Determining Multivalent Interactomes of Intrinsically Disordered Proteins in Phase-Separated Condensates in Live Cells

Authors: Qinyu Han[1], Yanghao Zhong[1,4], Baiyi Quan[2,4], Ting-Yu Wang[2], Tsui-Fen Chou[2,3], Shasha Chong[1]*

Affiliations:

[1] Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

[2] Proteome Exploration Laboratory, Beckman Institute, California Institute of Technology, Pasadena, CA 91125, USA

[3] Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125, USA

[4] These authors contributed equally.

*Correspondence to: schong@caltech.edu

Abstract: Many intrinsically disordered protein regions (IDRs) can form liquid-liquid phase separation (LLPS) condensates or related biomolecular assemblies via multivalent interactions, but how these interaction behaviors contribute to specific cellular functions remains unclear due to the unknown composition of the IDR-mediated assemblies. Here, we report **phase**-separation-induced **i**nteractome **d**etection (PhaseID), a new method that determines the protein components within the LLPS condensates driven by any given IDR in live human cells. Using PhaseID, we demonstrated that different IDRs with transactivation functions have distinct multivalent interactomes and thereby execute transactivation through distinct pathways. In-depth analyses of PhaseID-determined IDR interactomes revealed that LLPS tunes the interaction selectivity of IDRs, enables new roles of IDRs in specific cellular processes, and involves heterotypic interactions encoded by protein sequence grammars.

Main Text: Often referred to as the "dark proteome", intrinsically disordered protein regions (IDRs) do not fold into well-defined 3D structures and are therefore not amenable to classical structure-function analysis. Although IDRs constitute 44-54% of the eukaryotic and viral proteomes (*1*) and play essential roles in numerous cellular processes (*2*), how most IDRs perform specific functions remains unclear. This void represents a major hurdle for understanding pathological mechanisms and developing therapeutics for numerous human diseases, where over 20% of all disease mutations occur in IDRs (*3*). The past 15 years have witnessed the groundbreaking discovery that many IDRs can form liquid-liquid phase separation (LLPS) condensates via their multivalent interactions, i.e., protein-protein interactions with a variable, concentration-dependent stoichiometry (*4–6*). An explosive number of reports have documented that such interaction behaviors play important roles in cellular organization and many cellular functions (*7–13*). These works have opened a new frontier for studying IDRs, but substantial challenges remain in understanding an IDR's working mechanism. The function of a biomolecule is fundamentally determined by which partners it interacts with and how it interacts with them. Although many recent studies have clearly shown that an IDR has highly selective multivalent interaction partners (*5*, *14–17*), there is currently no versatile way to efficiently identify the interaction partners of any given IDR in its LLPS condensates in live cells. It thus remains unclear how most IDRs execute specific cellular functions. Equally challenging is to uncover how exactly the formation of IDR-mediated biomolecular assemblies, including LLPS condensates, contributes to cellular functions.

To fill these gaps, we establish a method that determines all the protein components within the LLPS condensates driven by any given IDR in live human cells, thereby identifying the IDR's multivalent interaction partners across the cell's proteome. This new method, Phase-separation-induced Interactome Detection (PhaseID), combines chemical induction of LLPS with proximity labeling mass spectrometry to enable high-throughput identification of a target IDR's multivalent protein partners *in situ* within its LLPS condensates in live cells. Robust and versatile by design, PhaseID is generally applicable to any IDR with an LLPS propensity and compatible with diverse cell types, providing a useful tool for understanding many cellular processes involving IDRs or biomolecular condensates.

We showcase the first application of PhaseID by using it to study mammalian transcriptional regulation. Many transcriptional regulators contain sizable IDRs. Preeminent examples are intrinsically disordered transactivation domains of DNA-binding transcription factors (TFs). These TF IDRs mediate the interactions between TFs and other regulatory proteins (*18*) to enable gene activation, but how the IDRs successfully execute such transactivation functions without well-defined protein structures remains unclear. Previously, using live-cell single-molecule imaging approaches, we found that TF IDRs undergo dynamic, multivalent, and selective interactions to drive the formation of local high-concentration TF hubs at target genomic loci, which can develop into LLPS condensates upon IDR overexpression. The multivalent IDR-IDR interactions play a crucial role in transactivation (*5*). Many other reports have also documented the functional roles of TF hubs or condensates in transcription (see a recent review (*19*)). However, without knowing the composition of a TF hub in live cells, it remains elusive which regulatory proteins a TF coordinates with to orchestrate transactivation and how IDR-mediated TF hubs enable the coordination. Moreover, more than 1,600 human TFs that contain distinct IDRs have been discovered to date (*18*). Whether and how these TF IDRs differ in their transactivation mechanisms is completely unexplored. We tackle these critical questions by using PhaseID to compare the multivalent interactomes of different TF IDRs and examining the functional relevance of PhaseID-detected IDR partners. We also explore the origin of proteins' selectivity in interacting with specific IDRs by computing diverse sequence-encoded physicochemical properties of the proteins enriched in and depleted from different LLPS condensates.

PhaseID determines an IDR's multivalent interactome in its LLPS condensates in live human cells

The challenge in characterizing the composition of LLPS condensates stems from the fact that multivalent interactions between IDRs, which drive condensate formation, are weak, transient, and highly sensitive to protein concentrations and other environmental parameters (*2*). These features are distinct from stoichiometric interactions between structured proteins. Thus, LLPS condensates formed in live cells are not readily preserved during biochemical purification and are not amenable to composition characterization *in vitro,* where the environment is drastically different from that within the cell. PhaseID overcomes these challenges by using a novel way to chemically induce LLPS of a target IDR in live cells and identifying all the protein components within the induced condensates *in situ.*

Specifically, PhaseID utilizes a chemically inducible heterodimerization system comprising FKBP and FRB, where the two proteins bind in the presence of rapalog (a rapamycin analog molecule) (*20*). The FKBP-FRB heterodimer associates an IDR of interest with FTH1, a 24-mer-forming protein (*21*), only in the presence of rapalog, which promotes LLPS of the IDR by increasing its interaction valence. The chemically induced heterodimer, meanwhile, associates the IDR with the biotin protein ligase TurboID (*22*). As a result, TurboID can biotinylate all proteins interacting with the IDR, which is primarily located in its LLPS condensates. The beauty of using rapalog (a small molecule) to control LLPS is that it allows the acquisition of a homogenous cell population, where each cell contains many condensates of the target IDR upon chemical induction, and the system readily scales to large numbers of cells or culture plates. Obtaining a sizable cell population of such is crucial for maximizing the detection sensitivity of in-condensate proteins, as many sizable condensates in each cell can significantly enrich these proteins, and many such cells ensure the production of a sufficiently large biotinylated protein sample for mass spectrometry. As such, the workflow of PhaseID begins with stable expression of two proteins, "core" and "bait", in the cell. The core protein is a fusion of FKBP, TurboID, EGFP, and FTH1, while the bait protein is a fusion of FRB, mCherry, and the target IDR. We next treat the cells with rapalog to induce LLPS of the target IDR, which can be monitored by fluorescence imaging of EGFP and mCherry. We then add biotin to the cells to trigger TurboID-mediated biotinylation of all proteins in the induced LLPS condensates, purify the biotinylated proteins using streptavidin-coated beads, and identify the proteins using quantitative mass spectrometry (Fig. 1A).

We first demonstrated PhaseID in HEK293 cells using the N-terminal IDR of TAF15 (*23*) (residues 2-205; referred to as TAF15 IDR below) as the model IDR, which is known to undergo LLPS under specific conditions (*24–26*). TAF15 IDR can execute transactivation when fused to a DNA-binding domain (*23*). Since transactivation occurs in the nucleus, and to enable comparison of the multivalent interactomes of TAF15 IDR and other transactivating IDRs, we performed PhaseID for these IDRs in the nucleus by fusing nuclear localization signal (NLS) peptides to both core and bait (fig. S1A). Whereas both the core and TAF15 IDR-containing bait were distributed homogeneously in the nucleoplasm of live HEK293 cells, the two proteins co-condensed into

numerous prominent and spherical puncta in the nucleus when we treated the cells with 500 nM rapalog (Fig. 1B). 70% of all the cells contained the puncta. As a control, we removed TAF15 IDR from the bait and kept all the other settings unchanged (fig. S1A). We observed no puncta formation after treating the cells expressing the "no IDR" bait with rapalog (Fig. 1B). These results suggest that the rapalog-induced puncta in the TAF15 IDR-bait-expressing cells are indeed LLPS condensates driven by TAF15 IDR.

