The oncogenic fusion protein EWS-FLI1 promotes premature ageing of biomolecular condensates by catalyzing fibril formation.

Emily E. Selig¹,², Alma K. Romero-Moreno¹,², Shivani Akula¹,², Xiaoping Xu¹,², David S. Libich¹,²*

1. Greehey Children’s Cancer Research Institute, The University of Texas Health Science Center at San Antonio, San Antonio, TX, 78229
2. Department of Biochemistry and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, TX, 78229

Email correspondence: libich@uthscsa.edu
ORCID: (Selig) https://orcid.org/0000-0002-3012-6814
ORCID: (Libich) https://orcid.org/0000-0001-6492-2803

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Abbreviations: ATP-dependent BRG1/BRM associated factor, BAF; Breast cancer type 1 susceptibility protein, BRCA1; chemical shift perturbation, CSP; C-terminal domain, CTD; DNA-binding domain, DBD; ethylenediaminetetraacetic acid, EDTA; Transcriptional regulator ERG, ERG; ETS translocation variant 1, ETV1; E-twenty-six transformation-specific, ETS; RNA-binding protein EWS, EWS; Ewing sarcoma, EwS; protein FEV, FEV; Friend leukemia integration 1, FLI1; fluorescence recovery after photobleaching, FRAP; fused in sarcoma, FUS; green fluorescent protein, GFP; high-affinity, HA; heteronuclear single quantum coherence, HSQC; intrinsically disordered region IDR; low-complexity domain, LCD; liquid-liquid phase separation, LLPS; nuclear magnetic resonance, NMR; primitive neuroectodermal tumor, PNET; transcription factor PU.1, PU.1; DNA-directed RNA polymerase II subunit RPB1, RNA Pol II; Arg-Gly-Gly, RGG; RNA-recognition motif, RRM; size-exclusion chromatography, SEC; TATA-binding protein associated factor 2N, TAF15; transmission electron microscopy, TEM; Tobacco Etch Virus, TEV; Thioflavin T, ThT; wild-type, WT.

Running Title: FLI1 DBD promotes fibril formation.
**Abstract**

Ewing sarcoma (EwS) is an aggressive pediatric cancer of bone and soft tissue. A chromosomal translocation that joins the low-complexity domain of EWS (EWS\(^{LCD}\)) with the DNA-binding domain of FLI1 (FLI1\(^{DBD}\)) creates EWS-FLI1, a fusion oncoprotein essential for EwS development and accounts for 85% of all EwS cases. EWS-FLI1 acts as an aberrant transcription factor and interferes with the normal functions of nucleic acid-binding proteins via multivalent interactions and biomolecular condensation. The FLI1\(^{DBD}\) was found to directly interact with the EWS\(^{LCD}\) causing enhanced phase separation and induced hardening of EWS\(^{LCD}\) condensates. Three related ETS DBDs (ERG, ETV1 and PU.1) also induced EWS\(^{LCD}\) condensate hardening. DNA binding blocked the interaction with the EWS\(^{LCD}\), and NMR spectroscopy confirmed that ETS DBDs interact with EWS\(^{LCD}\) via the DNA-binding interface. Our results provide a physical basis for the dominant-negative effect EWS-FLI1 exerts on EWS and highlight the need for further investigations of the FLI1\(^{DBD}\)-EWS\(^{LCD}\) interaction in vivo.
Introduction

The oncogenic EWS-FLI1 fusion protein is the archetypical example of a related group of fusion proteins characteristic of Ewing sarcoma (EwS), an aggressive pediatric bone and soft tissue cancer\(^1\). Arising from the t(11;22)(q12;q24) chromosomal translocation that fuses the N-terminal low-complexity domain (LCD) of the RNA-binding protein EWS (EWS) in frame with the DNA-binding domain (DBD) of the E-twenty-six transformation-specific (ETS) family transcription factor Friend leukemia integration 1 (FLI1), the resultant EWS-FLI1 fusion is responsible for approximately 85% of all EwS tumors\(^1\) (Fig. 1a). EWS-FLI1 acts as a pioneering factor aiding in chromatin opening, yet these observations do not fully explain its oncogenic role in EwS. There is mounting evidence that EWS-FLI1 exerts a dominant negative effect on the normal roles of EWS in transcriptional regulation and splicing\(^2\)-\(^5\). Indeed, it was recently noted that the presence of EWS-FLI1 at transcriptionally active sites prevents the release of Breast cancer type 1 susceptibility protein (BRCA1) from DNA-directed RNA polymerase II subunit RPB1 (RNA Pol II), resulting in elevated transcriptional stress and subsequent accumulation of unresolved R-loops\(^2\).

