Figure S1, related to Figure 1

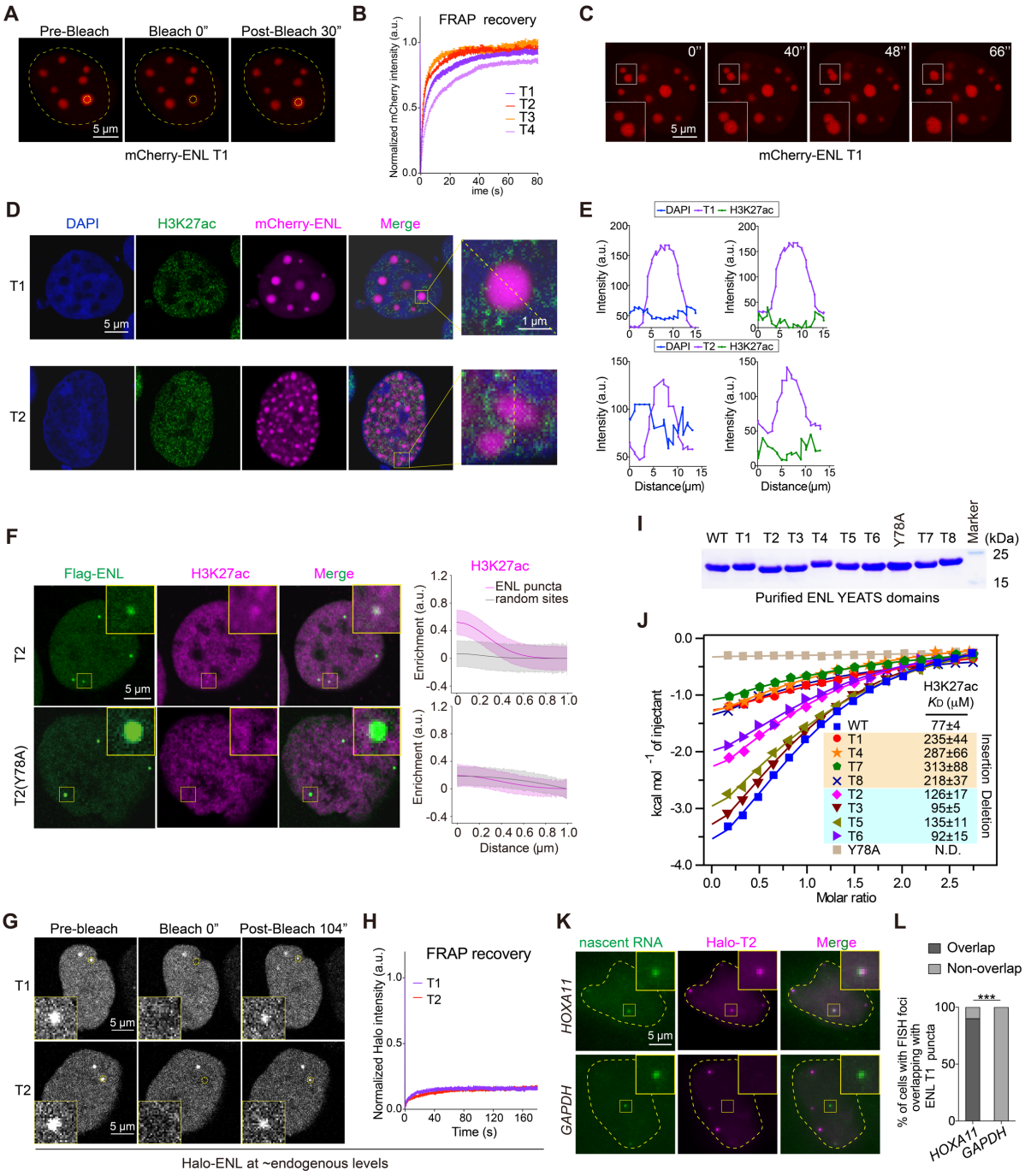

Figure S1. Hotspot mutations in the structured YEATS domain enhance ENL's intrinsic propensity to form condensates, Related to Figure 1.

(A) Representative fluorescence images for FRAP assay in HEK293 cells expressing mCherry-ENL-T1. The big yellow dashed circle indicates the nucleus, and the small yellow dashed circle indicates punctum subjected to bleaching. Scale bar, 5 μm.

(B) Average FRAP curves from areas inside mCherry-ENL puncta formed by indicated ENL mutants. Bleaching occurred at $t = 0$ s. Data represent Mean ± S.E.M.; $n = 11, 7, 10, 12$ for T1, T2, T3, and T4, respectively. One punctum was bleached in each cell.

(C) Representative images showing fusion of mCherry-ENL-T1 puncta. Scale bar, 5 μm.

(D) IF staining of H3K27ac (green) and mCherry-ENL mutants (magenta) demonstrating DNA (DAPI) (blue) and H3K27ac are excluded by puncta formed by markedly overexpressed mCherry-ENL in HEK293 cells. Scale bar, 5 μm. Scale bar for zoom-in image, 1 μm.

(E) Line plot of the dotted line in (D).

(F) IF staining of Flag-ENL (green) and H3K27ac (magenta) in HEK293 cells expressing indicated Flag-ENL constructs at near endogenous levels. The enrichment of H3K27ac at ENL puncta (magenta line) and random sites (gray line) was quantified. Puncta number = 78, 51 for T2, T2(Y78A), respectively. Scale bar, 5 μm.