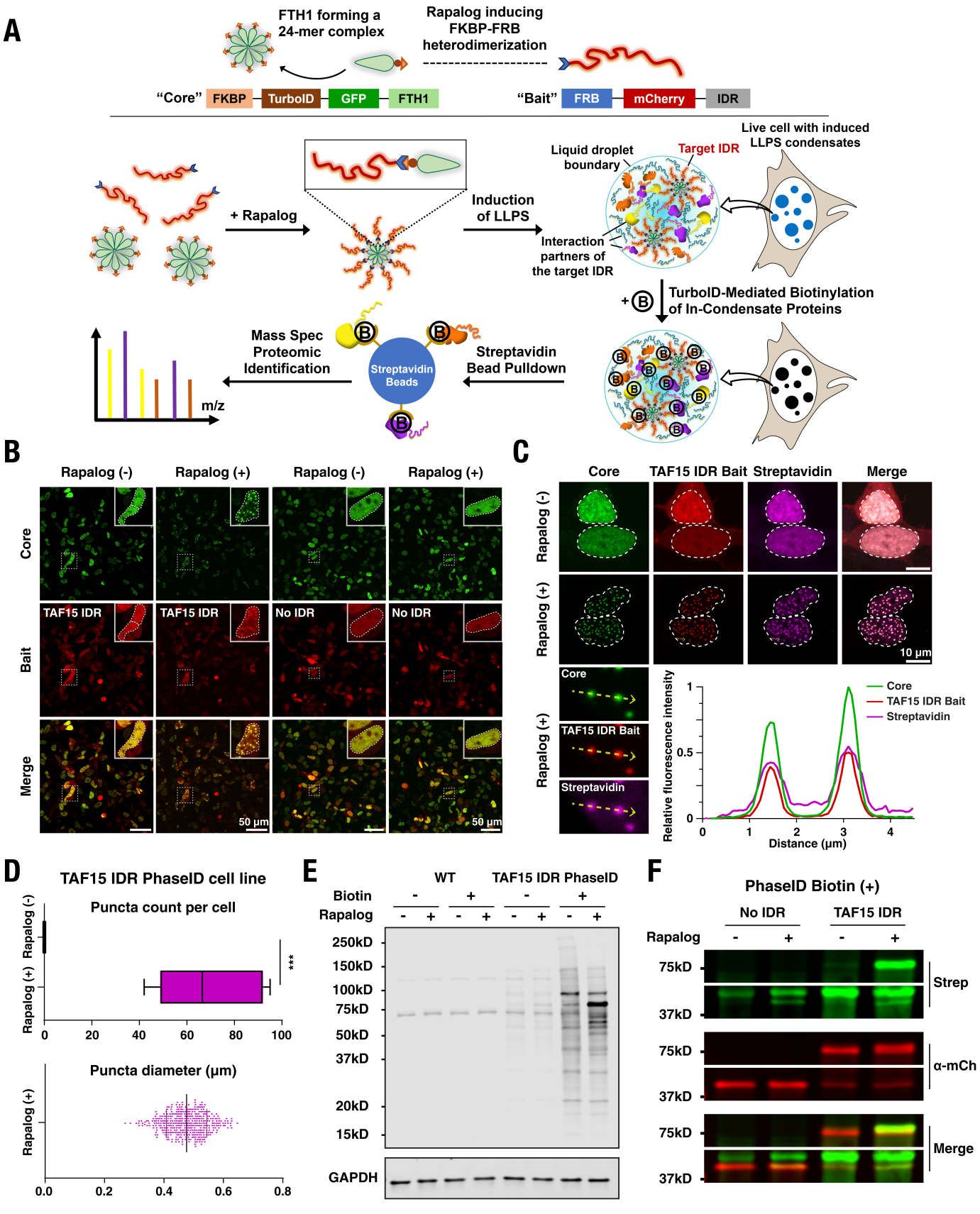

Fig. 1. PhaseID enables chemically induced LLPS and specific in-condensate biotinylation in live cells.
(A) Schematic of the PhaseID workflow. We stably express two fluorescently tagged fusion proteins, core and bait, in the cell. LLPS of the IDR of interest occurs upon rapalog addition to the cells, which induces the association of core and bait via FKBP-FRB heterodimerization. TurboID mediates proximity biotinylation of proteins within the LLPS condensates. The biotinylated proteins are then isolated by streptavidin pull-down and identified by mass spectrometry. We also perform a control PhaseID without an IDR in the bait to identify the proteins binding to the domains of core and bait other than the target IDR.
(B) Confocal fluorescence images of modified HEK293 cells expressing the TAF15 IDR or no-IDR control PhaseID constructs before and after rapalog treatment (500 nM, 10.5 hrs). Rapalog induces LLPS of TAF15 IDR in live cells. The distributions of core and bait are visualized in the EGFP (green) and mCherry (red) channels, respectively. A representative cell under each condition is zoomed in. A white dashed contour outlines the cell nucleus. Scale bars, 50 µm.
(C) Higher-magnification views of rapalog-induced TAF15 IDR condensates in HEK293 cells. After the cells were treated with biotin (50 µM, 1 hr), immunostaining with AF750-labeled streptavidin (magenta) showed that protein biotinylation predominantly occurs in the condensates. A white dashed contour outlines the cell nucleus. Scale bars, 10 µm. Line profiles show a strong co-localization of the core, bait, and streptavidin signals within the condensates.
(D) Quantification of puncta number per cell (top) and puncta diameter (bottom) across six cells, showing rapalog-dependent TAF15 IDR condensate formation. Puncta number per cell is presented as a Tukey box plot. Each dot in the bottom plot represents a punctum. Statistical significance: $p < 0.001$ (***); Welch's two-sample *t*-test.
(E) Streptavidin immunoblots of whole-cell lysates from wild-type and TAF15 IDR PhaseID cells before and after treatment of rapalog (500 nM, 10.5 hrs) and biotin (50 µM, 1 hr). The overall biotinylation levels in TAF15 IDR PhaseID cells increase significantly upon rapalog-induced LLPS. All lanes were loaded with equal amounts of total protein according to Ponceau S staining of the same membrane (data not shown).
(F) Streptavidin (Strep) and anti-mCherry (α-mCh) blots showing rapalog-dependent biotinylation of the mCherry-labeled TAF15 IDR bait (~61.7 kDa) and no-IDR bait (~39.4 kDa), confirming successful core-bait association upon chemical induction of LLPS.

We next incubated the rapalog-treated cells with 50 µM biotin for 1 hour to enable TurboID-mediated proximity labeling. We found that protein biotinylation predominantly occurs in the induced TAF15 IDR condensates, as determined by immunofluorescence using fluorescently labeled streptavidin (Fig. 1C). Each cell has 69 ±22 biotinylated TAF15 IDR condensates with an average diameter of 0.476 ± 0.0679 µm (Fig. 1D). In contrast, cells expressing the control bait with no IDR have biotinylated proteins distributed nearly homogeneously with no detectable puncta throughout the nuclei (fig. S1B). Using Western blots, we observed an increased overall level of protein biotinylation in the TAF15 IDR-bait-expressing cells after rapalog treatment (Fig. 1E). The newly biotinylated proteins included the mCherry-labeled TAF15 IDR bait (Fig. 1F). Similarly, in cells expressing the control bait with no IDR, the bait was biotinylated only after the cells were treated with rapalog (Fig. 1F), though the overall protein biotinylation level only minimally increased compared with the TAF15 IDR-bait-expressing cells upon rapalog treatment (fig. S1D). By driving LLPS, TAF15 IDR recruits many protein molecules into its condensates and enables their biotinylation, thereby enhancing the overall biotinylation levels of the cell. These results are consistent with our expectation that, only upon rapalog treatment of the cells, the bait and its interaction partners become proximal to TurboID and get biotinylated. Regardless of rapalog treatment and the presence of TAF15 IDR in the bait, the GFP-labeled core that contains TurboID is always biotinylated as expected (fig. S1E).

To identify proteins whose biotinylation levels are sensitive to LLPS induction by rapalog, we performed proximity labeling and mass spectrometry under both rapalog induction and no rapalog conditions, calculated the abundance fold change of each detected biotinylated protein upon rapalog induction compared with the no rapalog condition, and identified proteins with a fold change above 1.5 and below 0.67 with statistical significance as those enriched in and depleted from the induced TAF15 IDR condensates, respectively. Since proteins can be enriched in the condensates due to their interactions with other domains in the core and bait besides TAF15 IDR, we performed a control PhaseID in the cells expressing the bait with no IDR to identify the nonspecific partners. Proteins enriched and depleted in this control were removed from the condensate-enriched and depleted hits in the TAF15 IDR PhaseID, respectively, to define the multivalent interactome of TAF15 IDR shown in fig. S2.

Orthogonal fluorescence imaging assays validate the PhaseID-determined multivalent interactome

We employed cell imaging approaches orthogonal to mass spectrometry to validate the results obtained with PhaseID. Since PhaseID requires overexpressing the target IDR and associating it with several other proteins (mCherry, EGFP, FTH1, and the FKBP-FRB heterodimer), making an environment different from that of the endogenous IDR-containing protein, it is critical to confirm that the PhaseID-detected protein-protein interactions occur between endogenous proteins in the cell. We previously generated reagents useful in imaging the interaction behaviors of an endogenous IDR-containing protein, EWS::FLI1, which is an oncogenic TF known to cause

Ewing sarcoma (*27*) and produced by chromosomal translocation that fuses the N-terminal IDR of EWSR1 (*28*), an RNA-binding protein, to the C-terminal DNA-binding domain of FLI1, a TF that plays roles in development and homeostasis (*29*). We performed PhaseID for EWS IDR (residues 2-264 of EWS::FLI1; referred to as EWS IDR below) and leveraged our previously established cell imaging assays to evaluate the PhaseID-identified partners of EWS IDR under both overexpression and native conditions.

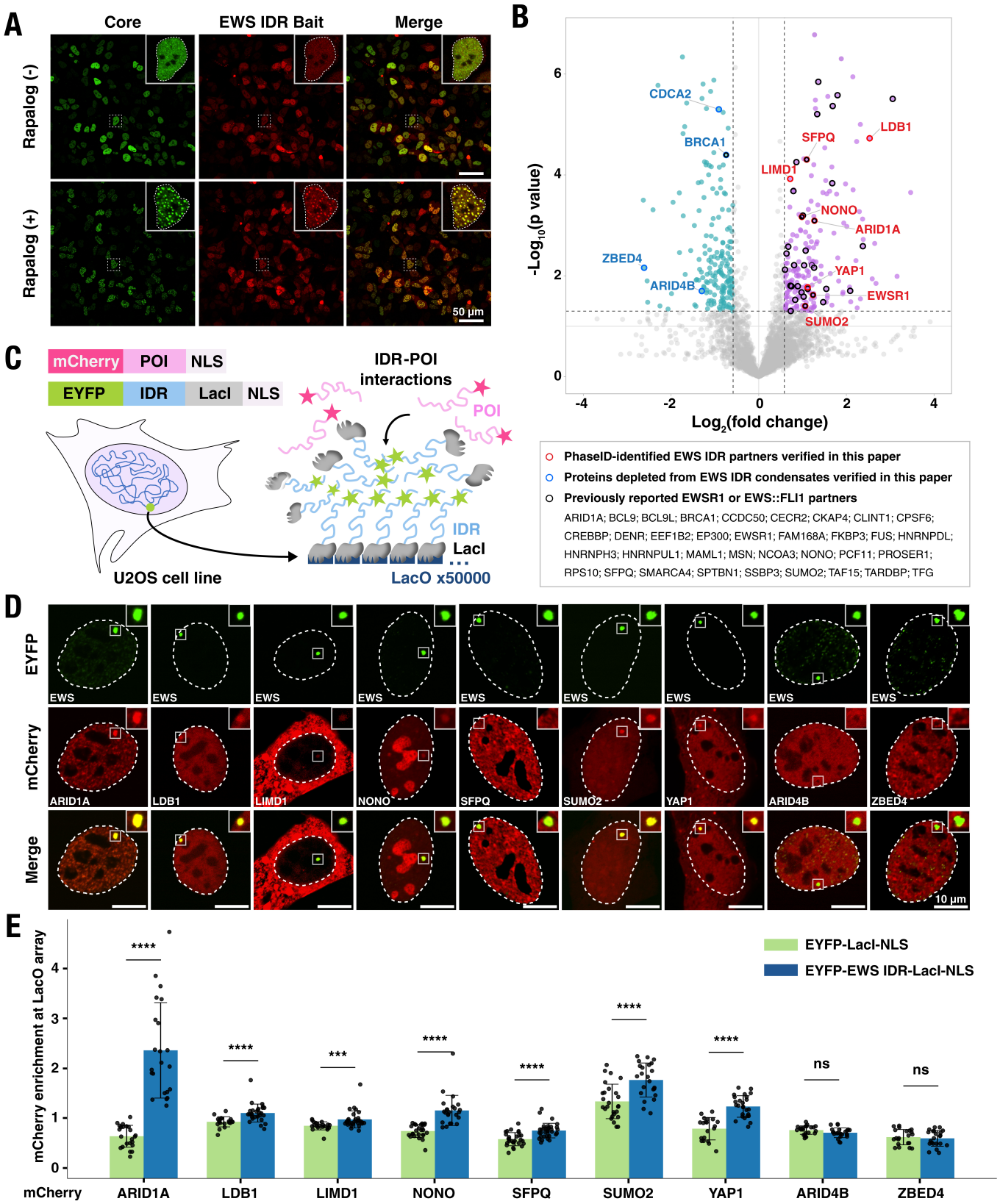

Fig. 2. PhaseID determines the interactome of EWS IDR, which is validated by the LacO array assay.
(A) Confocal fluorescence images of modified HEK293 cells expressing the EWS IDR PhaseID constructs before and after rapalog treatment (500 nM, 2 hrs). Rapalog induces LLPS of EWS IDR in live cells. The distributions of core and bait are visualized in EGFP (green) and mCherry (red) channels, respectively. A representative cell under each condition is zoomed in. A white dashed contour outlines the cell nucleus. Scale bars, 50 µm.
(B) Volcano plot showing the PhaseID-determined EWS IDR interactome, based on nine biological replicates per condition (before and after rapalog treatment). Each protein is represented by a dot in the plot. Proteins significantly enriched in and depleted from the rapalog-induced EWS IDR condensates are shown in magenta and blue, respectively. Proteins verified in this study using orthogonal assays are highlighted with red or blue circles. Previously reported EWSR1 or EWS::FLI1 partners are highlighted with black circles (*98*, *99*). The x-axis represents the fold change of the detected protein abundance upon rapalog induction compared with no rapalog condition. The y-axis represents the significance of the fold change. The dashed horizontal line shows the threshold *p* value of statistical significance (0.05). The two dashed vertical lines show the thresholds of abundance fold change (1.5 or 0.67).
(C) Schematic of the LacO array assay where we express EYFP-IDR-LacI and mCherry-tagged protein of interest (POI) in the LacO-containing U2OS cells. Colocalization of the two proteins at the LacO array suggests LCD-POI interactions.
(D) Confocal fluorescence images of LacO-containing U2OS cells co-expressing EYFP-EWS IDR-LacI and mCherry-labeled proteins identified by PhaseID. The region surrounding the LacO array is zoomed in. Scale bars, 10 µm.
(E) Quantification of mCherry-POI enrichment at the LacO array bound by EYFP-LacI or EYFP-EWS IDR-LacI. The enrichment was calculated as the peak mCherry fluorescence at the array divided by the average intensity immediately surrounding the array. Higher mCherry-POI enrichment at the array bound by EYFP-EWS IDR-LacI than by EYFP-LacI suggests EWS IDR–POI interactions. Error bars represent standard deviations. Statistical significance: $p < 0.001$ (***), $p < 0.0001$ (****), not significant (ns); Welch's two-sample *t*-test.

Similar to TAF15 IDR PhaseID, we successfully induced LLPS of EWS IDR in the nucleus of live HEK293 cells by treating the cells with 500 nM rapalog (Fig. 2A) and acquired the multivalent interactome of EWS IDR (Fig. 2B). We found that PhaseID identified multiple known interaction partners of EWSR1 (*5*, *30–34*) (Fig. 2B). We then employed the "LacO array assay" (*5*) that can characterize the multivalent interactions between any pair of proteins by quantitative cell imaging, to validate new EWS IDR partners identified by PhaseID. The LacO array assay exploits a large synthetic array of lac operators (LacOs) incorporated into the genome of U2OS cells (*35*). The LacO array becomes a local high-concentration region for any IDR of interest when it is fused to lac repressor (LacI) and EYFP and expressed in the cell. To detect potential interactions between the IDR and another protein of interest (POI), we co-express in the cell POI labeled with mCherry, and examine whether mCherry-POI is enriched at the LacO array bound by EYFP-IDR-LacI using fluorescence microscopy (Fig. 2C). An enrichment of mCherry fluorescence signal at the LacO array would suggest IDR-POI interactions, and the degree of mCherry enrichment is proportional to the IDR-POI interaction affinity. Using the LacO array assay, we confirmed the interactions between EWS IDR and several PhaseID-identified EWS IDR partners, including ARID1A (*36*), LDB1 (*37*), LIMD1 (*38*), NONO (*39*), SUMO2 (*40*), and YAP1 (*41*) (Fig. 2D). We observed that these partners interact with EWS IDR with different affinities, as shown by different degrees of mCherry enrichment at the LacO array (Fig. 2E). We also confirmed that ARID4B (*42*) and ZBED4 (*43*), proteins depleted from the induced EWS IDR condensates in PhaseID, do not interact with EWS IDR (Fig. 2D and E). We note that the PhaseID hits examined using the LacO array assay were randomly selected from the enriched and depleted protein lists. These results demonstrate the validity of PhaseID in determining the multivalent interactome of a target IDR.