The FET family of RNA-binding proteins is named after its three members: fused-in-sarcoma (FUS), EWS, and TATA-binding protein associated factor 2N (TAF15). All FET proteins are implicated in neurodegenerative diseases amyotrophic lateral sclerosis and frontotemporal dementia, and various cancers including prostate, leukemia and sarcoma\(^6\)-\(^9\). FET proteins contain an N-terminal LCD (also known as the transactivation domain) and a C-terminal RNA binding domain (Fig. 1a, Supplementary Fig. 1). The LCD has low sequence complexity, is characterized by a degenerate repeated motif, SYGQ, and lacks stable secondary structure. These properties confer a propensity for self-association and liquid-liquid phase separation (LLPS)\(^10\)-\(^12\).

The ETS transcription factor family has 28 members characterized by a highly conserved DBD with a winged helix-turn-helix fold that recognizes an eight nucleotide consensus with a core GGAA/T sequence\(^13\). ETS transcription factors regulate genes involved in processes that can be tumorigenic when dysregulated such as cell cycle control, proliferation, migration, invasion, apoptosis, and angiogenesis\(^14\)-\(^17\). For reasons that are not entirely clear, FLI1, transcriptional regulator ERG (ERG), and protein FEV (FEV) most commonly participate in the FET-ETS fusions characteristic of EwS. However, rare chromosomal rearrangements involving FET proteins with other ETS transcription factors have been identified in EwS and in the related family of primitive neuroectodermal tumors (PNETs)\(^18\).

EWS-FLI1 alters normal genetic programs through aberrant DNA binding at GGAA microsatellites and recruitment of chromatin remodelers, epigenetic modifiers, and transcriptional machinery\(^11\),\(^19\)-\(^21\). The FLI1 portion of the fusion enables binding to both FLI1 consensus sites and GGAA microsatellites in
enhancer regions of EWS-FLI1-responsive genes. However, the EWS LCD contributes to GGAA microsatellite binding and both domains are essential for recruitment of the ATP-dependent BRG1/BRM associated factor (BAF) chromatin remodeling complex and for transcriptional activation\textsuperscript{19,22-27}. Transcriptional activation is achieved through the recruitment of RNA Pol II via its C-terminal domain (CTD) in a phosphorylation-dependent manner\textsuperscript{11,12,20}.

In recent years, biomolecular condensation, a process in which proteins and nucleic acids demix from the aqueous phase via LLPS to form membraneless organelles, has emerged as a sub-cellular organizational paradigm\textsuperscript{28-30}. These membraneless compartments regulate essential cellular processes such as transcription\textsuperscript{31,32}, splicing\textsuperscript{33}, DNA damage repair\textsuperscript{34} and the stress response\textsuperscript{35}. Intrinsically disordered proteins or proteins with intrinsically disordered regions (IDRs), including FET family proteins, are crucial components of biomolecular condensates\textsuperscript{10}. Indeed, oncogenic fusions of EWS-FLI1 appear to form dynamic yet specific assemblies with other LCD-containing proteins in cells such as EWS\textsuperscript{12,36} and the CTD of RNA Pol II via multivalent interactions mediated by its LCD\textsuperscript{37}. Furthermore, self-association stabilizes the binding of EWS-FLI1 to GGAA microsatellites and is required transcriptional activation, but the exact nature of this self-association remains contentious\textsuperscript{11,12,19}.