(G) Representative images for FRAP assay in HEK293 cells expressing indicated Halo-ENL constructs at near endogenous levels. The yellow dashed circle indicates punctum subjected to bleaching. Scale bar, 5 μm.

(H) Average FRAP curves from areas of Halo-ENL puncta formed by indicated ENL mutants. Bleaching occurred at $t = 0$ s. Data represent Mean ± S.E.M.; $n = 29, 35$ for T1 and T2, respectively. One punctum was bleached in each cell.

(I) Coomassie staining of indicated purified ENL YEATS domain proteins.

(J) ITC titration fitting curves for purified ENL YEATS domain proteins (as shown in (I)), using a histone H3 peptide acetylated at lysine 27 (H3(17-28) K27ac).

(K) Representative images indicating the co-localization of Halo-ENL-T2 puncta with *HOXA11* nascent RNA FISH foci. Scale bar, 5 μm.

(L) Quantification of the percentage of cells ($n = 20$) in which one or more RNA FISH foci for indicated genes overlap with Halo-ENL-T2 puncta. Chi-square test, *** $P < 0.001$.

Figure S2, related to Figure 1

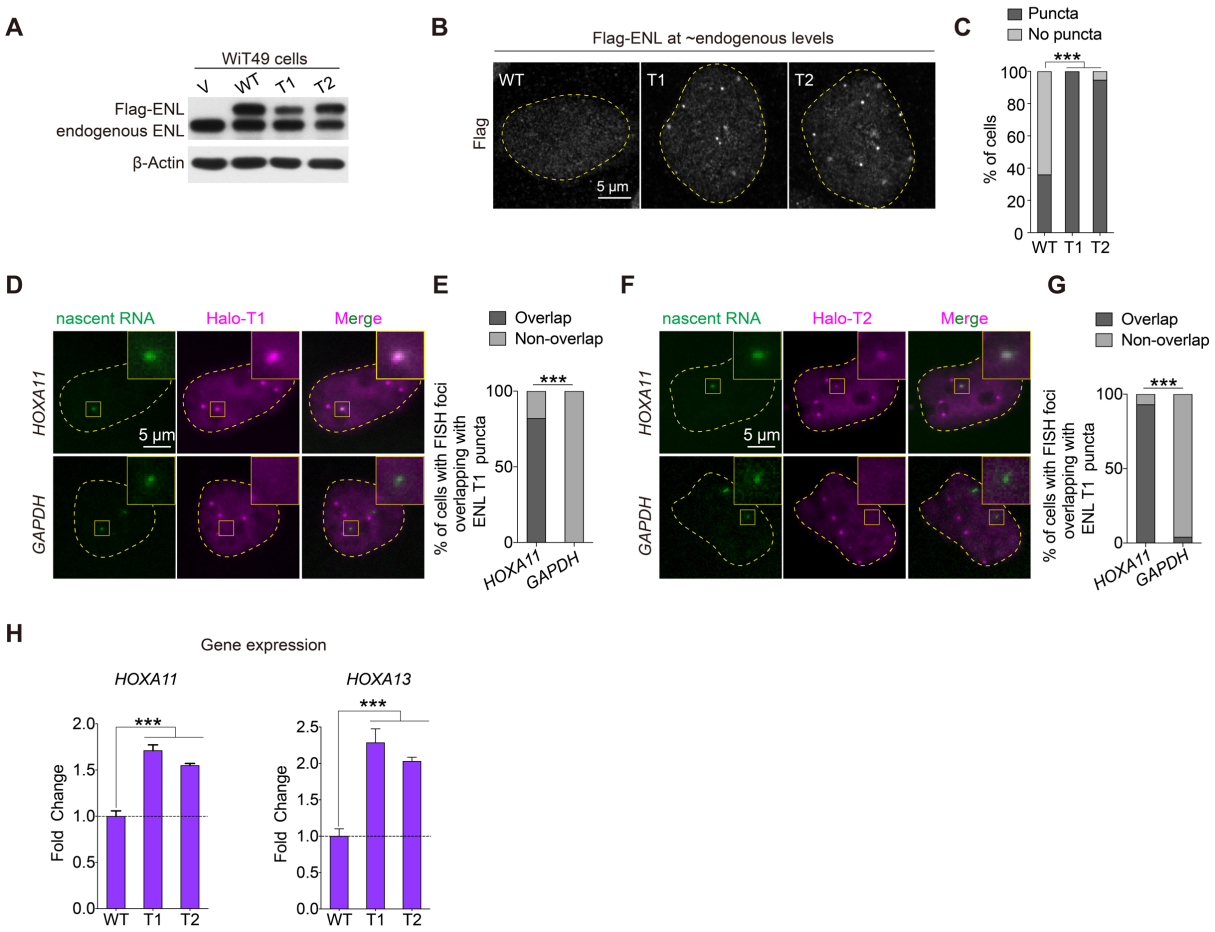

Figure S2. Near endogenous levels of ENL mutants form submicron-sized condensates at native genomic targets in the Wilms' tumor cell line WiT49, Related to Figure 1.

(A) Western blotting showing Flag-tagged WT and ENL mutant transgenes were expressed at near endogenous levels in WiT49 cells. V, empty vector.

(B) IF imaging of Flag-ENL in WiT49 cells expressing indicated Flag-ENL transgenes. Scale bar, 5 μm.

(C) Quantification of the percentage of nuclei with and without Flag-ENL puncta in each nucleus of WiT49 cells. $n = 25, 38, 50$ cells from left to right. Chi-square test; *** $P < 0.001$.