We next examined whether PhaseID-identified EWS IDR partners interact with endogenous EWS::FLI1 in the Ewing sarcoma cell line A673. We exploited a knock-in A673 cell line that we previously generated using CRISPR-mediated genome editing, in which a HaloTag labels endogenous EWS::FLI1 without disrupting its functions (*5*). Since HaloTag can covalently bind to a fluorescent ligand with excellent photophysical properties (*44*, *45*), such labeling allows live-cell imaging of endogenous EWS::FLI1 at its native expression levels and with single-molecule resolution. We previously visualized the formation of small, transient, and EWS IDR-mediated EWS::FLI1 hubs at endogenous target genes in these cells (*5*). Here, we focused on several PhaseID-identified EWS IDR interaction partners (YAP1, EWSR1, SFPQ (*46*), and SUMO2) and imaged each endogenous protein by immunofluorescence while simultaneously imaging endogenous EWS::FLI1-Halo (Fig. 3A and fig. S4A). We detected significant enrichment of each partner at endogenous EWS::FLI1 hubs (Fig. 3B and C). In contrast, BRCA1 (*47*) and CDCA2 (*48*), proteins depleted from the induced EWS IDR condensates in PhaseID, were not enriched in endogenous EWS::FLI1 hubs (Fig. 3B and C, and fig. S4A). We note that our choice of the examined PhaseID hits was based on our access to reliable, immunofluorescence-compatible antibodies that target these proteins. Our results suggest that PhaseID, despite involving target IDR overexpression in the workflow, detects

protein-protein interactions of the endogenously expressed target IDR in its native cellular context. As such, PhaseID determines functionally relevant multivalent interactomes.

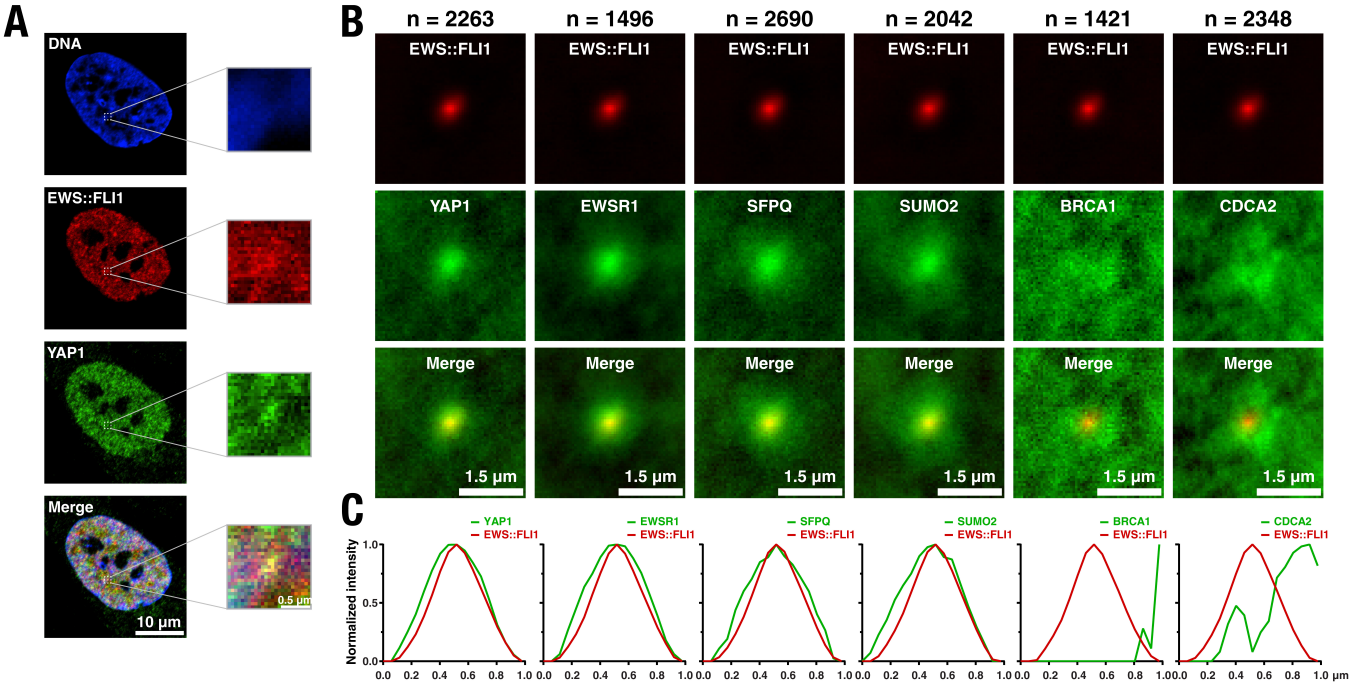

Fig. 3. PhaseID-identified EWS IDR interaction partners are enriched in endogenous EWS::FLI1 condensates.
(A) Confocal fluorescence images of a knock-in A673 cell nucleus showing DNA stained with Hoechst (blue), endogenous EWS::FLI1-Halo labeled with the HaloTag ligand JFX646 (*100*) (red), and endogenous YAP1 immunostained with a primary YAP1 antibody and an AF488-labeled secondary antibody (green). The zoom-in images show a single EWS::FLI1 hub and fluorescence of the same field of view in all channels.
(B) Averaged two-color confocal fluorescence images showing endogenous EWS::FLI1-Halo hubs (red) and immunostained endogenous proteins (green) detected by EWS IDR PhaseID. Four EWS IDR partners (YAP1, EWSR1, SFPQ, and SUMO2) and two proteins depleted from the EWS IDR condensates (BRCA1 and CDCA2) are investigated. The number of analyzed EWS::FLI1 hubs for each protein is provided above the respective two-color image (11–20 cells analyzed for each protein).
(C) Normalized intensity profiles of the central region (1 µm × 1 µm square) of the two-color fluorescence images in (B). The profiles were generated by averaging pixel intensities along each column within the central region.

Multivalent interactomes of transactivating IDRs indicate different transactivation mechanisms

We applied PhaseID to understand how different IDRs execute transactivation. To this end, we measured the multivalent interactomes of three transactivating IDRs, i.e., TAF15 IDR, EWS IDR, and the IDR of Sp1 (residues 2-507; referred to as Sp1 IDR below), a TF involved in regulating many cellular processes (*49*, *50*). Similar to TAF15 and EWS IDRs, we induced LLPS of Sp1 IDR in the nucleus of live HEK293 cells with 500 nM rapalog (fig. S5B and C) and determined its multivalent interactome in the induced condensates (fig. S5D). By comparing the PhaseID results for all three IDRs, we found that their interactomes are distinct from each other with only small overlaps (Fig. 4A). 284 out of the 348 interaction partners of TAF15 IDR are unique to TAF15 IDR, 141 out of the 204 partners of EWS IDR are unique to EWS IDR, and 92 out of the 120 partners of Sp1 IDR are unique to Sp1 IDR. The IDR interactomes overlap to different degrees. EWS IDR shares 50 partners with TAF15 IDR and only 14 partners with Sp1 IDR. TAF15 IDR only shares 15 partners with Sp1 IDR. This result suggests that Sp1 IDR has a multivalent interactome that is most distinct from those of EWS IDR and TAF15 IDR, whereas the latter two better resemble each other despite the distinctions between them. We also found that endogenous TAF15, FUS, and EWSR1 but not Sp1, are significantly enriched in the EWS and TAF15 IDR condensates. In contrast, endogenous Sp1 but not TAF15, FUS, or EWSR1 is significantly enriched in the Sp1 IDR condensates (fig. S6). This result is consistent with previously reported findings by us and others that Sp1 homotypically interacts, and the FET family proteins homotypically interact and heterotypically interact within the family, all via their IDRs (*5*, *26*). The successful detection of the expected IDR-mediated interactions provides additional validation of the PhaseID-determined interactomes.

The fact that the three transactivating IDRs have distinct multivalent interactomes supports a model in which the IDRs each enable the corresponding TFs to form a different "flavor" of hubs with a distinct composition, recruiting a particular group of regulatory proteins to target genes and executing transactivation through a distinct pathway. Based on this new model, we hypothesize that a protein uniquely interacting with the IDR of one TF only contributes to the functions of that TF and does not affect the functions of other TFs with which it does not interact. We focused on two TFs, EWS::FLI1 and Sp1, to test this hypothesis. Specifically, we examined how the gene activation functions of EWS::FLI1 and Sp1 change in A673 cells, where both TFs are expressed endogenously, upon knockdown of specific PhaseID-identified interaction partners of EWS IDR or Sp1 IDR. The investigated protein partners unique to EWS IDR were HNRNPUL1, SFPQ, TAF15, LDB1, SUMO2, MN1, and LIMD1, while those unique to Sp1 IDR were ZMYM2, PRDM16, and HSF2. To characterize the transactivation functions of EWS::FLI1 and Sp1, we focused on their known target genes, i.e., *CYP4F22*, *UGT3A2*, *PPP1R1A*, and *NR0B1* for EWS::FLI1 (*5*, *51*), and *BIRC5*, *B4GALT5*, *MMP14*, and *IGF1R* for Sp1 (*52–55*). Using RT-qPCR, we examined how transcription of these genes changes upon siRNA knockdown of each of the above interaction partners of EWS IDR or Sp1 IDR. We found transcription of the EWS::FLI1 target genes was significantly reduced upon knockdown of the EWS IDR partners, and it was largely unaffected upon knockdown of the Sp1 IDR partners. On the other hand, transcription of the Sp1 target genes was significantly reduced upon knockdown of the Sp1 IDR partners, and it was unaffected upon knockdown of the EWS IDR partners (Fig. 4B). These results suggest that many PhaseID-identified interaction partners of one TF IDR indeed specifically contribute to the transactivation function of that TF. This finding verifies our hypothesis and supports our model that different TFs can form hubs with distinct compositions, and each TF interacts with a unique group of partners within its hubs to activate genes, as shown in Fig. 6.

LLPS can tune an IDR's selectivity in interacting with proteins involved in diverse cellular processes

Previous reports have characterized the interactomes of intrinsically disordered proteins in the cell using classical proximity labeling mass spectrometry (*16*, *56*) without having the target protein predominantly located in its LLPS condensates or related IDR-mediated assemblies. We asked whether the interactome of an IDR can alter when it undergoes LLPS by comparing IDR interactomes measured by PhaseID and conventional TurboID-mediated proximity labeling mass spectrometry (*57*). For the latter, we fused TurboID and mCherry directly to a target IDR (fig. S9A), stably expressed the fusion protein in HEK293 cells, and performed TurboID-mediated proximity labeling followed by mass spectrometry to identify all the proteins proximal to the IDR. We also performed a "no IDR" control experiment in the cells stably expressing TurboID fused to mCherry only. The control dataset was used as the reference for calculating abundance fold changes, and proteins significantly enriched relative to this control were defined as partners of the target IDR.

We confirmed that none of EWS, Sp1, and TAF15 IDRs form prominent puncta in the cells expressing the IDR TurboID constructs (fig. S9B). After we added biotin to the cells, TurboID-mediated biotinylation was nearly homogeneously distributed across the cell nucleus (fig. S9C), distinct from the predominantly in-condensate biotinylation distribution in PhaseID (Fig. 1C). We compared the TurboID- and PhaseID-identified interactomes for EWS, Sp1, and TAF15 IDRs. Interestingly, for each IDR, its interactomes identified by TurboID and PhaseID only partially overlap, and a significant portion of its interaction partners identified by one method are not recognized by the other (Fig. 4C). In addition, a subset of protein partners detected by TurboID are excluded from the induced condensates of the target IDR according to PhaseID (fig. S10). This indicates that LLPS of an IDR can tune its interaction selectivity.

