also indicated for each condition.



◀ **Figure EV5. KE motifs 1, 2, and 3 control BtRho condensation.**

(A) Heatmap of the amino acid charge per residue (EMBOSS (Rice et al, 2000), sliding window 11 amino acids) for wild-type BtRho and the indicated variants. (B) Amino acid sequence of wild-type KE motifs 1 and 2 and of the indicated variants. (C) DIC microscopy of in vitro condensation assays of wild-type BtRho and variants (2.5 μ M) in the presence of increasing RNA amounts corresponding to total RNA extract from *B. thetaiotaomicron*. Three independent experiments were performed, and a representative experiment is shown. Scale bar: 5 μ m. (D) Size quantification of droplets formed in (C) for each variant. For each condition, the droplets counted were from three different fields of view of the same sample. The median (M) value is also indicated for each condition. (E) In vivo BtRho condensation calculated as % Clustering in *B. thetaiotaomicron* strains expressing HA-tagged versions of the indicated proteins (wild type: AK600, Δ IDR: AK602, KE motifs 1 + 2 + 3 Shuffled: AK660, and KE motifs 1 + 2 + 3 Neutral: AK658). Bacteria were grown in glucose (+) until mid-exponential phase and then shifted to a media without any carbon source for 30 min (–). Data points represent clustering values of individual cells from three independent experiments ($n = 90$), black bars are mean values and error bars represent SEM. Dashed lines are used as visual aids to indicate the levels of clustering for wild-type BtRho in carbon starvation and for Δ IDR BtRho. One-way ANOVA was performed, followed by pairwise comparisons between wild-type and the mutant variants for the same growth condition or between the two conditions for the same strain. P values < 0.05 are shown. Šidák's test was used to correct for multiple comparisons.

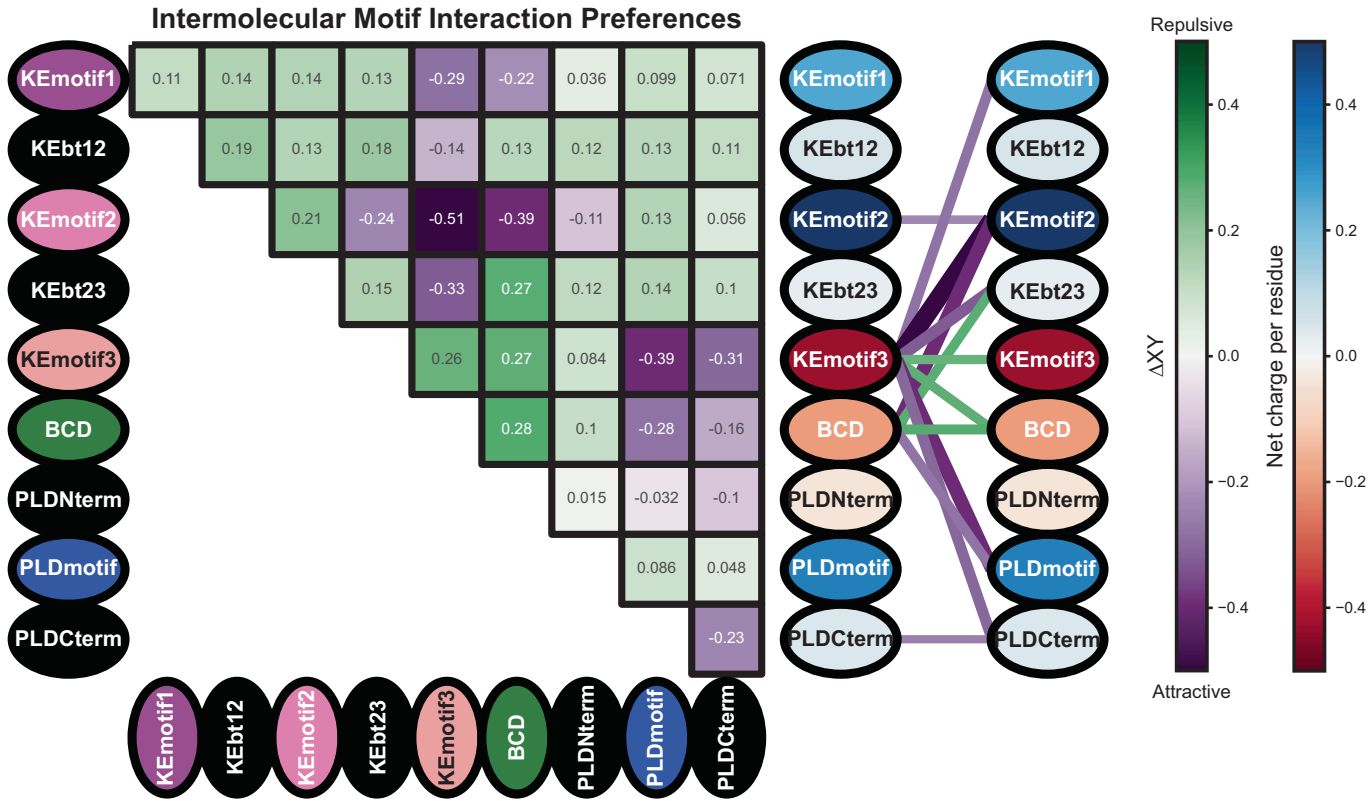


Figure EV6. Two molecule simulations reveal intermolecular interactions between the identified motifs in *BtRho*.