(D and F) Representative images indicating the co-localization of Halo-ENL-T1 (D) or T2 (F) puncta with *HOXA11* nascent RNA FISH foci. Scale bar, 5 μm.

(E and G) Quantification of the percentage of cells in which one or more RNA FISH foci for indicated genes overlap with Halo-ENL-T1 (E) (*HOXA11*, $n = 44$ cells; *GAPDH*, $n = 20$ cells) or Halo-ENL-T2 puncta (G) (*HOXA11*, $n = 38$ cells; *GAPDH*, $n = 26$ cells). Chi-square test, *** $P < 0.001$.

(H) mRNA expression (normalized to *GAPDH*) of *HOXA11* or *HOXA13* in WiT49 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Data shown are Mean ± S.D.. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

Figure S3, related to Figure 3

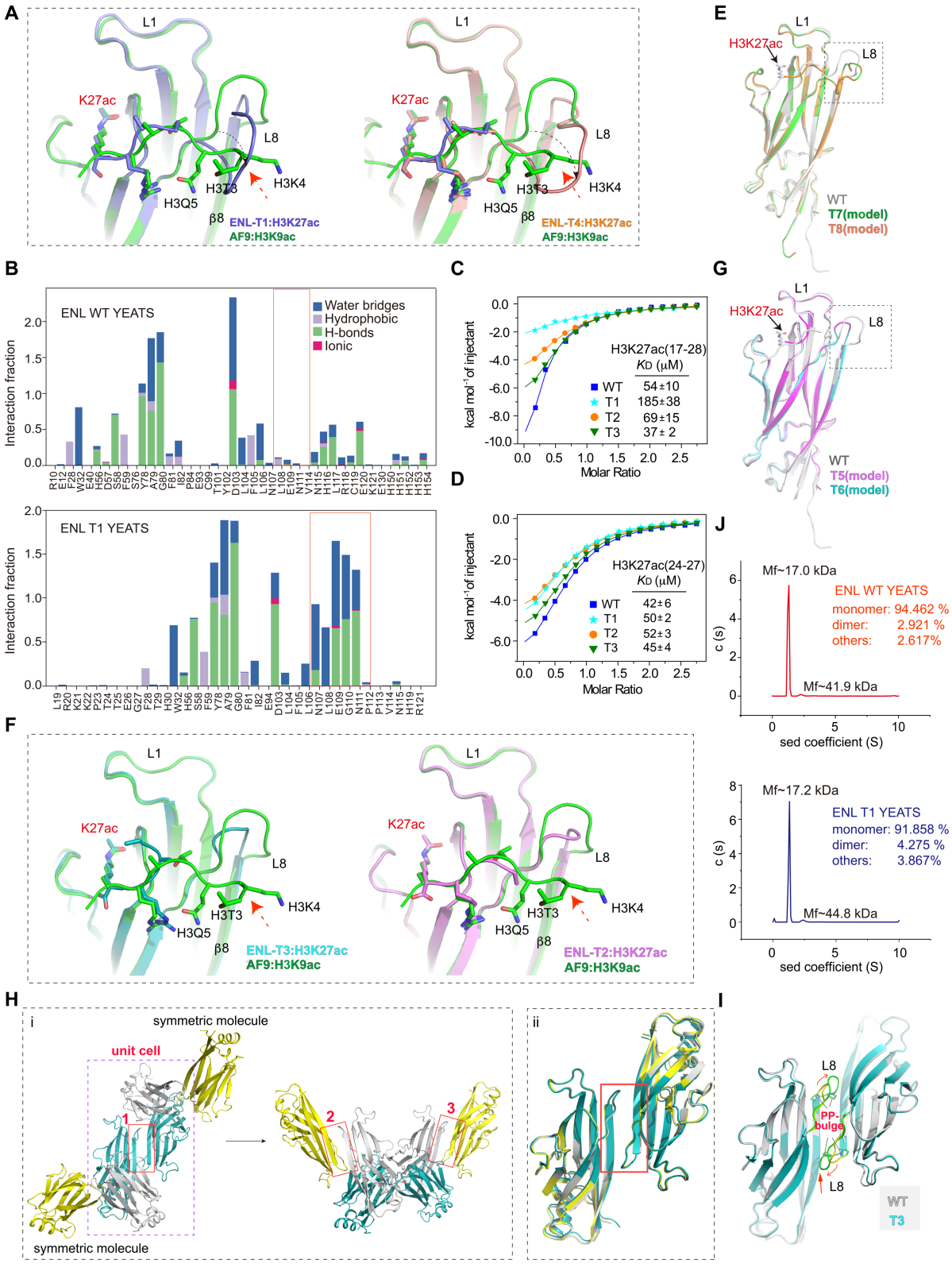

Figure S3. Insertion and deletion mutations induce consensus structural changes in the ENL YEATS domain, Related to Figure 3.

(A) Structural alignment between AF9:H3K9ac (PDB: 4TMP) and H3K27ac-bound ENL T1 (left) or T4 (right) showing steric clash between H3 N-terminal segment and the loop L8 in T1/T4.

(B) Molecular dynamics simulation results of ENL WT (top) and T1 (bottom) YEATS. Protein interactions are categorized by type and summarized. The stacked bar charts are normalized over the course of the trajectory. The residues on loop L8 are boxed.

(C and D) ITC titration fitting curves for the indicated ENL YEATS domains using a long H3(17-28) K27ac (C) or a short H3(24-27) K27ac (D) peptide.