Since the oncogenic function of EWS-FLI1 requires features of both the EWS\textsuperscript{LCD} and the FLI1\textsuperscript{DBD}, the interplay between these two domains was investigated. The properties of biomolecular condensates of EWS, EWS\textsuperscript{LCD} in the presence and absence of EWS-FLI1, FLI1\textsuperscript{DBD} and ETS DBDs were investigated with biophysical approaches. Fluorescent recovery after photo bleaching (FRAP) and Thioflavin T (ThT) assays revealed that the dynamics of intermolecular interactions are altered in EWS-FLI1 condensates relative to EWS and EWS\textsuperscript{LCD} condensates. FLI1\textsuperscript{DBD} catalyzed rapid hardening of EWS\textsuperscript{LCD} condensates, an effect that was inhibited by DNA binding, which inhibited colocalization of FLI1\textsuperscript{DBD} to EWS\textsuperscript{LCD} condensates. The rapid ageing effect was a general feature of ETS domains. NMR confirmed that ETS DBDs transiently interact with EWS\textsuperscript{LCD} via two loops involved in DNA binding. Both the charge density and the relative conformation of the loops was inferred to be important for the EWS\textsuperscript{LCD} interaction. These findings provide a structural explanation for the observed dominant-negative effect of EWS-FLI1 as well as help to explain the apparent toxicity of the fusion, even to EwS tumor cells. Considering these data, further structural investigations as to how the interaction between EWS\textsuperscript{LCD} and ETS DBDs might affect intermolecular interactions of FET-ETS fusions are warranted.

**Results**
**EWS-FLI1 has altered condensation properties.** Phase diagrams for full-length FUS, full-length EWS and full-length EWS-FLI1 were constructed from turbidity measurements. Phase separation of EWS and FUS was promoted at increased protein and low NaCl concentrations, consistent with previous reports for FUS, where it was determined that phase separation is driven by electrostatic interactions between Arg residues in the Arg-Gly-Gly (RGG) motifs and Tyr residues in the LCD\textsuperscript{38,39} (Fig. 1b). Conversely, phase separation of EWS-FLI1 was promoted by high protein and NaCl concentrations (Fig. 1b), suggesting hydrophobic interactions drive condensation. EWS and EWS-FLI1 are known to interact in vivo, likely via multivalent LCD-LCD interactions in biomolecular condensates\textsuperscript{36}. As a way of examining these LCD-LCD interactions, biomolecular condensates were prepared by mixing 25 µM EWS\textsuperscript{LCD}, which is common to both EWS and EWS-FLI1 (EWS 1-264, Fig. 1a, Table 1) with 9 µM wild-type (WT) EWS, 5 µM EWS-FLI1, or 25 µM EWS\textsuperscript{LCD} (Fig. 1c). WT EWS and EWS-FLI1 readily form dual-component condensates with EWS\textsuperscript{LCD}, similar to in vivo observations in a EwS cell line A673\textsuperscript{40}. Condensate dynamics were assessed by monitoring fluorescence recovery after photobleaching (FRAP) of the fluorescently labelled molecules within the condensates (Fig. 1c). Condensates containing WT EWS and EWS\textsuperscript{LCD} alone showed rapid FRAP, while condensates containing EWS-FLI1 recovered slower (Fig. 1c), indicating that EWS-FLI1 alters the dynamics of EWS\textsuperscript{LCD} molecules within condensates and that EWS-FLI1 condensates are less fluid and more gel-like.

**FLI1\textsuperscript{DBD} alters the physical properties of EWS\textsuperscript{LCD} condensates.** The FRAP experiments revealed that FLI1\textsuperscript{DBD} may adversely affect EWS condensate properties. The effect of FLI1\textsuperscript{DBD} on the phase separation propensity of EWS\textsuperscript{LCD} was tested using turbidity measurements and microscopy (Fig. 2a). The addition of equimolar concentrations of FLI1\textsuperscript{DBD} (25 µM) significantly increased the turbidity of the sample, indicating an increase in phase separation, and dramatically altered the morphology of the condensates as visualized by bright-field microscopy (Fig. 2a). These condensates no longer coalesce and instead appear fusion-defective (Supplementary Movies 1 and 2). To determine whether this effect was specific to FLI1\textsuperscript{DBD}, equimolar concentrations of constructs including the RNA-recognition motif (RRM) of EWS (EWS\textsuperscript{RRM}, or EWS\textsuperscript{RRM-RGG2}, 25 µM) were also tested (Fig. 2a, Table 1, Supplementary Fig. 1). The EWS\textsuperscript{RRM} construct induced a slight increase in turbidity and resulted in EWS\textsuperscript{LCD} condensates that appeared more spherical than those formed in the absence of RRM (Fig. 2a). The EWS\textsuperscript{RRM-RGG2} construct induced an increase in turbidity that was less than that observed for FLI1\textsuperscript{DBD}, and induced spherical EWS\textsuperscript{LCD} condensates (Fig. 2a). In contrast to FLI1, EWS\textsuperscript{LCD} condensates formed with the EWS\textsuperscript{RRM} and EWS\textsuperscript{RRM-RGG2} constructs retained the ability to fuse (Supplementary Movies 3 and 4). Therefore, FLI1\textsuperscript{DBD} increases the phase separation propensity of EWS\textsuperscript{LCD}, has a different effect on
the morphology of the condensates compared to the RNA-binding domains that are naturally found in WT EWS, and changes the material properties of the condensates such that they are fusion-defective.