Two molecule simulations were run by splitting up the *BtRho* IDR into nine pieces (KE motif 1, KEbt12 (region between motifs 1 + 2), KE motif 2, KEbt23 (region between motifs 2 and 3), KE motif 3, BCD, PLD Nterm, PLDmotif, PLD Cterm) and then determining the effective interaction between each pair of pieces (ΔXY). Negative values imply attraction and positive values imply repulsion. Lines are drawn between two pieces if $|\Delta XY| \geq 0.23$ and weighted by their interaction strength.

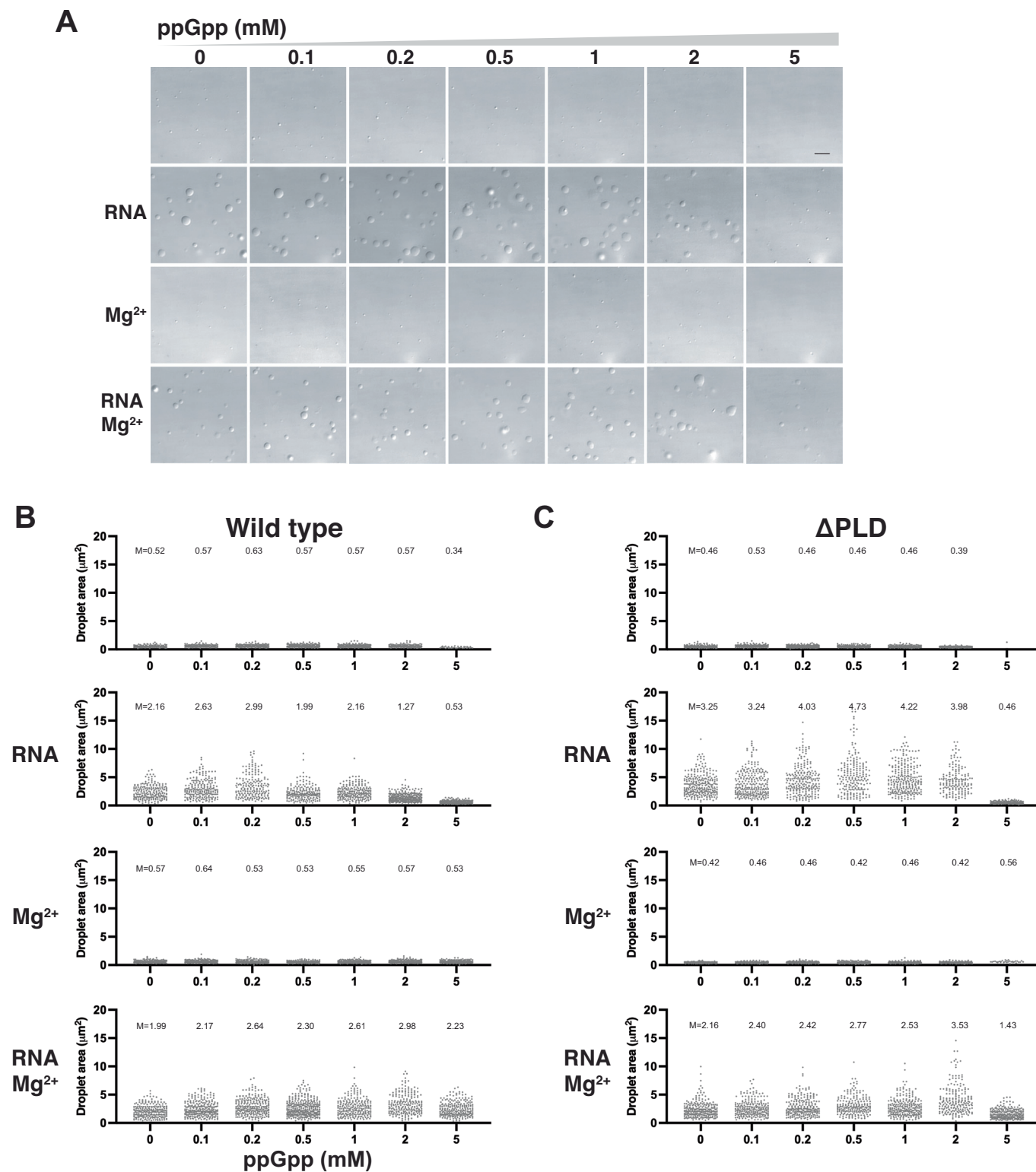


Figure EV7. ppGpp promotes RNA-dependent BtRho phase separation in vitro.

(A) DIC microscopy of in vitro condensation of the BtRho ΔPLD protein (2.5 μM) in the presence of increasing ppGpp concentration, RNA (12.5 ng/μl), and/or MgCl₂ (2.5 mM). Three independent experiments were performed, and a representative experiment is shown. Scale bar: 5 μm. (B) Size quantification of droplets formed in Fig. 6F for each condition. (C) Size quantification of droplets formed in (A) for each condition. For each graph, the droplets counted were from three different fields of view of the same sample. The median (M) value is also indicated for each condition.