To identify all the cellular functions that may be mediated or impacted by LLPS of EWS and Sp1 IDRs, we performed Gene Ontology (GO) enrichment analysis for all PhaseID-identified interaction partners of each IDR using DAVID Bioinformatics Resources (*58*) (Fig. 4D). Besides transcription, which is expected to involve many partners of EWS and Sp1 IDRs due to their transactivation functions (Fig. 4E and F), several other biological processes are also overrepresented for the partners of both IDRs, including SUMOylation, chromatin remodeling, stress response, DNA damage response and repair, RNA splicing, and translation. On the other hand, processes such as DNA replication regulation, protein folding, nuclear export, and cell cycle are overrepresented only for Sp1 IDR partners. In contrast, processes such as RNA localization and stabilization, cell differentiation, immune response and regulation, circadian rhythm, and deubiquitination are overrepresented only for EWS IDR partners (Fig. 4D). At the same time, chromatin, nuclear speckles, and PML bodies are overrepresented cellular

components for the partners of both EWS and Sp1 IDRs. However, components such as the SWI/SNF complex are overrepresented only for EWS IDR partners, whereas components like the mitotic apparatus and ribosome are overrepresented only for Sp1 IDR partners. These results suggest significant functional differences between EWS and Sp1 IDR condensates. Although Sp1, EWSR1, or EWS::FLI1 have been previously reported to play a role in some of the overrepresented biological processes and cellular components (*59–68*), this is the first time that their IDRs are found to interact with specific proteins involved in many of the processes or components in the context of LLPS. While the PhaseID-determined interactions between each IDR and many of its partners still require rigorous validation using orthogonal assays, which is out of the scope of this paper, our work opens the door to future mechanistic studies to understand how Sp1, EWSR1, or EWS::FLI1 may contribute to diverse cellular functions.

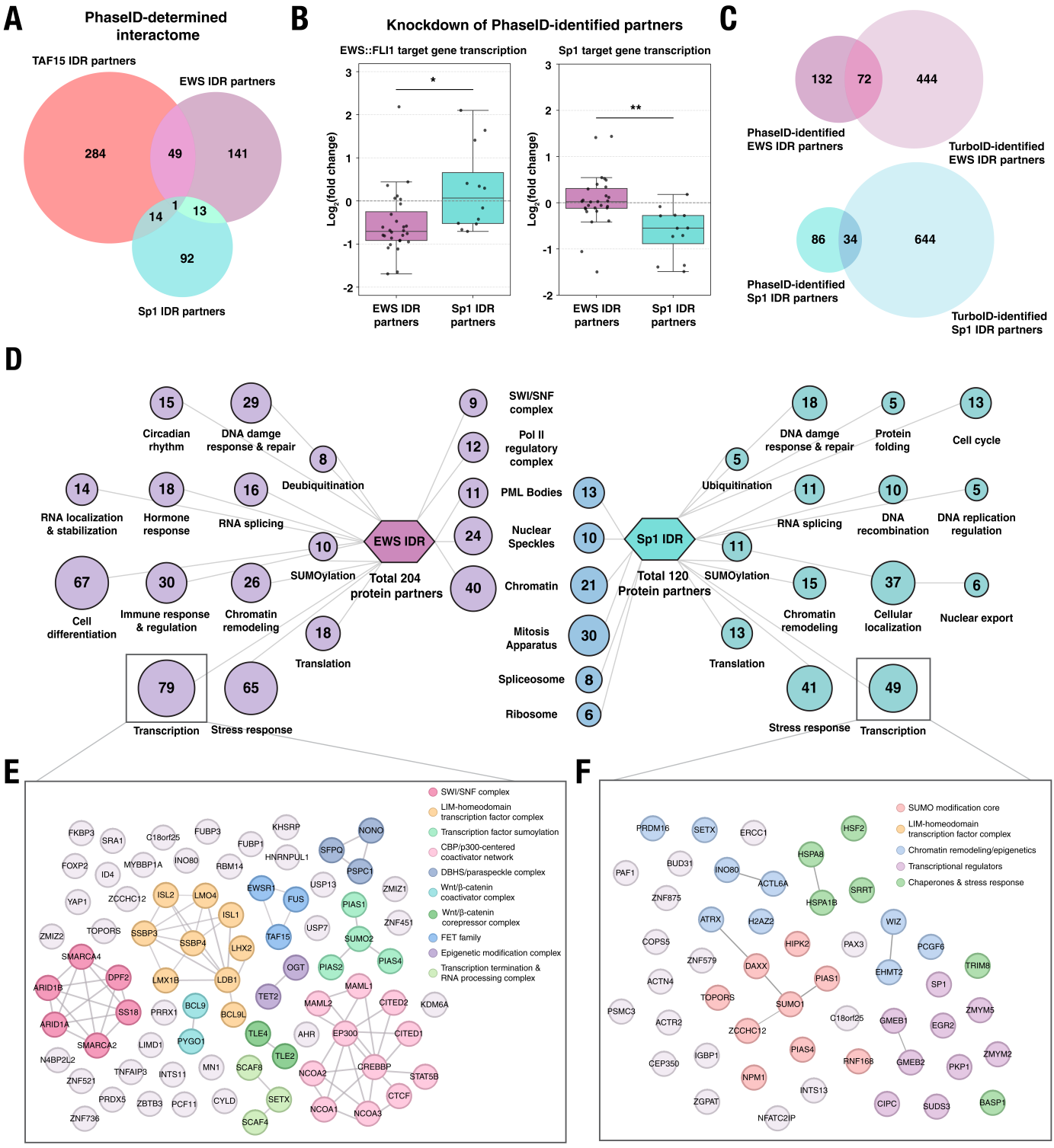

Fig. 4. PhaseID reveals that different transactivation IDRs have distinct, functionally relevant multivalent interactomes.
(A) Venn diagram comparing the PhaseID-determined interactomes of TAF15, EWS, and Sp1 IDRs.
(B) siRNA-mediated knockdown of PhaseID-identified IDR partners in A673 cells specifically affects transcription activated by the corresponding IDR-containing transcription factor. Left, knocking down EWS IDR partners (HNRNPUL1, SFPQ, TAF15, LDB1, SUMO2, MN1, or LIMD1) but not Sp1 IDR partners (ZMYM2, PRDM16, or HSF2) reduces transcription of EWS::FLI1 target genes (*CYP4F22*, *CCK*, *PPP1R1A*, and *NR0B1*). Right, knocking down Sp1 IDR partners but not EWS IDR partners listed above reduces transcription of Sp1 target genes (*BIRC5*, *B4GALT5*, *MMP14*, and *IGF1R*). The transcription levels were measured by RT-qPCR. The y-axis represents the fold change of the transcription level upon siRNA knockdown compared with that in wild-type A673 cells. Data presented as Tukey box plots. Each dot represents the fold change of each target gene upon knockdown of one partner. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**); Welch's two-sample *t*-test.
(C) Venn diagrams comparing the PhaseID- and TurboID-determined interactomes of EWS IDR (top) and Sp1 IDR (bottom).
(D) Gene Ontology (GO) enrichment analysis of the PhaseID-identified EWS IDR (left) and Sp1 IDR (right) interaction partners. The center nodes represent significantly enriched "Cellular Component" terms among the IDR partners, and the side nodes represent enriched "Biological Process" terms among the IDR partners.
(E-F) Network representation of PhaseID-identified EWS IDR partners (E) and Sp1 IDR partners (F) that are associated with transcription in the GO enrichment analysis.

To further identify the cellular functions that are particularly sensitive to LLPS, we compared the overrepresented GO terms for the interaction partners of each IDR identified by PhaseID and TurboID. While biological processes including transcription, chromatin remodeling, stress response, DNA damage response and repair, cell cycle, cell differentiation, and RNA splicing stand out for the partners identified by both methods, some processes, such as SUMOylation, translation, and deubiquitination, are overrepresented for the EWS or Sp1 IDR partners identified only by PhaseID (Fig. 4D and fig. S11). This result suggests that the EWS or Sp1 IDR partners involved in these processes only interact with the respective IDR when it forms LLPS condensates or related assemblies. This highlights the particular importance of LLPS or assembly formation, as the assembly behaviors likely enable new roles for Sp1, EWSR1, or EWS::FLI1 in these processes via IDR-mediated interactions. Our finding supports the previously reported functional relationship between protein SUMOylation and LLPS, where SUMOylation within IDRs is implicated in the regulation of LLPS, and LLPS can also facilitate protein SUMOylation (*69–71*). In addition, the fact that deubiquitination is overrepresented for EWS IDR partners identified only by PhaseID suggests that the previously reported role of deubiquitinating enzymes in the regulation and oncogenic functions of EWS::FLI1 (*72–74*) likely requires the formation of EWS::FLI1 hubs, and this regulation might not occur to EWS::FLI1 molecules outside the hubs.

Proteins recruited to Sp1 IDR and EWS IDR condensates have distinct sequence features

An increasing number of reports have shown that a "sequence grammar" may underlie the interaction selectivity of some IDRs, that is, specific physicochemical properties encoded in the amino acid sequences of an IDR or its protein partners may be required for their selective interactions (*5*, *16*, *17*, *75*). To understand the physicochemical origin of IDR interaction selectivity observed with PhaseID, we computed 140 sequence parameters (*76–82*) for each protein that is significantly enriched in or depleted from the induced EWS or Sp1 IDR condensates, compared the parameter values of each condensate-enriched or depleted protein group to those of the mass spectrometry detected proteome, and identified the sequence parameters that have their values for the protein groups significantly different from the proteome values.

The interesting sequence parameters we identified include the fractions of specific residues and residue patches (consecutive occurrence) as well as parameters describing the charge content and distribution (Fig. 5). First, all condensate-enriched and depleted protein groups have higher fractions of disorder-promoting residues than the proteome, indicating that IDRs of these proteins play a role in their abilities to partition in LLPS condensates selectively. We then examined the sequence parameters of specific protein groups. Compared to the proteome, proteins enriched in Sp1 IDR condensates have higher fractions of charged residues, including aspartic acid, glutamate, and lysine, higher fractions and lengths of DE and DEKR patches (D, aspartic acid; E, glutamate; K, lysine; R, arginine), and an overall higher fraction of charged residues (FCR). These proteins also exhibit higher δ and κ values, suggesting their more uneven charge distributions and greater charge segregation levels than the proteome (*77*). In contrast, the values of the same charge parameters in proteins depleted from Sp1 IDR condensates are lower than or comparable to those in the proteome. Intriguingly, proteins enriched in and depleted from EWS IDR condensates exhibit lower and higher values of these charge parameters, respectively, than the proteome values, showing a trend opposite to that observed for the Sp1 IDR interactome. On the other hand,

compared to the proteome, proteins enriched in EWS IDR condensates have higher fractions of specific non-charged residues, including methionine, asparagine, glutamine, glycine, tyrosine, and higher fractions and lengths of Q and NQ patches (Q, glutamine; N, asparagine). Proteins depleted from EWS IDR condensates and enriched in Sp1 IDR condensates both exhibit lower values or no significant difference in these sequence parameters compared to the proteome (Fig. 5). Collectively, these results suggest that proteins selectively recruited to Sp1 IDR and EWS IDR condensates exhibit many opposite sequence features. For each IDR, proteins enriched in and depleted from its condensates often exhibit opposite features as well. In short, the selectivity of proteins in binding to specific condensates is strongly correlated with their particular physicochemical properties encoded in the amino acid sequences.