Biomolecular condensates formed by LCD-containing proteins similar in composition to EWS LCD “ripen” or “age” spontaneously over time becoming more gel-like\(^4^1\). Cross-β structure has been shown to stabilize the condensed state of the closely related FET protein, FUS\(^4^2\), and condensate ageing is concurrent with the formation of fibrillar structures that sometimes protrude from the condensates\(^4^1\). To investigate the effect of the FLI1 DBD on the formation of cross-β structure in EWS LCD condensates, ThioflavinT (ThT) fluorescence assays were developed. Under non-phase-separating conditions (no NaCl), ThT fluorescence was not observed for EWS LCD alone, or in the presence of FLI1 DBD (Fig. 2b). Bright-field microscopy revealed that EWS LCD remains in the dilute phase, but the addition of FLI1 DBD was associated with the appearance of several small condensates (Fig. 2b), consistent with FLI1 DBD increasing the phase separation propensity of EWS LCD (Fig. 2a), however no measurable increase in turbidity was observed under these conditions. Under phase-separating conditions (150 mM NaCl), EWS LCD condensates age slowly over approximately 10 hours, with a small concomitant increase in ThT fluorescence (Fig. 2c), an effect similar to that reported for FUS\(^4^3,^4^4\). The addition of FLI1 DBD, even at sub-stoichiometric concentrations (1:10 molar ratio), to EWS LCD condensates significantly accelerated condensate ageing in a manner dependent on the concentration of FLI1 DBD (Fig. 2c). FLI1 DBD also increased ThT fluorescence of WT EWS under phase-separating conditions (Supplementary Fig. 2a). An EWS-FLI1 truncation mutant corresponding to the minimal region of the fusion required for oncogenic transformation\(^1^9\) (Table 1, Supplementary Fig. 1) rapidly became ThT positive under phase-separating conditions (Supplementary Fig. 2b).

Aged, ThT positive samples of EWS LCD and EWS LCD with FLI1 DBD were analyzed via transmission electron microscopy (TEM) to assess amyloid fibril formation. Although fibrils formed by EWS have been observed\(^4^5,^4^6\) and fibril formation within FET protein condensates has also been reported\(^1^0,^4^1\), the observed structures did not display the typical morphological features of amyloid fibrils. Instead, heterogeneously branched structures that appeared intertwined were observed that are consistent with a pre-fibrillar or proto-fibril state\(^4^5\) (Fig. 2c, Supplementary Fig. 2c). Therefore, the observed increase in ThT fluorescence arises from the formation of cross-β structure that has not had time or is incapable of organizing into bona fide amyloid fibrils. As amyloid fibrils are not known to be associated with EwS, this line of investigation was not pursued and instead ThT fluorescence provided a convenient assay for probing the equilibrium between dilute and condensed phase EWS LCD.
DNA binding to FLI1 inhibits EWSLCD condensate aging. The ability of FLI1DBD to alter EWSLCD condensate properties suggests that the two proteins directly interact. To determine if the interaction site involves the FLI1 DNA-binding site, ThT assays were conducted in the presence a double stranded high-affinity consensus sequence for ETS DBDs47 (HA DNA, Table 2). HA DNA inhibited FLI1DBD-induced aging of EWSLCD condensates in a concentration-dependent manner (Fig. 3a). At the highest concentrations of HA DNA tested (50 µM corresponding to a 10:1 DNA:FLI1DBD ratio), the effect of FLI1 on EWSLCD condensates was almost entirely abolished (Supplementary Fig. 3a). A double stranded DNA oligonucleotide containing 10 tandem GGAA repeats, which are known to bind FLI1DBD in vitro27,48, also inhibited the effect FLI1DBD exerts on EWSLCD condensates (Fig. 3b, Table 2). A scrambled dsDNA control sequence had no inhibitory effect, demonstrating that the inhibitory effect of DNA in these ThT assays is due to DNA binding by FLI1 (Fig. 3b, Table 2).