(E) Superimposition of modeled ENL T7 (green) and T8 (orange) YEATS structures with WT:H3K27ac (gray).

(F) Structural alignment between AF9:H3K9ac (PDB: 4TMP) and H3K27ac-bound ENL T2 (right) or T3 (left) showing no steric clash between H3 N-terminal segment and loop L8 in T2/T3.

(G) Superimposition of modeled ENL T5 (magenta) and T6 (cyan) YEATS structures with WT:H3K27ac (gray).

(H) Analysis of crystal packing of H3K27-bound T3 YEATS domain. (i, left) there are four copies in the unit cell shown in the purple dashed box. For better understanding, two copies with a β8-β8 interface are colored in cyan, and the other two are shown in gray; another two symmetric YEATS molecules are generated and shown in yellow; the β8-β8 interface is highlighted in red box 1; (i, right) another view of (i, left). Yellow molecules can also form β8-β8 anti-parallel sheet with the gray molecules and the β8-β8 interface is highlighted in red box 2 and 3; (ii) three YD-YD 'dimer' mediated by each β8-β8 interface (1, 2, 3 shown in i) are superposed showing that they are identical and thus the β8-β8 association is a consensus dimer interface under crystal packing conditions.

(I) Overlay of WT (gray) and T3 (cyan) YEATS dimer showing that PP-bulge can act as blocker and cause steric clash incompatible with β8-β8 association. For loop L8, WT is in green and T3 is in yellow.

(J) Sedimentation coefficient c(s) distributions for ENL WT (top) and T1 (bottom) YEATS domains. The estimated molecular weight is labeled above the peaks. The percentage of monomer and dimer is calculated and annotated.

Figure S4, related to Figure 4

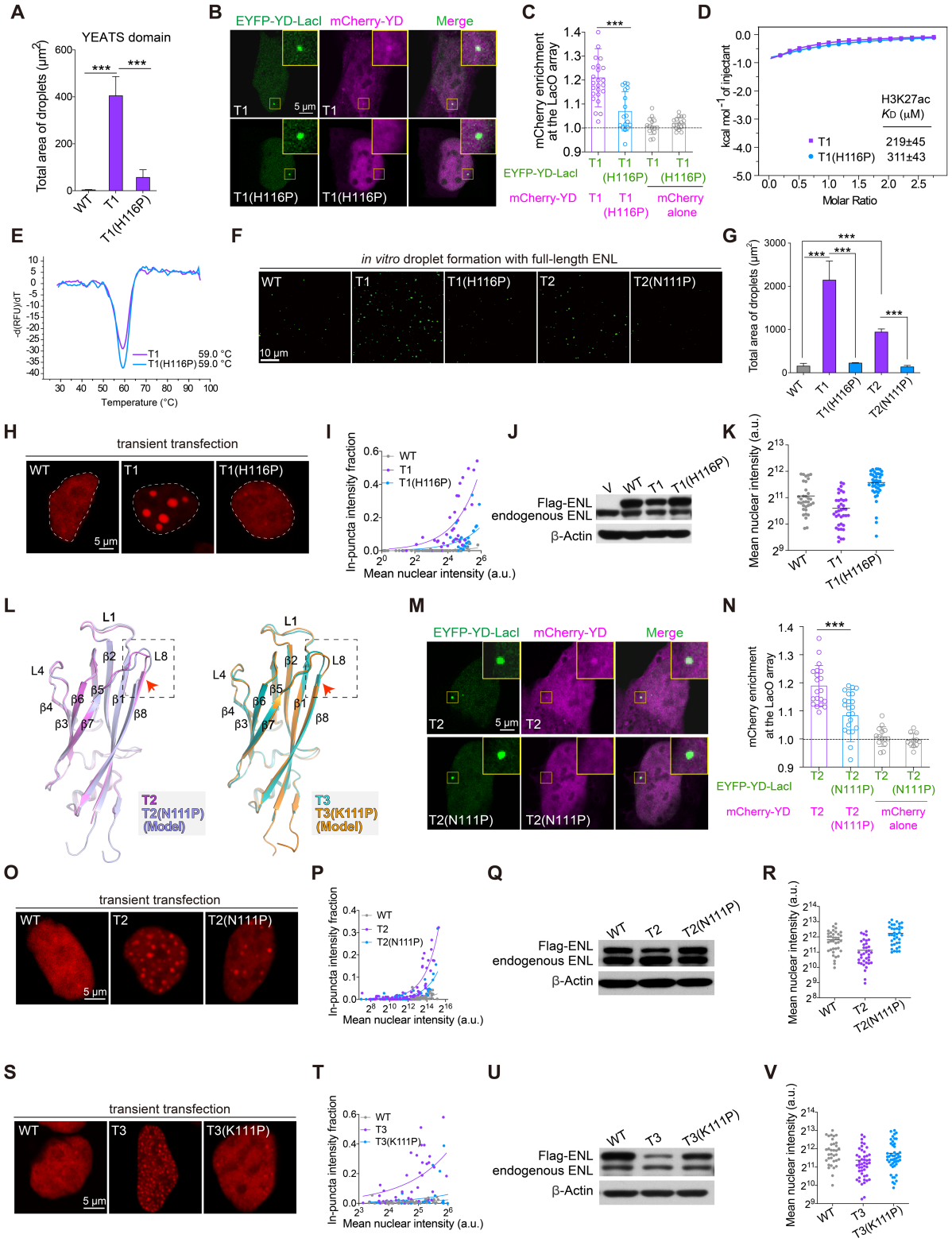

Figure S4. Reverting ENL mutation-induced structural changes abolishes condensate formation and function, Related to Figure 4.