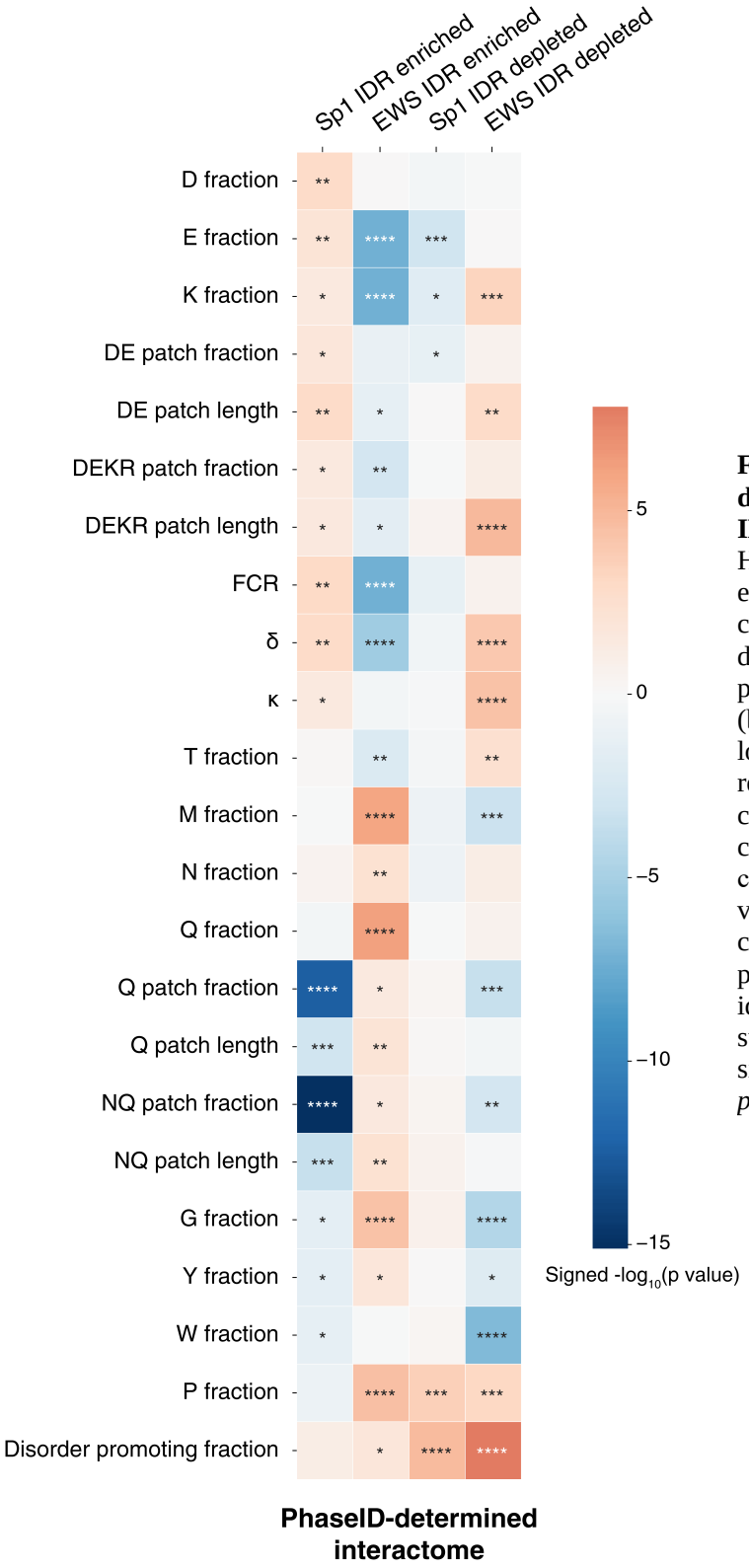

Fig. 5. Sequence feature analysis of the PhaseID-determined multivalent interactomes of EWS and Sp1 IDRs.
Heatmap of signed *p* values showing that proteins enriched in or depleted from EWS IDR or Sp1 IDR condensates have specific sequence parameter values distinct from those of the mass spectrometry detected proteome. A positive value (red) and a negative value (blue) denotes that the sequence parameter has higher and lower values for the protein group than for the proteome, respectively. The sequence parameter values were computed using localCIDER (*76*). FCR, fraction of charged residues. κ quantifies the linear patterning of charged residues along a protein sequence. δ is the variance of local charge asymmetry from the overall charge asymmetry across defined residue windows in a protein sequence. Patches are contiguous runs of ≥5 identical or chemically similar residues. See supplemental methods for details. Statistical significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****); Welch's two-sample *t*-test.

We next asked whether this sequence feature-dependent interaction selectivity in the PhaseID-determined IDR interactomes is also observed in the TurboID-determined IDR interactomes, where the protein partners do not interact with the target IDR in its LLPS condensates. To this end, we computed 140 sequence parameters for each protein that is detected by TurboID as interacting with EWS or Sp1 IDR and compared the parameter values of each IDR partner group with those of the mass spectrometry detected proteome. Although these protein groups show significant deviations from the proteome for many sequence parameters, their sequence features differ markedly from those of PhaseID-identified IDR partners (Fig. 5 and fig. S12). For example, the interaction partners of Sp1 IDR identified by TurboID have lower fractions of lysine and similar fractions of aspartic acid and glutamate compared to the proteome, features distinct from those of proteins enriched in Sp1 IDR condensates identified by PhaseID. Importantly, TurboID-identified partners of Sp1 IDR do not exhibit opposite sequence features to those of EWS IDR partners as we observed for Sp1 and EWS IDR partners in respective condensates using PhaseID. These findings suggest that the weak, IDR-mediated multivalent interactions occurring specifically in LLPS condensates have the interaction selectivity encoded by a better-defined protein sequence grammar than the IDR-mediated interactions in the absence of condensates. Since the in-condensate interactions can be comprehensively determined by PhaseID but not by conventional proximity labeling mass spectrometry methods like TurboID, our findings highlight the unique power of PhaseID in decoding the sequence grammar underlying the selectivity in IDR-mediated multivalent interactions in the context of LLPS or related biomolecular assembly.

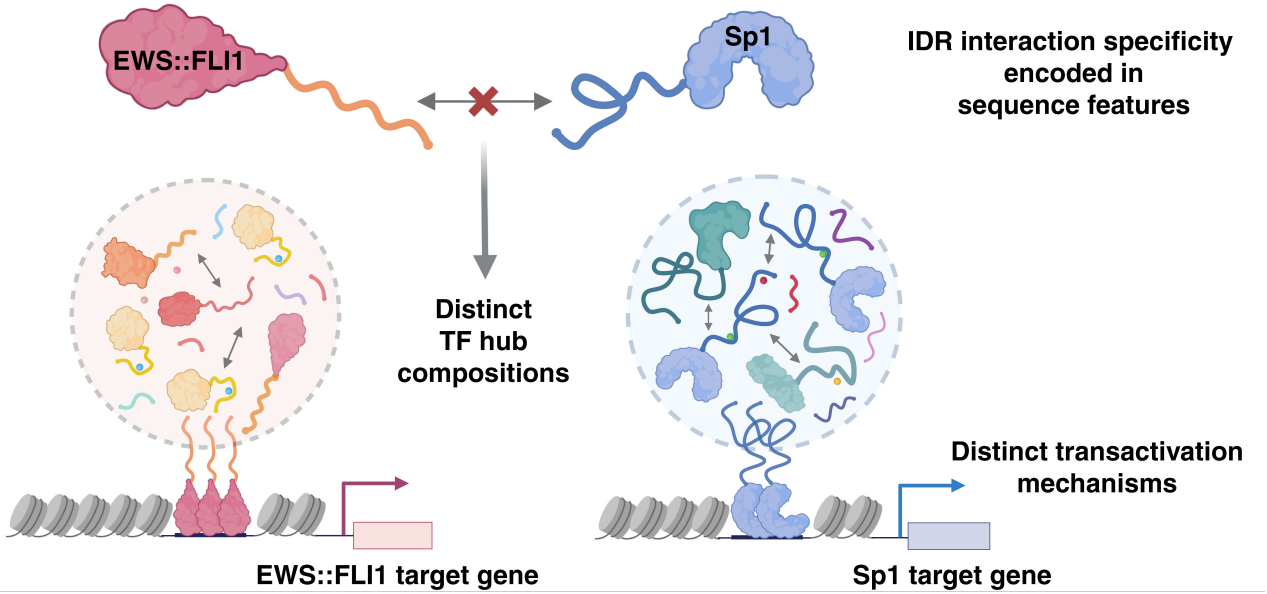

Fig. 6. A model for transactivation by different types of transcription factor (TF) hubs.
IDR-mediated hubs of EWS::FLI1 and Sp1 feature distinct compositions due to the distinct interactomes of their IDRs, which can lead to different transactivation mechanisms by the two TFs. The selectivity of proteins in binding to specific hubs is encoded in their amino acid sequence features.

Discussion

An increasing number of IDRs have been reported to form biomolecular assemblies, including LLPS condensates, via their selective multivalent interactions. Although such interaction behaviors have been clearly shown to be important for the functions of IDRs in many cellular processes, an in-depth mechanistic understanding of how assembly formation contributes to IDR functions is often challenging due to a lack of tools to identify the selective interaction partners of IDRs in the assemblies. Such tools are also highly desirable because of the important roles that LLPS plays in cellular organization and many cellular functions. Identifying the biomolecules assembled into LLPS condensates in the cell is critical for uncovering how exactly LLPS contributes to specific functions. Here, we fill the gaps by establishing the first method (PhaseID) to determine all the protein components of LLPS condensates driven by any given IDR in any mammalian cell type. Given the near-ubiquitous involvement of IDRs and biomolecular condensates in cell biology, PhaseID provides a powerful tool for understanding numerous cellular processes and diseases.

Historically, the challenge in characterizing the composition of LLPS condensates stems from the fact that multivalent interactions between IDRs within condensates, unlike stoichiometric interactions between structured proteins, are weak, transient, and sensitively dependent on protein concentrations and other environmental parameters (*2*). Thus, LLPS condensates formed in live cells cannot be readily preserved during biochemical purification or amenable to composition characterization *in vitro*, where the environment is drastically different from that in the cell. PhaseID overcomes this obstacle by combining chemical induction of LLPS with proximity labeling mass spectrometry, which enables identifying all the protein components of LLPS condensates *in situ* in live cells. Using a small molecule to induce LLPS allows the acquisition of a large, homogenous cell population where each cell contains many condensates, sufficiently enriching each protein component for mass spectrometry identification.

Several recent studies have characterized the composition of condensates nucleated by alternative approaches, including *in vitro* droplet formation, optogenetics, and an array of lac operators (LacO). The protocols based on *in vitro* and optogenetic LLPS assays often require significant modification or reinvention for studying each new LLPS-driving protein and are, therefore, not generally applicable methods. For example, determining the composition of MED1 IDR condensates based on *in vitro* droplet formation relies on an essential property of purified MED1 IDR, i.e., it forms LLPS condensates when added to a soluble nuclear extract (*17*). However, many IDRs are difficult to purify, and purified IDRs often require drastically different conditions to undergo LLPS *in vitro* (*83*). As such, many IDRs do not spontaneously form LLPS condensates in the cell extract. Although optogenetic LLPS activation applies to many more IDR-containing proteins, and it, combined with proximity labeling mass spectrometry, has enabled compositional characterization of TopBP1 and RNA Polymerase II condensates (*84*, *85*), light-induced condensates are known to dissolve progressively during downstream steps of in-condensate protein identification, and the protocol of maintaining condensates is dependent on the LLPS-driving protein and needs to be developed on a case-by-case basis (*86*). A modified U2OS cell line containing a LacO array in its genome has been used to nucleate condensates of an IDR of interest when it is fused to lac repressor. This system has recently been combined with proximity labeling mass spectrometry to identify interaction partners of MED1 and FUS IDRs (*87*). Although the LacO array can create a local high-concentration region for any IDR, and thus in principle should allow compositional characterization for nuclear condensates driven by any IDR with a LLPS propensity, the characterization can only be performed in U2OS cells, unless significant efforts are spent in re-establishing a LacO array in the genome of a different cell line. Additionally, there is only one condensate per nucleus nucleated by the LacO array, which occupies a tiny fraction of the nuclear volume and recruits a small number of interaction partners of the target IDR, thereby limiting the downstream detection efficiency of the protein partners by mass spectrometry. Besides, the LacO-array-based method cannot characterize condensates in the cytoplasm. Distinct from existing methods, PhaseID is designed to be sufficiently robust and sensitive and does not require significant protocol changes to characterize the composition of LLPS condensates driven by a new target protein or in a new cell type. Leveraging chemical induction of many LLPS condensates per cell, PhaseID should detect the target IDR's protein partners in the condensates with a significantly higher efficiency than the LacO-array-based method. Although in this work, we intentionally induced the formation of nuclear condensates of EWS, TAF15, and Sp1 IDRs because they execute transactivation functions in the nucleus, PhaseID can readily induce LLPS of a target IDR in the cytoplasm and determine the cytoplasmic condensate composition by removing the NLS from core and bait. Overall, the unprecedented versatility and robustness that PhaseID offers will make it universally helpful in studying IDR-mediated biomolecular assemblies in diverse cell types and for understanding how numerous IDR-containing proteins perform functions.

We recognize a limitation of PhaseID - it requires protein overexpression, chemical induction of LLPS, and significantly higher levels of multivalent interactions than those of endogenous proteins. This design ensures high efficiency in recruiting protein partners of the target IDR to its LLPS condensates, thereby maximizing the detection sensitivity of even the weakest interaction partners using downstream proximity labeling mass spectrometry. Due to the synthetic promotion of LLPS in PhaseID, the prominent puncta of a target IDR (Fig. 1B, 1C, and 2A) should not be considered functionally comparable to biomolecular assemblies formed by endogenous proteins in the cell. Our orthogonal cell imaging experiments have demonstrated that PhaseID-detected protein-protein interactions occur between endogenous proteins (Fig. 3). Novel microscopy approaches, e.g., proximity-assisted photoactivation (PAPA), which is capable of measuring IDR-mediated interactions between endogenous

proteins with single-molecule resolution in live cells (*88*), may provide a valuable tool to further validate PhaseID-identified IDR partners in the future. Besides, further methodological development to enable direct compositional characterization of any IDR-mediated endogenous biomolecular assemblies will be of great interest. Recent work characterizing endogenous RNA Polymerase II condensates in mouse embryonic stem cells may provide a promising direction for the development of such methods in the future.