To quantify if FLI1DBD associates with EWSLCD condensates or remains in the dilute phase, aged samples (24 hrs old) from ThT assays were pelleted. Though condensates are readily visible via microscopy, the majority (> 95 %) of EWSLCD remains in the dilute phase (supernatant) in the absence of FLI1DBD (Fig. 3c). However, in the presence of FLI1DBD, the partitioning of EWSLCD into the pellet fraction was noticeably higher (~ 50 % of total EWSLCD, Fig. 3c), consistent with FLI1DBD enhancing EWSLCD phase separation (Fig. 2a). FLI1DBD also partitioned into the pellet fraction (~ 55 % of total FLI1DBD), indicating direct association with EWSLCD. Addition of HA DNA and FLI1DBD reduced the partitioning of both EWSLCD and FLI1DBD (~ 90 % of each protein remains in the dilute phase) into the pellet further indicating that DNA inhibits the EWSLCD-FLI1DBD interaction. HA DNA alone had no effect on EWSLCD phase separation and FLI1DBD incubated alone remained in the dilute phase (Fig. 3c). Furthermore, the partitioning of FLI1DBD with EWSLCD in the pellet was a specific phenomenon as EWSRRM-RGG2 remained mostly in the supernatant fraction when incubated with EWSLCD condensates under identical conditions (Supplementary Fig. 3b).

Fluorescence microscopy was used to characterize multicomponent EWSLCD condensates. EWSLCD,650 (labeled with DyLight 650) was mixed with FLI1DBD,488 (labeled with DyLight 488) under phase-separating conditions. Fluorescence from FLI1DBD,488 spatially overlapped with fluorescence arising from EWSLCD,650 condensates, consistent with colocalization of FLI1DBD in EWSLCD in condensates (Fig. 3d). FLI1DBD,488 does not phase separate under these conditions. This colocalization is specific and further reinforces the notion that EWSLCD and FLI1DBD interact since unrelated proteins such as green fluorescent protein (GFP) do not colocalize to EWSLCD condensates unless fused to EWSLCD (Supplementary Fig. 4a). Surprisingly, mixing EWSLCD,488 condensates with unlabeled FLI1DBD and HA DNA650 revealed that HA DNA was mostly excluded from the condensates (Fig. 3d).
Exclusion of DNA from EWS<sup>LCD</sup> condensates may be due to a lack of charge neutralization of the DNA phosphate backbone within the condensates since out of 264 residues the EWS<sup>LCD</sup> contains only two positively charged residues in contrast with six negatively charged and 27 tyrosines with a delocalized electron in their sidechains. DNA exclusion from the condensate indicated that the inhibitory effect of DNA in the ThT assays may arise from decreased partitioning of FLI1<sup>DBD</sup> into EWS<sup>LCD</sup> condensates due DNA binding. To test this, fluorescence microscopy experiments using EWS<sup>LCD</sup>, FLI1<sup>DBD</sup>, and increasing concentrations of unlabeled HA DNA were conducted (Fig. 3e). A fluorescence intensity ratio (\(I_{in}/I_{out}\)) was calculated to quantify partitioning of FLI1<sup>DBD</sup> in the condensates (Fig. 3e). In the absence of DNA, \(I_{in}/I_{out}\) was \(\sim 5.8 \pm 2.0\). As the concentration of HA DNA increased, the intensity of FLI1<sup>DBD</sup> in condensates became noticeably dimmer, and \(I_{in}/I_{out}\) reduced to \(1.6 \pm 0.2\) for EWS<sup>LCD</sup>,50 with FLI1<sup>DBD</sup> and 50 \(\mu\)M HA DNA (Fig. 3e). Therefore, FLI1 DNA binding out-competes the interactions between EWS<sup>LCD</sup> and FLI1<sup>DBD</sup> that drive condensate colocalization. Exclusion of HA DNA from EWS<sup>LCD</sup> condensates was also observed when full-length EWS-FLI1 was substituted for FLI1<sup>DBD</sup> (Supplementary Fig. 4b).