(A) Quantification of the total area of droplets formed by indicated purified ENL YEATS domain proteins (60 μM). Data represent Mean ± S.D., two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

(B) Representative images of LacO-containing U2OS cells that co-expressed indicated mCherry-ENL(YD) and EYFP-ENL(YD)-LacI constructs. YD, YEATS domain. Yellow squares indicate the LacO array. Scale bar, 5 μm.

(C) Quantification of mCherry-ENL(YD) enrichment at the LacO array bound by indicated EYFP-ENL(YD)-LacI proteins. Enrichment of mCherry above 1 suggests YD-YD self-association. Data represents Mean ± S.D.; $n = 23, 17, 14, 17$ cells from left to right. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

(D) ITC titration fitting curves for ENL YEATS domain with the T1 or T1(H116P) mutation, using an H3(17-28) K27ac peptide.

(E) Thermal shift assay showing similar protein stability of purified T1 and T1(H116P) YEATS domains.

(F and G) Representative images (F) and quantification (G) of droplets formed by indicated full-length ENL variants (375 nM) *in vitro*. Scale bar, 10 μm. G, Data represent Mean ± S.D., two-tailed unpaired Student's *t*-test; *** $P < 0.001$. The data was collected from the same batch of experiments shown in Figure 1E.

(H) Representative images of HEK293 cells transiently transfected with indicated mCherry-ENL constructs. All cells shown expressed similar levels of mCherry-ENL proteins. Scale bar, 5 μm.

(I) Fraction of in-puncta fluorescence intensity in the nucleus of HEK293 cells transfected with indicated mCherry-ENL constructs as a function of mean nuclear intensity. Each dot indicates one cell (WT, $n = 42$; T1, $n = 42$; T1(H116P), $n = 40$).

(J) Western blotting showing WT and mutant Flag-ENL transgenes expressed at near endogenous levels in HEK293. V, empty vector.

(K) Mean Flag-ENL nuclear intensity in HEK293 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Each dot indicates one cell (WT, $n = 34$; T1, $n = 48$; T1(H116P), $n = 43$). Center lines indicate medians.

(L) Left, superimposition of modeled T2(N111P) YEATS structure (purple) with T2 YEATS:H3K27ac structure (magenta). Right, superimposition of modeled ENL T3(K111P) YEATS structure (orange) with T3 YEATS:H3K27ac structure (cyan).

(M) Representative images of LacO-containing U2OS cells that co-expressed indicated mCherry-ENL(YD) and EYFP-ENL(YD)-LacI constructs. Yellow squares indicate the LacO array. Scale bar, 5 μm.

(N) Quantification of mCherry-ENL(YD) enrichment at the LacO array bound by indicated EYFP-ENL(YD)-LacI proteins. Enrichment of mCherry above 1 suggests YD-YD association. Data represent Mean ± S.D.; $n = 20, 22, 14, 11$ cells from left to right. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

(O and S) Representative images of HEK293 cells transiently transfected with indicated mCherry-ENL constructs. All cells shown expressed similar levels of mCherry-ENL proteins. Scale bar, 5 μm.

(P and T) Fraction of in-puncta fluorescence intensity in the nucleus of HEK293 cells transfected with indicated mCherry-ENL constructs as a function of mean nuclear intensity. Each dot indicates one cell (*n* at the range of 37~89).

(Q and U) Western blotting showing Flag-ENL transgenes expressed at near endogenous levels in HEK293 cells.

(R and V) Quantification of nuclear intensity in HEK293 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Center lines indicate medians. Each dot indicates one cell (*n* at the range of 36~48).