One of the IDRs we studied using PhaseID is EWS IDR, part of the oncogenic TF EWS::FLI1 that drives the development of Ewing sarcoma, an aggressive and metastatic pediatric cancer that is poorly managed with standard treatments. The survival rate for Ewing sarcoma patients with metastases or recurrence remains extremely low (*27*). While several targeted therapies for Ewing sarcoma have been proposed over the past two decades (*89*), none of them have shown promising results in clinical trials. New, effective therapeutic strategies are thus urgently needed. EWS::FLI1 is long considered an attractive yet notoriously difficult target for drug development as it plays a critical role in driving the oncogenic transcription program in Ewing sarcoma (*90*), with mechanisms insufficiently understood, mainly due to its disordered nature. We previously showed that EWS::FLI1 forms pathological hubs at its target genes via multivalent interactions of EWS IDR, and this interaction behavior plays a crucial role in both transcription and transformation functions of EWS::FLI1 (*5*, *91*). However, the compositional characterization of the EWS::FLI1 hubs was lacking due to a lack of suitable tools. Here, we fill in the gap by using PhaseID to identify all the multivalent interaction partners of EWS IDR in its induced LLPS condensates in HEK293 cells and validating the presence of multiple identified partners in endogenous EWS::FLI1 hubs in Ewing sarcoma cells. In the future, it will be of great interest to comprehensively validate all the PhaseID-detected EWS IDR partners in terms of their interactions with EWS::FLI1 and the roles they play in EWS::FLI1 functions in Ewing sarcoma cells. Together, the current and future works hold the promise of identifying new essential regulatory factors of EWS::FLI1 and new therapeutic targets for Ewing sarcoma that are otherwise inaccessible, which will pave the way for the development of novel therapeutics. Moreover, aberrant IDR-containing proteins and IDR-mediated pathological assemblies are involved in numerous diseases besides Ewing sarcoma (*92–95*). Using PhaseID to characterize the composition of these pathological assemblies in the future will likely shed new light on both the mechanisms and therapeutics of these diseases.

Leveraging the versatility of PhaseID, we discovered that three transactivating IDRs have distinct multivalent interactomes in respective LLPS condensates. In particular, focusing on EWS and Sp1 IDRs, we found that many PhaseID-identified, unique partners of each IDR specifically contribute to the transactivation function of the corresponding TF. These results suggest that TFs may form IDR-mediated hubs with distinct compositions, thereby executing transactivation through different pathways. This represents a new model of IDR-interactome-dependent transactivation (Fig. 6). Here, we only characterized how particular IDR partners affect gene activation by TFs. It will be of future interest to comprehensively investigate the roles that the newly identified IDR partners play in TF functions besides gene activation, such as chromatin binding location and dynamics, chromatin regulation, and cell phenotype determination. The PhaseID-determined IDR interactomes provide a uniquely valuable resource for understanding the molecular mechanisms underlying transcriptional control in the lens of TF hub formation. Although here we only focus on EWS::FLI1 and Sp1, PhaseID combined with established functional assays will be useful for studying the IDR-mediated functions of numerous other TFs in the future.

An interesting observation we have had from comparing IDR interactomes measured by PhaseID and conventional TurboID-mediated proximity labeling mass spectrometry is that an IDR can alter its interaction selectivity when undergoing LLPS. This is the first time that this property of LLPS has been discovered to our knowledge. The ability of LLPS in tuning the interaction selectivity of IDRs may cause a protein to perform new functions by interacting with new partners in its condensates. Consistently, GO enrichment analyses showed that specific biological processes are overrepresented among PhaseID-identified partners of an IDR but not among its TurboID-identified partners, suggesting that proteins containing the IDR may play roles in these processes only upon their LLPS or the formation of related biomolecular assemblies. We speculate that the LLPS dependence of IDR interaction selectivity is due to the IDR's high local concentration in its condensates, as IDR-mediated protein-protein interactions are often multivalent, i.e., having a variable, concentration-dependent stoichiometry and thus an affinity that increases with protein concentrations (*4*). The concentration dependence of homotypic multivalent interactions has been well characterized (*7*). Here, our findings indicate that the selectivity and affinity of IDR-mediated heterotypic multivalent interactions are likely dependent on protein concentrations as well. Since endogenous proteins often form IDR-mediated biomolecular assemblies with higher local concentrations

of the IDR compared to the surrounding cellular environment, even though these concentrations might be lower than those in its LLPS condensates, we speculate that the IDR interactome is at least partially different inside and outside such biomolecular assemblies. It will be of future interest to characterize how an IDR's interactome may be quantitatively dependent on its concentration and how the concentration dependence may contribute to specific cellular functions. The concentration dependence of IDR interaction selectivity may also explain phenomena such as the requirement for optimal local IDR concentrations for efficient transcriptional activation of endogenous target genes by the IDR-containing TF (*56*, *91*, *96*).

Another finding from analyzing PhaseID-determined IDR interactomes is that the selectivity of proteins in binding to specific IDR condensates is encoded in particular protein sequence features. Intriguingly, the fact that proteins in EWS and Sp1 IDR condensates have distinct sequence features was revealed by PhaseID but not by TurboID measurements, suggesting that these sequence features play an important role in the IDR interaction selectivity of proteins within LLPS condensates, and not in the absence of LLPS. Thus, PhaseID provides a uniquely valuable tool for future studies that decode the sequence grammar underlying the interaction selectivity of numerous IDRs in the context of LLPS or related biomolecular assemblies. Combining PhaseID and the continuously advancing computational tools for protein sequence feature analysis (*75*, *76*, *97*) will pave the way for building a comprehensive sequence-interaction-function paradigm for the dark proteome.

References

1. N. Perdigão, J. Heinrich, C. Stolte, K. S. Sabir, M. J. Buckley, B. Tabor, B. Signal, B. S. Gloss, C. J. Hammang, B. Rost, A. Schafferhans, S. I. O'Donoghue, Unexpected features of the dark proteome. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 15898–15903 (2015).
2. S. Chong, M. Mir, Towards Decoding the Sequence-Based Grammar Governing the Functions of Intrinsically Disordered Protein Regions. *J. Mol. Biol.* **433**, 166724 (2021).
3. V. Vacic, P. R. L. Markwick, C. J. Oldfield, X. Zhao, C. Haynes, V. N. Uversky, L. M. Iakoucheva, Disease-associated mutations disrupt functionally important regions of intrinsic protein disorder. *PLoS Comput. Biol.* **8**, e1002709 (2012).
4. P. Li, S. Banjade, H.-C. Cheng, S. Kim, B. Chen, L. Guo, M. Llaguno, J. V. Hollingsworth, D. S. King, S. F. Banani, P. S. Russo, Q.-X. Jiang, B. T. Nixon, M. K. Rosen, Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**, 336–340 (2012).
5. S. Chong, C. Dugast-Darzacq, Z. Liu, P. Dong, G. M. Dailey, C. Cattoglio, A. Heckert, S. Banala, L. Lavis, X. Darzacq, R. Tjian, Imaging dynamic and selective low-complexity domain interactions that control gene transcription. *Science* **361**, eaar2555 (2018).
6. X. Darzacq, R. Tjian, Weak multivalent biomolecular interactions: a strength versus numbers tug of war with implications for phase partitioning. *RNA N. Y. N* **28**, 48–51 (2022).
7. S. F. Banani, H. O. Lee, A. A. Hyman, M. K. Rosen, Biomolecular condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* **18**, 285–298 (2017).
8. Y. Shin, C. P. Brangwynne, Liquid phase condensation in cell physiology and disease. *Science* **357**, eaaf4382 (2017).
9. D. M. Mitrea, R. W. Kriwacki, Phase separation in biology; functional organization of a higher order. *Cell Commun. Signal. CCS* **14**, 1 (2016).
10. S. Alberti, A. Gladfelter, T. Mittag, Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell* **176**, 419–434 (2019).
11. S. Mehta, J. Zhang, Liquid-liquid phase separation drives cellular function and dysfunction in cancer. *Nat. Rev. Cancer* **22**, 239–252 (2022).
12. S. Boeynaems, S. Alberti, N. L. Fawzi, T. Mittag, M. Polymenidou, F. Rousseau, J. Schymkowitz, J. Shorter, B. Wolozin, L. Van Den Bosch, P. Tompa, M. Fuxreiter, Protein Phase Separation: A New Phase in Cell Biology. *Trends Cell Biol.* **28**, 420–435 (2018).
13. B. Wang, L. Zhang, T. Dai, Z. Qin, H. Lu, L. Zhang, F. Zhou, Liquid-liquid phase separation in human health and diseases. *Signal Transduct. Target. Ther.* **6**, 290 (2021).
14. M. W. Parker, M. Bell, M. Mir, J. A. Kao, X. Darzacq, M. R. Botchan, J. M. Berger, A new class of disordered elements controls DNA replication through initiator self-assembly. *eLife* **8**, e48562 (2019).
15. C. H. Li, E. L. Coffey, A. Dall'Agnese, N. M. Hannett, X. Tang, J. E. Henninger, J. M. Platt, O. Oksuz, A. V. Zamudio, L. K. Afeyan, J. Schuijers, X. S. Liu, S. Markoulaki, T. Lungjangwa, G. LeRoy, D. S. Svoboda,