FRAP was used to determine whether colocalization of FLI1<sup>DBD</sup> to EWS<sup>LCD</sup> condensates results in a reduction in the dynamics of EWS<sup>LCD</sup> within the condensates, as observed for EWS-FLI1 (Fig. 1c). Freshly prepared EWS<sup>LCD</sup> condensates underwent rapid recovery after photobleaching, indicating that the condensates are liquid-like (Fig. 3f). In contrast, freshly prepared condensates formed in the presence of FLI1<sup>DBD</sup> displayed slower fluorescence recovery, indicating that the dynamics of EWS<sup>LCD</sup> molecules within the condensates change in the presence of FLI1<sup>DBD</sup> (Fig. 3f). When EWS<sup>LCD</sup> condensates were prepared with FLI1<sup>DBD</sup> and increasing concentrations of HA DNA, fluorescence recovered more rapidly in a DNA concentration-dependent manner, consistent with DNA binding inhibiting the effect of FLI1<sup>DBD</sup> on EWS<sup>LCD</sup> (Figs. 3e and f).

**The FLI1 DNA-binding and dimer interfaces are not involved in the interaction with EWS<sup>LCD</sup>**. The ThT assays and fluorescent colocalization data indicate that FLI1<sup>DBD</sup> interacts directly with EWS<sup>LCD</sup>. The DNA recognition helix (\(\alpha 3\)) harbors two highly conserved arginine residues that contact core bases of the ETS consensus sequence in all solved crystal structures of ETS DBDs complexed with DNA<sup>49-53</sup> (Fig. 4A, Supplementary Fig. 5a). To test if these arginines participate in Arg-Tyr stacking interactions with EWS<sup>LCD</sup>, both arginines were mutated to leucines (R2L2). This mutant is incapable of binding HA DNA (Supplementary Fig. 6a) and its effect on EWS<sup>LCD</sup> condensates was assessed using ThT assays (Fig. 4b). Surprisingly, the R2L2 mutant retained the ability to enhance the rate of EWS<sup>LCD</sup> condensate
ageing (Fig. 4b). Together these results suggest that the positively charged DNA recognition helix of FLI1 may not be involved in the EWS_{LCD}-FLI1_{DBD} interaction.

The structures of ETS DBDs solved to date share the same fold (Supplementary Fig. 5b), however the precise margins of the ETS domain vary in the existing literature. In some studies an 85 amino acid construct comprising only three α-helices is used\(^4\,54-56\), while other studies used a longer construct with a fourth α-helix\(^4,22,50\). A recent study crystalized FLI1_{DBD} as a dimer with the fourth α-helix participating in the dimer interface\(^50\). The FLI1_{DBD} construct used in the ThT assays includes this fourth α-helix (residues 362 - 369) as well as ~30 C-terminal disordered residues. To determine whether this fourth α-helix with exposed hydrophobic residues interacted with EWS_{LCD}, a FLI1_{DBD} construct truncated at residue 361 was subjected to our ThT assays (Fig. 4a, Table 1, Supplementary Fig. 1). As anticipated, this construct retained DNA binding activity as judged by electrophoretic mobility shift assays (Supplementary Fig. 6b). Further, the shorter FLI1_{DBD} construct exerted the same effect as the longer construct on EWS_{LCD} condensates in ThT assays (Fig. 4c). Together, these observations further delineate the EWS_{LCD} interaction site that is responsible for inducing EWS_{LCD} ageing in condensates to the folded core of FLI1, between residues 276 and 361 but excluding the DNA recognition helix (α3, Fig. 4a).