Figure S5, related to Figure 5

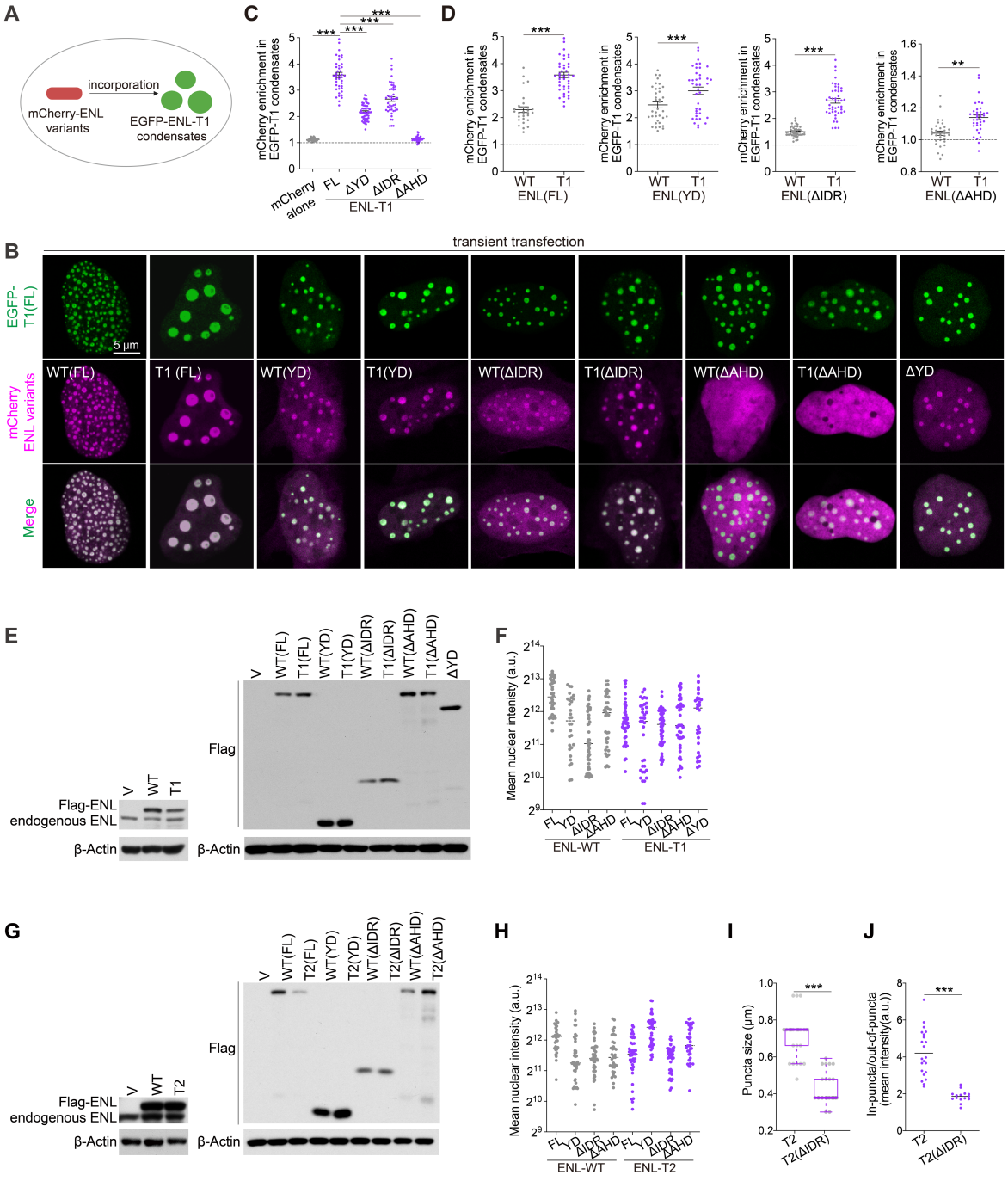

Figure S5. Multiple regions of ENL mutants are required for condensate formation and function, Related to Figure 5.

(A) Schematic depicting strategy to determine the contribution of each ENL region to intermolecular interactions. Full-length EGFP-ENL-T1 and mCherry-ENL deletion variants were co-transfected into U2OS cells. Degree of mCherry enrichment in EGFP-ENL-T1 condensates reflects intermolecular interactions.

(B) Representative images showing the co-localization of full-length EGFP-ENL-T1 (green) with indicated mCherry-ENL constructs (magenta). Scale bar, 5 μm.

(C) Quantification of mCherry enrichment in EGFP-ENL-T1 condensates. Enrichment of mCherry above 1 suggests interactions of indicated mCherry-ENL constructs with EGFP-ENL-T1. Each dot indicates one cell (*n* at the range of 30~52). Data represent Mean ± S.E.M.; two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

(D) Comparison of incorporation of indicated mCherry-ENL constructs with or without T1 mutation into EGFP-ENL-T1 condensates. Each dot indicates one cell (*n* at the range of 32~48). Data represent Mean ± S.E.M.; two-tailed unpaired Student's *t*-test, *** $P < 0.001$, ** $P < 0.01$.

(E and G) Western blotting showing indicated Flag-ENL transgenes expressed at near endogenous levels in HEK293 cells. V, empty vector.

(F and H) Mean Flag-ENL nuclear intensity in HEK293 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Each dot indicates one cell (F: *n* at the range of 30~60, H: *n* at the range of 33~48). Center lines indicate medians.

(I and J) Quantification of the puncta size (I) and the ratio of in-puncta/out-of-puncta intensity (J) in HEK293 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Each dot indicates one punctum (I: T2, $n = 26$; T2(ΔIDR2), $n = 30$; J: T2, $n = 22$; T2(ΔIDR2), $n = 15$). Center lines indicate median (I and J) and box limits (I) are set to the 25th and 75th percentiles. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