E. Wogram, T. I. Lee, R. Jaenisch, R. A. Young, MeCP2 links heterochromatin condensates and neurodevelopmental disease. *Nature* **586**, 440–444 (2020).
16. A. Patil, A. R. Strom, J. A. Paulo, C. K. Collings, K. M. Ruff, M. K. Shinn, A. Sankar, K. S. Cervantes, T. Wauer, J. D. St Laurent, G. Xu, L. A. Becker, S. P. Gygi, R. V. Pappu, C. P. Brangwynne, C. Kadoch, A disordered region controls cBAF activity via condensation and partner recruitment. *Cell* **186**, 4936-4955.e26 (2023).
17. H. Lyons, R. T. Veettil, P. Pradhan, C. Fornero, N. De La Cruz, K. Ito, M. Eppert, R. G. Roeder, B. R. Sabari, Functional partitioning of transcriptional regulators by patterned charge blocks. *Cell* **186**, 327-345.e28 (2023).
18. S. A. Lambert, A. Jolma, L. F. Campitelli, P. K. Das, Y. Yin, M. Albu, X. Chen, J. Taipale, T. R. Hughes, M. T. Weirauch, The Human Transcription Factors. *Cell* **172**, 650–665 (2018).
19. G. Pei, H. Lyons, P. Li, B. R. Sabari, Transcription regulation by biomolecular condensates. *Nat. Rev. Mol. Cell Biol.* **26**, 213–236 (2025).
20. J. Choi, J. Chen, S. L. Schreiber, J. Clardy, Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science* **273**, 239–242 (1996).
21. G. Bellapadrona, M. Elbaum, Supramolecular protein assemblies in the nucleus of human cells. *Angew. Chem. Int. Ed Engl.* **53**, 1534–1537 (2014).
22. K. F. Cho, T. C. Branon, N. D. Udeshi, S. A. Myers, S. A. Carr, A. Y. Ting, Proximity labeling in mammalian cells with TurboID and split-TurboID. *Nat. Protoc.* **15**, 3971–3999 (2020).
23. I. Kwon, M. Kato, S. Xiang, L. Wu, P. Theodoropoulos, H. Mirzaei, T. Han, S. Xie, J. L. Corden, S. L. McKnight, Phosphorylation-regulated binding of RNA polymerase II to fibrous polymers of low-complexity domains. *Cell* **155**, 1049–1060 (2013).
24. M.-T. Wei, Y.-C. Chang, S. F. Shimobayashi, Y. Shin, A. R. Strom, C. P. Brangwynne, Nucleated transcriptional condensates amplify gene expression. *Nat. Cell Biol.* **22**, 1187–1196 (2020).
25. S. Irgen-Gioro, S. Yoshida, V. Walling, S. Chong, Fixation can change the appearance of phase separation in living cells. *eLife* **11**, e79903 (2022).
26. M. Altmeyer, K. J. Neelsen, F. Teloni, I. Pozdnyakova, S. Pellegrino, M. Grøfte, M.-B. D. Rask, W. Streicher, S. Jungmichel, M. L. Nielsen, J. Lukas, Liquid demixing of intrinsically disordered proteins is seeded by poly(ADP-ribose). *Nat. Commun.* **6**, 8088 (2015).
27. T. G. P. Grünewald, F. Cidre-Aranaz, D. Surdez, E. M. Tomazou, E. de Álava, H. Kovar, P. H. Sorensen, O. Delattre, U. Dirksen, Ewing sarcoma. *Nat. Rev. Dis. Primer* **4**, 5 (2018).
28. J. Lee, P. T. Nguyen, H. S. Shim, S. J. Hyeon, H. Im, M.-H. Choi, S. Chung, N. W. Kowall, S. B. Lee, H. Ryu, EWSR1, a multifunctional protein, regulates cellular function and aging via genetic and epigenetic pathways. *Biochim. Biophys. Acta Mol. Basis Dis.* **1865**, 1938–1945 (2019).
29. Y. Li, H. Luo, T. Liu, E. Zacksenhaus, Y. Ben-David, The ets transcription factor Fli-1 in development, cancer and disease. *Oncogene* **34**, 2022–2031 (2015).
30. D. Zhang, A. J. Paley, G. Childs, The transcriptional repressor ZFM1 interacts with and modulates the ability of EWS to activate transcription. *J. Biol. Chem.* **273**, 18086–18091 (1998).
31. T. Rolland, M. Taşan, B. Charloteaux, S. J. Pevzner, Q. Zhong, N. Sahni, S. Yi, I. Lemmens, C. Fontanillo, R. Mosca, A. Kamburov, S. D. Ghiassian, X. Yang, L. Ghamsari, D. Balcha, B. E. Begg, P. Braun, M. Brehme, M. P. Broly, A.-R. Carvunis, D. Convery-Zupan, R. Corominas, J. Coulombe-Huntington, E. Dann, M. Dreze, A. Dricot, C. Fan, E. Franzosa, F. Gebreab, B. J. Gutierrez, M. F. Hardy, M. Jin, S. Kang, R. Kiros, G. N. Lin, K. Luck, A. MacWilliams, J. Menche, R. R. Murray, A. Palagi, M. M. Poulin, X. Rambout, J. Rasla, P. Reichert, V. Romero, E. Ruyssinck, J. M. Sahalie, A. Scholz, A. A. Shah, A. Sharma, Y. Shen, K. Spirohn, S. Tam, A. O. Tejeda, S. A. Wanamaker, J.-C. Twizere, K. Vega, J. Walsh, M. E. Cusick, Y. Xia, A.-L. Barabási, L. M. Iakoucheva, P. Aloy, J. De Las Rivas, J. Tavernier, M. A. Calderwood, D. E. Hill, T. Hao, F. P. Roth, M. Vidal, A proteome-scale map of the human interactome network. *Cell* **159**, 1212–1226 (2014).
32. J. Wang, K. Huo, L. Ma, L. Tang, D. Li, X. Huang, Y. Yuan, C. Li, W. Wang, W. Guan, H. Chen, C. Jin, J. Wei, W. Zhang, Y. Yang, Q. Liu, Y. Zhou, C. Zhang, Z. Wu, W. Xu, Y. Zhang, T. Liu, D. Yu, Y. Zhang, L. Chen, D. Zhu, X. Zhong, L. Kang, X. Gan, X. Yu, Q. Ma, J. Yan, L. Zhou, Z. Liu, Y. Zhu, T. Zhou, F. He, X. Yang, Toward an understanding of the protein interaction network of the human liver. *Mol. Syst. Biol.* **7**, 536 (2011).

33. N. Araya, K. Hirota, Y. Shimamoto, M. Miyagishi, E. Yoshida, J. Ishida, S. Kaneko, M. Kaneko, T. Nakajima, A. Fukamizu, Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. *J. Biol. Chem.* **278**, 5427–5432 (2003).
34. J.-F. Rual, K. Venkatesan, T. Hao, T. Hirozane-Kishikawa, A. Dricot, N. Li, G. F. Berriz, F. D. Gibbons, M. Dreze, N. Ayivi-Guedehoussou, N. Klitgord, C. Simon, M. Boxem, S. Milstein, J. Rosenberg, D. S. Goldberg, L. V. Zhang, S. L. Wong, G. Franklin, S. Li, J. S. Albala, J. Lim, C. Fraughton, E. Llamosas, S. Cevik, C. Bex, P. Lamesch, R. S. Sikorski, J. Vandenhaute, H. Y. Zoghbi, A. Smolyar, S. Bosak, R. Sequerra, L. Doucette-Stamm, M. E. Cusick, D. E. Hill, F. P. Roth, M. Vidal, Towards a proteome-scale map of the human protein-protein interaction network. *Nature* **437**, 1173–1178 (2005).
35. S. M. Janicki, T. Tsukamoto, S. E. Salghetti, W. P. Tansey, R. Sachidanandam, K. V. Prasanth, T. Ried, Y. Shav-Tal, E. Bertrand, R. H. Singer, D. L. Spector, From silencing to gene expression: real-time analysis in single cells. *Cell* **116**, 683–698 (2004).
36. D. Barisic, C. R. Chin, C. Meydan, M. Teater, I. Tsialta, C. Mlynarczyk, A. Chadburn, X. Wang, M. Sarkozy, M. Xia, S. E. Carson, S. Raggiri, S. Debek, B. Pelzer, C. Durmaz, Q. Deng, P. Lakra, M. Rivas, C. Steidl, D. W. Scott, A. P. Weng, C. E. Mason, M. R. Green, A. Melnick, ARID1A orchestrates SWI/SNF-mediated sequential binding of transcription factors with ARID1A loss driving pre-memory B cell fate and lymphomagenesis. *Cancer Cell* **42**, 583-604.e11 (2024).
37. N. G. Aboreden, J. C. Lam, V. Y. Goel, S. Wang, X. Wang, S. C. Midla, A. Quijano, C. A. Keller, B. M. Giardine, R. C. Hardison, H. Zhang, A. S. Hansen, G. A. Blobel, LDB1 establishes multi-enhancer networks to regulate gene expression. *Mol. Cell* **85**, 376-393.e9 (2025).
38. Y. Wang, C. Zhang, W. Yang, S. Shao, X. Xu, Y. Sun, P. Li, L. Liang, C. Wu, LIMD1 phase separation contributes to cellular mechanics and durotaxis by regulating focal adhesion dynamics in response to force. *Dev. Cell* **56**, 1313-1325.e7 (2021).
39. X.-J. Fan, Y.-L. Wang, W.-W. Zhao, S.-M. Bai, Y. Ma, X.-K. Yin, L.-L. Feng, W.-X. Feng, Y.-N. Wang, Q. Liu, M.-C. Hung, X.-B. Wan, NONO phase separation enhances DNA damage repair by accelerating nuclear EGFR-induced DNA-PK activation. *Am. J. Cancer Res.* **11**, 2838–2852 (2021).
40. H. Saitoh, J. Hinchey, Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* **275**, 6252–6258 (2000).
41. S. Piccolo, T. Panciera, P. Contessotto, M. Cordenonsi, YAP/TAZ as master regulators in cancer: modulation, function and therapeutic approaches. *Nat. Cancer* **4**, 9–26 (2023).
42. R.-C. Wu, M. Jiang, A. L. Beaudet, M.-Y. Wu, ARID4A and ARID4B regulate male fertility, a functional link to the AR and RB pathways. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 4616–4621 (2013).
43. V. V. Mokhonov, V. P. Theendakara, Y. E. Gribanova, N. B. Ahmedli, D. B. Farber, Sequence-specific binding of recombinant Zbed4 to DNA: insights into Zbed4 participation in gene transcription and its association with other proteins. *PloS One* **7**, e35317 (2012).
44. J. B. Grimm, B. P. English, J. Chen, J. P. Slaughter, Z. Zhang, A. Revyakin, R. Patel, J. J. Macklin, D. Normanno, R. H. Singer, T. Lionnet, L. D. Lavis, A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nat. Methods* **12**, 244–250, 3 p following 250 (2015).
45. J. B. Grimm, B. P. English, H. Choi, A. K. Muthusamy, B. P. Mehl, P. Dong, T. A. Brown, J. Lippincott-Schwartz, Z. Liu, T. Lionnet, L. D. Lavis, Bright photoactivatable fluorophores for single-molecule imaging. *Nat. Methods* **13**, 985–988 (2016).
46. D. Yu, C.-J. Huang, H. O. Tucker, Established and Evolving Roles of the Multifunctional Non-POU Domain-Containing Octamer-Binding Protein (NonO) and Splicing Factor Proline- and Glutamine-Rich (SFPQ). *J. Dev. Biol.* **12**, 3 (2024).
47. S. C. Moser, J. Jonkers, Thirty Years of BRCA1: Mechanistic Insights and Their Impact on Mutation Carriers. *Cancer Discov.* **15**, 461–480 (2025).
48. X. Lin, Z. Zou, J. Zhong, T. Wang, W. Ma, T. Hu, W. Sun, Y. Xu, A. M. M. Eggermont, Y. Chen, The Role of CDCA2 in tumor genesis, prognosis and future treatments. *Eur. J. Cancer* **211**, 114308 (2024).
49. J. T. Kadonaga, K. A. Jones, R. Tjian, Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* **11**, 20–23 (1986).
50. L. O'Connor, J. Gilmour, C. Bonifer, The Role of the Ubiquitously Expressed Transcription Factor Sp1 in Tissue-specific Transcriptional Regulation and in Disease. *Yale J. Biol. Med.* **89**, 513–525 (2016).

51. G. Boulay, G. J. Sandoval, N. Riggi, S. Iyer, R. Buisson, B. Naigles, M. E. Awad, S. Rengarajan, A. Volorio, M. J. McBride, L. C. Broye, L. Zou, I. Stamenkovic, C. Kadoch, M. N. Rivera, Cancer-Specific Retargeting of BAF Complexes by a Prion-like Domain. *Cell* **171**, 163-178.e19 (2017).
52. F. Li, D. C. Altieri, Transcriptional analysis of human *survivin* gene expression. *Biochem. J.* **344**, 305–311 (1999).
53. T. Sato, K. Furukawa, Transcriptional regulation of the human beta-1,4-galactosyltransferase V gene in cancer cells: essential role of transcription factor Sp1. *J. Biol. Chem.* **279**, 39574–39583 (2004).
54. M. A. Alfonso-Jaume, R. Mahimkar, D. H. Lovett, Co-operative interactions between NFAT (nuclear factor of activated T cells) c1 and the zinc finger transcription factors Sp1/Sp3 and Egr-1 regulate MT1-MMP (membrane type 1 matrix metalloproteinase) transcription by glomerular mesangial cells. *Biochem. J.* **380**, 735–747 (2004).
55. D. Beitner-Johnson, H. Werner, C. T. Roberts, D. LeRoith, Regulation of insulin-like growth factor I receptor gene expression by Sp1: physical and functional interactions of Sp1 at GC boxes and at a CT element. *Mol. Endocrinol.* **9**, 1147–1156 (1995).
56. L. Chen, Z. Zhang, Q. Han, B. K. Maity, L. Rodrigues, E. Zboril, R. Adhikari, S.-H. Ko, X. Li, S. R. Yoshida, P. Xue, E. Smith, K. Xu, Q. Wang, T. H.-M. Huang, S. Chong, Z. Liu, Hormone-induced enhancer assembly requires an optimal level of hormone receptor multivalent interactions. *Mol. Cell* **83**, 3438-3456.e12 (2023).
57. T. C. Branon, J. A. Bosch, A. D. Sanchez, N. D. Udeshi, T. Svinkina, S. A. Carr, J. L. Feldman, N. Perrimon, A. Y. Ting, Efficient proximity labeling in living cells and organisms with TurboID. *Nat. Biotechnol.* **36**, 880–887 (2018).
58. B. T. Sherman, M. Hao, J. Qiu, X. Jiao, M. W. Baseler, H. C. Lane, T. Imamichi, W. Chang, DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res.* **50**, W216–W221 (2022).
59. E. Deniaud, J. Baguet, R. Chalard, B. Blanquier, L. Brinza, J. Meunier, M.-C. Michallet, A. Laugraud, C. Ah-Soon, A. Wierinckx, M. Castellazzi, J. Lachuer, C. Gautier, J. Marvel, Y. Leverrier, Overexpression of Transcription Factor Sp1 Leads to Gene Expression Perturbations and Cell Cycle Inhibition. *PLoS ONE* **4**, e7035 (2009).
60. E. Grinstein, F. Jundt, I. Weinert, P. Wernet, H.-D. Royer, Sp1 as G1 cell cycle phase specific transcription factor in epithelial cells. *Oncogene* **21**, 1485–1492 (2002).
61. M. L. Swift, K. Beishline, S. Flashner, J. Azizkhan-Clifford, DSB repair pathway choice is regulated by recruitment of 53BP1 through cell cycle-dependent regulation of Sp1. *Cell Rep.* **34**, 108840 (2021).
62. K. Beishline, C. M. Kelly, B. A. Olofsson, S. Koduri, J. Emrich, R. A. Greenberg, J. Azizkhan-Clifford, Sp1 Facilitates DNA Double-Strand Break Repair through a Nontranscriptional Mechanism. *Mol. Cell. Biol.* **32**, 3790–3799 (2012).
63. W. J. Turner, M. E. Woodworth, DNA Replication Efficiency Depends on Transcription Factor-Binding Sites. *J. Virol.* **75**, 5638–5645 (2001).
64. M. I. Khalil, J. Hay, W. T. Ruyechan, Cellular Transcription Factors Sp1 and Sp3 Suppress Varicella-Zoster Virus Origin-Dependent DNA Replication. *J. Virol.* **82**, 11723–11733 (2008).
65. S. Komura, K. Semi, F. Itakura, H. Shibata, T. Ohno, A. Hotta, K. Woltjen, T. Yamamoto, H. Akiyama, Y. Yamada, An EWS-FLI1-Induced Osteosarcoma Model Unveiled a Crucial Role of Impaired Osteogenic Differentiation on Osteosarcoma Development. *Stem Cell Rep.* **6**, 592–606 (2016).
66. N. Riggi, B. Knoechel, S. M. Gillespie, E. Rheinbay, G. Boulay, M. L. Suvà, N. E. Rossetti, W. E. Boonseng, O. Oksuz, E. B. Cook, A. Formey, A. Patel, M. Gymrek, V. Thapar, V. Deshpande, D. T. Ting, F. J. Hornicek, G. P. Nielsen, I. Stamenkovic, M. J. Aryee, B. E. Bernstein, M. N. Rivera, EWS-FLI1 Utilizes Divergent Chromatin Remodeling Mechanisms to Directly Activate or Repress Enhancer Elements in Ewing Sarcoma. *Cancer Cell* **26**, 668–681 (2014).
67. S. Eliazer, J. Spencer, D. Ye, E. Olson, R. L. Ilaria, Alteration of Mesodermal Cell Differentiation by *EWS / FLI - 1* , the Oncogene Implicated in Ewing's Sarcoma. *Mol. Cell. Biol.* **23**, 482–492 (2003).
68. V. Verdile, F. Svetoni, P. La Rosa, G. Ferrante, E. Cesari, C. Sette, M. P. Paronetto, EWS splicing regulation contributes to balancing *Foxp1* isoforms required for neuronal differentiation. *Nucleic Acids Res.* **50**, 3362–3378 (2022).
69. X. Cheng, Protein SUMOylation and phase separation: partners in stress? *Trends Biochem. Sci.* **48**, 417–419 (2023).

70. E. Gutierrez-Morton, Y. Wang, The role of SUMOylation in biomolecular condensate dynamics and protein localization. *Cell Insight* **3**, 100199 (2024).
71. J. Keiten-Schmitz, L. Röder, E. Hornstein, M. Müller-McNicoll, S. Müller, SUMO: Glue or Solvent for Phase-Separated Ribonucleoprotein Complexes and Molecular Condensates? *Front. Mol. Biosci.* **8**, 673038 (2021).
72. S. Wang, X. Huo, Y. Yang, Y. Mo, R. K. Kollipara, R. Kittler, Ablation of EWS-FLI1 by USP9X inhibition suppresses cancer cell growth in Ewing sarcoma. *Cancer Lett.* **552**, 215984 (2023).
73. S. Su, J. Chen, Y. Jiang, Y. Wang, T. Vital, J. Zhang, C. Laggner, K. T. Nguyen, Z. Zhu, A. W. Prevatte, N. K. Barker, L. E. Herring, I. J. Davis, P. Liu, *SPOP* and *OTUD7A* Control *EWS–FLI1* Protein Stability to Govern Ewing Sarcoma Growth. *Adv. Sci.* **8**, 2004846 (2021).
74. M. E. Gierisch, G. Pedot, F. Walser, L. A. Lopez-Garcia, P. Jaaks, F. K. Niggli, B. W. Schäfer, USP19 deubiquitinates EWS-FLI1 to regulate Ewing sarcoma growth. *Sci. Rep.* **9**, 951 (2019).
75. G. M. Ginell, R. J. Emenecker, J. M. Lotthammer, A. T. Keeley, S. P. Plassmeyer, N. Razo, E. T. Usher, J. F. Pelham, A. S. Holehouse, Sequence-based prediction of intermolecular interactions driven by disordered regions. *Science* **388**, eadq8381 (2025).
76. A. S. Holehouse, R. K. Das, J. N. Ahad, M. O. G. Richardson, R. V. Pappu, CIDER: Resources to Analyze Sequence-Ensemble Relationships of Intrinsically Disordered Proteins. *Biophys. J.* **112**, 16–21 (2017).
77. R. K. Das, R. V. Pappu, Conformations of intrinsically disordered proteins are influenced by linear sequence distributions of oppositely charged residues. *Proc. Natl. Acad. Sci.* **110**, 13392–13397 (2013).
78. J. Kyte, R. F. Doolittle, A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132 (1982).
79. V. N. Uversky, Natively unfolded proteins: A point where biology waits for physics. *Protein Sci.* **11**, 739–756 (2002).
80. A. H. Mao, S. L. Crick, A. Vitalis, C. L. Chicoine, R. V. Pappu, Net charge per residue modulates conformational ensembles of intrinsically disordered proteins. *Proc. Natl. Acad. Sci.* **107**, 8183–8188 (2010).
81. W. Austin Elam, T. P. Schrank, A. J. Campagnolo, V. J. Hilser, Evolutionary conservation of the polyproline II conformation surrounding intrinsically disordered phosphorylation sites. *Protein Sci.* **22**, 405–417 (2013).
82. A. L. Rucker, C. T. Pager, M. N. Campbell, J. E. Qualls, T. P. Creamer, Host-guest scale of left-handed polyproline II helix formation. *Proteins Struct. Funct. Bioinforma.* **53**, 68–75 (2003).
83. D. T. McSwiggen, M. Mir, X. Darzacq, R. Tjian, Evaluating phase separation in live cells: diagnosis, caveats, and functional consequences. *Genes Dev.* **33**, 1619–1634 (2019).
84. C. Frattini, A. Promonet, E. Alghoul, S. Vidal-Eychenie, M. Lamarque, M.-P. Blanchard, S. Urbach, J. Basbous, A. Constantinou, TopBP1 assembles nuclear condensates to switch on ATR signaling. *Mol. Cell* **81**, 1231-1245.e8 (2021).
85. C. Lee, A. Quintana, I. Suppanz, A. Gomez-Auli, G. Mittler, I. I. Cissé, Light-induced targeting enables proteomics on endogenous condensates. *Cell* **187**, 7079-7090.e17 (2024).
86. E. Alghoul, J. Basbous, A. Constantinou, An optogenetic proximity labeling approach to probe the composition of inducible biomolecular condensates in cultured cells. *STAR Protoc.* **2**, 100677 (2021).
87. N. De La Cruz, P. Pradhan, R. T. Veettil, B. A. Conti, M. Oppikofer, B. R. Sabari, Disorder-mediated interactions target proteins to specific condensates. *Mol. Cell* **84**, 3497-3512.e9 (2024).
88. A. A. Abidi, G. M. Dailey, R. Tjian, T. G W Graham, Collective unstructured interactions drive chromatin binding of transcription factors. [Preprint] (2025). https://doi.org/10.1101/2025.05.16.654615.
89. K. I. Pishas, S. L. Lessnick, Recent advances in targeted therapy for Ewing sarcoma. *F1000Research* **5**, F1000 Faculty Rev-2077 (2016).
90. S. L. Lessnick, M. Ladanyi, Molecular pathogenesis of Ewing sarcoma: new therapeutic and transcriptional targets. *Annu. Rev. Pathol.* **7**, 145–159 (2012).
91. S. Chong, T. G. W. Graham, C. Dugast-Darzacq, G. M. Dailey, X. Darzacq, R. Tjian, Tuning levels of low-complexity domain interactions to modulate endogenous oncogenic transcription. *Mol. Cell* **82**, 2084-2097.e5 (2022).
92. V. N. Uversky, V. Davé, L. M. Iakoucheva, P. Malaney, S. J. Metallo, R. R. Pathak, A. C. Joerger, Pathological unfoldomics of uncontrolled chaos: intrinsically disordered proteins and human diseases. *Chem. Rev.* **114**, 6844–6879 (2014).

93. S. Tripathi, H. K. Shirnekhi, S. D. Gorman, B. Chandra, D. W. Baggett, C.-G. Park, R. Somjee, B. Lang, S. M. H. Hosseini, B. J. Pioso, Y. Li, I. Iacobucci, Q. Gao, M. N. Edmonson, S. V. Rice, X. Zhou, J. Bollinger, D. M. Mitrea, M. R. White, D. J. McGrail, D. F. Jarosz, S. S. Yi, M. M. Babu, C. G. Mullighan, J. Zhang, N. Sahni, R. W. Kriwacki, Defining the condensate landscape of fusion oncoproteins. *Nat. Commun.* **14**, 6008 (2023).
94. A. Boija, I. A. Klein, R. A. Young, Biomolecular Condensates and Cancer. *Cancer Cell* **39**, 174–192 (2021).
95. A. Zbinden, M. Pérez-Berlanga, P. De Rossi, M. Polymenidou, Phase Separation and Neurodegenerative Diseases: A Disturbance in the Force. *Dev. Cell* **55**, 45–68 (2020).
96. J. Trojanowski, L. Frank, A. Rademacher, N. Mücke, P. Grigaitis, K. Rippe, Transcription activation is enhanced by multivalent interactions independent of phase separation. *Mol. Cell* **82**, 1878-1893.e10 (2022).
97. M. C. Cohan, M. K. Shinn, J. M. Lalmansingh, R. V. Pappu, Uncovering Non-random Binary Patterns Within Sequences of Intrinsically Disordered Proteins. *J. Mol. Biol.* **434**, 167373 (2022).
98. R. Oughtred, J. Rust, C. Chang, B. Breitkreutz, C. Stark, A. Willems, L. Boucher, G. Leung, N. Kolas, F. Zhang, S. Dolma, J. Coulombe-Huntington, A. Chatr-aryamontri, K. Dolinski, M. Tyers, The BIOGRID database: A comprehensive biomedical resource of curated protein, genetic, and chemical interactions. *Protein Sci.* **30**, 187–200 (2021).
99. D. J. Elzi, M. Song, K. Hakala, S. T. Weintraub, Y. Shiio, Proteomic Analysis of the EWS-Fli-1 Interactome Reveals the Role of the Lysosome in EWS-Fli-1 Turnover. *J. Proteome Res.* **13**, 3783–3791 (2014).
100. J. B. Grimm, L. Xie, J. C. Casler, R. Patel, A. N. Tkachuk, N. Falco, H. Choi, J. Lippincott-Schwartz, T. A. Brown, B. S. Glick, Z. Liu, L. D. Lavis, A General Method to Improve Fluorophores Using Deuterated Auxochromes. *JACS Au* **1**, 690–696 (2021).
101. J. Goedhart, M. S. Luijsterburg, VolcaNoseR is a web app for creating, exploring, labeling and sharing volcano plots. *Sci. Rep.* **10**, 20560 (2020).
102. P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **13**, 2498–2504 (2003).

Acknowledgments:
We thank Jiapei Miao, Michael Xiong, and Jonathan Bahn for participation in early investigation, Zikun Zhu and William Rosencrans for helpful discussions, Diana Huynh and Lai Chai Foong for instrument training, Luke Lavis for providing the fluorescent HaloTag ligand, and the Proteome Exploration Laboratory and Biological Imaging Facility at California Institute of Technology for providing helpful equipment and expertise. Schematics of the model illustrated in Figure 6 were created using BioRender (http://biorender.com). This work is supported by the Shurl and Kay Curci Foundation Research Grant (S.C.), the Pew-Stewart Scholar for Cancer Research Award (to S.C.), the Searle Scholar Award (to S.C.), the Merkin Innovation Seed Grant (S.C.), the Mallinckrodt Research Grant (S.C.), the Margaret E. Early Medical Research Trust Grants (S.C.), the Alex's Lemonade Stand Foundation Innovation Grant under award number 1260879 (to S.C.), and a grant from the Jonsson Comprehensive Cancer Center (to S.C.). The Proteome Exploration Laboratory is partially supported by Caltech Beckman Institute Endowment Funds.