Figure S6, related to Figure 6

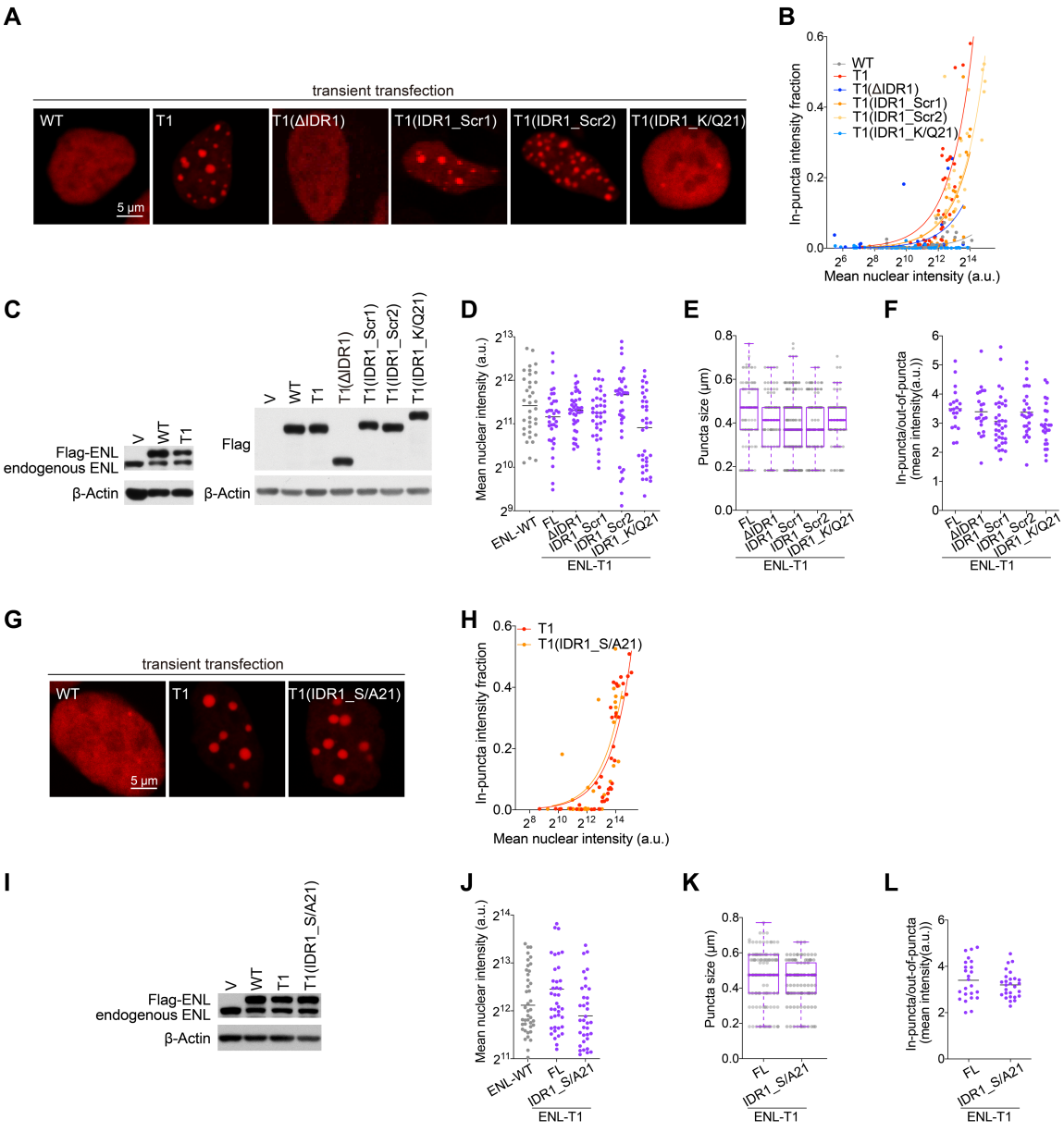

Figure S6. Positive charge-dependent interactions conferred by IDR1 regulate the initiation of ENL mutant condensates, Related to Figure 6.

(A and G) Representative images of HEK293 cells transiently transfected with indicated mCherry-ENL constructs. All cells shown expressed similar levels of mCherry-ENL proteins. Scr, primary sequence was scrambled. K/Q, lysine residues were substituted with glutamine. S/A, all serines in IDR1 residues were substituted with alanines. Scale bar, 5 μm.

(B and H) Fraction of in-puncta fluorescence intensity in the nucleus of HEK293 cells transfected with indicated mCherry-ENL constructs as a function of mean nuclear intensity. Each dot indicates one cell (*n* at the range of 30~59).

(C and I) Western blotting showing indicated Flag-ENL transgenes expressed at near endogenous levels in HEK293 cells. V, empty vector.

(D and J) Mean Flag-ENL nuclear intensity in HEK293 cells expressing indicated Flag-ENL at near endogenous levels. Each dot indicates one cell (*n* at the range of 35~51). Center lines indicate medians.

(E and K) Quantification of the size of Flag-ENL puncta in HEK293 cells expressing near endogenous level of indicated Flag-ENL transgenes. Each dot indicates one punctum (*n* at the range of 78~250). Center lines indicate median and box limits are set to the 25^{th} and 75^{th} percentiles. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

(F and L) Quantification of the ratio of in-puncta/out-of-puncta intensity in HEK293 cells expressing near endogenous levels of indicated Flag-ENL transgenes. Each dot indicates one punctum (*n* at the range of 20~36). Center lines indicate median. Two-tailed unpaired Student's *t*-test; *** $P < 0.001$.

Figure S7, related to Figures 6 and 7

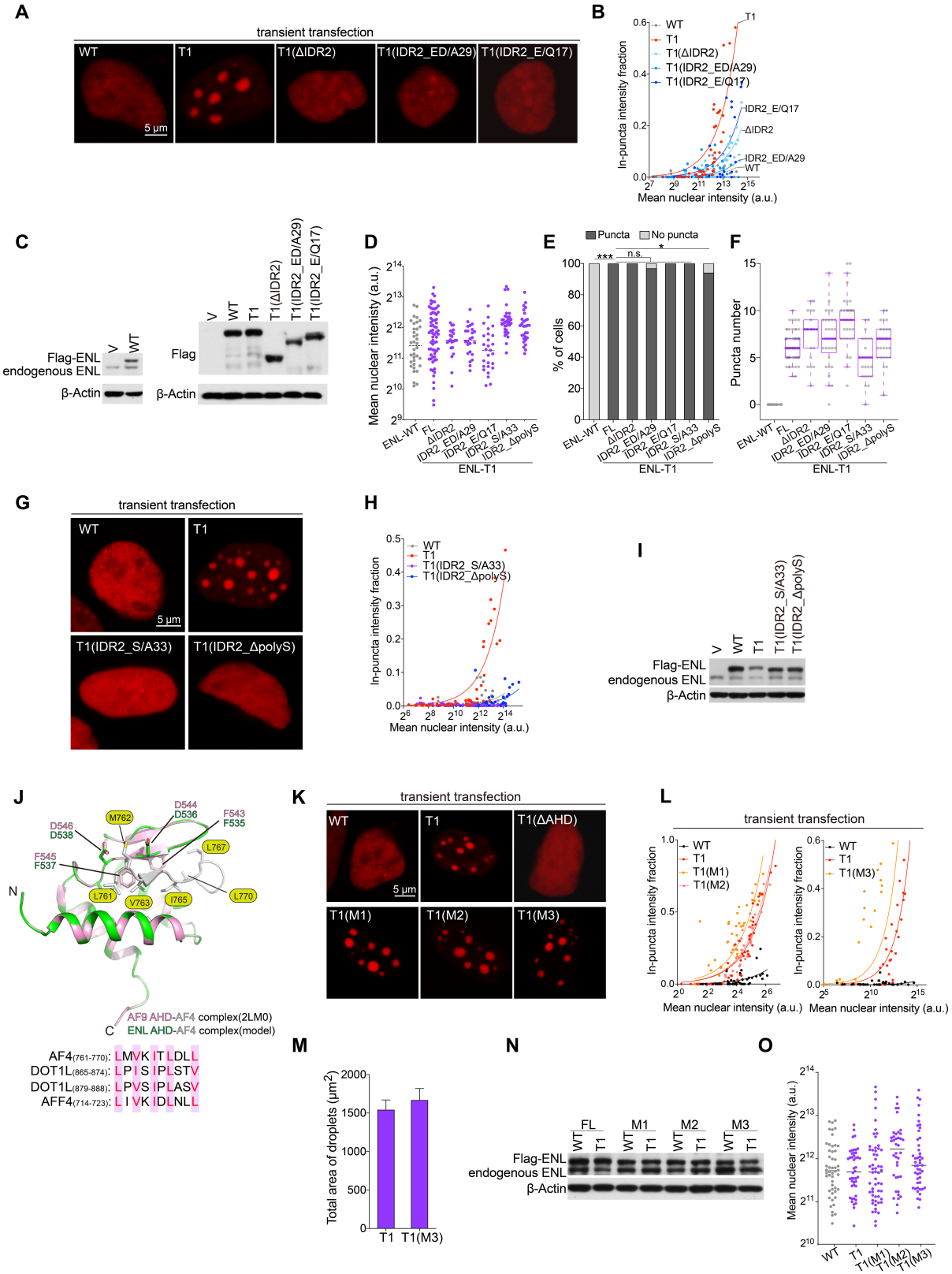

Figure S7. Negative charge- and serine-dependent interactions conferred by IDR2 regulate the growth of ENL mutant condensates; Heterotypic interactions with extrinsic factors are required for the formation and function of ENL mutant condensates, Related to Figures 6 and 7.

(A and G) Representative images of HEK293 cells transiently transfected with indicated mCherry-ENL constructs. All cells shown expressed similar levels of mCherry-ENL proteins. Scale bar, 5 μm.

(B and H) Fraction of in-puncta fluorescence intensity in the nucleus of HEK293 cells transfected with indicated mCherry-ENL constructs as a function of mean nuclear intensity. Each dot indicates one cell (*n* at the range of 32~78).

(C and I) Western blotting showing indicated Flag-ENL transgenes expressed at near endogenous levels in HEK293. V, empty vector.

(D–F) Mean Flag-ENL nuclear intensity (D), the percentage of nuclei with and without Flag-ENL puncta (E), and the number of puncta (F) in each nucleus of HEK293 cells expressing indicated Flag-ENL at near endogenous levels. Center lines indicate medians (D and F) and box limits (F) are set to the 25^{th} and 75^{th} percentiles. Each dot indicates one cell (D) or punctum (F). D and E, cell number at the range of 20~67; F, puncta number at the range of 109~436. E, Chi-square test. *** $P < 0.001$; n.s., no significance.

(J) Top, superimposition of modeled ENL AHD:AF4 structure with AF9 AHD:AF4 structure (PDB: 2LM0). AF9, pink; ENL, green; AF4, grey. Key residues predicted to mediate interactions of AF9/ENL AHD with SEC and DOT1L are shown. Bottom, sequence alignment of AF4, AFF4, and DOT1L. Key residues mediating interaction with AF9/ENL are in red.

(K) Representative images of HEK293 cells transiently transfected with indicated mCherry-ENL constructs. All cells shown expressed similar levels of mCherry-ENL proteins. Scale bar, 5 μm.

(L) Fraction of in-puncta fluorescence intensity in the nucleus of HEK293 cells transfected with indicated mCherry-ENL constructs as a function of mean nuclear intensity. Each dot indicates one cell (*n* at the range of 43~53).

(M) Quantification of total area of droplets formed by 500 nM purified full-length ENL T1 or T1(M3) proteins. Data represent Mean ± S.D..

(N) Western blotting showing indicated Flag-ENL transgenes expressed at near endogenous levels in HEK293 cells.

(O) Mean Flag-ENL nuclear intensity in HEK293 cells expressing indicated Flag-ENL at near endogenous levels. Each dot indicates one cell (*n* at the range of 40~55). Center lines indicate medians.