**Enhancement of EWS_{LCD} condensate ageing is a general property of ETS DBDs.** The evidence presented above indicate a specific interaction between EWS_{LCD} and FLI1_{DBD} is responsible for condensate ageing. Aside from FLI1, other EWS-ETS fusions have been identified as driver mutations in EwS. Therefore, two other ETS DBDs that participate in EwS (ERG, ETS translocation variant 1, ETV1), a non-EwS associated ETS DBD transcription factor PU.1 (PU.1), and the EWS_{RRM} and EWS_{RRM-RGG2} control proteins were subjected to ThT assays (Fig. 5, Supplemental Figs. 5b and c). All recombinant constructs were properly folded as judged from circular dichroism spectra (Supplementary Fig. 7). Bright-field microscopy images were acquired for each sample at the end of the ThT assay, (T > 10 hrs) (Fig. 5). The control EWS_{RRM} and EWS_{RRM-RGG2} constructs had minimal effects on the rate at which ThT fluorescence increased for EWS_{LCD} condensates and the condensates remained well dispersed and predominantly spherical (Fig. 5). In contrast, all four ETS DBDs significantly enhanced the rate at which the condensates aged (Fig. 5). Furthermore, EWS_{LCD} condensates formed with ETS DBDs were irregularly shaped, most notably in the presence of PU.1_{DBD} and ETV1_{DBD} (Fig. 5). At higher concentrations of FLI1_{DBD} (> 50 µM), EWS_{LCD} condensates were highly irregularly shaped, even at T ~ 0 hours (Supplementary Fig. 8).

The addition of HA DNA along with ERG_{DBD} and PU.1_{DBD} revealed that DNA binding had the same inhibitory effect on EWS_{LCD} condensate ageing as was observed for FLI1_{DBD}, suggesting a
common mechanism for ETS domains (Supplementary Fig. 9a). Additionally, the ETS DBD constructs, EWS$^{RRM}$ or EWS$^{RRM-RGG2}$ constructs alone were incapable of forming ThT-positive structures (Supplementary Fig. 9b). Potential contribution of the 8x His-tag used for purification of ETS DBDs, EWS$^{RRM}$ and EWS$^{RRM-RGG2}$ proteins was assessed with a His-tag free version of PU.1$^{DBD}$ (Supplementary Fig. 9c). Since the EWS$^{RRM}$ and EWS$^{RRM-RGG2}$ constructs also retained His-tags but did not induce condensate ageing and the His-tag free PU.1$^{DBD}$ induced condensate ageing at the same rate as His-tagged PU.1$^{DBD}$, the His-tag did not non-specifically induce ageing of EWS$^{LCD}$ condensates (Supplementary Fig. 9c). These finding suggest that the effect FLI1$^{DBD}$ exerts on EWS$^{LCD}$ condensates is conserved for members of the ETS TF family.

**ETS DBDs interact with the EWS$^{LCD}$ via residues adjacent to the DNA-binding face.** Solution NMR was used to map the ETS DBD interface with the EWS$^{LCD}$. The interaction between the proteins was predicted to be transient, and significant technical challenges arise from using NMR to study an aggregation-prone system. PU.1$^{DBD}$ was chosen for NMR since it induced the most rapid increase in ThT fluorescence, indicative of a stronger interaction between PU.1$^{DBD}$ and EWS$^{LCD}$ (Fig. 5). Backbone resonances of PU.1 were assigned using standard approaches. $^1$H,$^{15}$N heteronuclear single quantum coherence (HSQC) spectra were recorded for each addition of unlabeled EWS$^{LCD}$ to $^{15}$N PU.1$^{DBD}$ up to a molar ratio of ~ 3:1 (Supplementary Fig. 10). Addition of higher concentrations of EWS$^{LCD}$ were not possible because, PU.1$^{DBD}$ induced EWS$^{LCD}$ self-association and phase separation. Nevertheless, small chemical shift perturbations (CSP) ($\delta\Delta \sim 0.04$ ppm) were observed for PU.1 resonances (Fig. 6a, Supplementary Fig. 10). The signal intensity uniformly decreased between the initial and last titration points likely due to co-aggregation of PU.1 with EWS$^{LCD}$ condensates (Fig. 6b). However, a few peaks were differentially broadened, and coincided with or were located near residues with CSPs (Figs. 6a and b). Residues with CSPs greater than one standard deviation and residues with differential signal intensities less than one standard deviation were plotted onto the AlphaFold$^{58}$ structure of human PU.1 (Fig. 6c). Notably, these residues clustered to one face of the DBD, primarily incorporating residues in the ETS DBD “wings” (Loops 4 and 6) that contact the phosphate backbone of DNA$^{51}$, however no shifts or differential broadening were observed for the DNA recognition helix, supporting our earlier hypothesis that it is not involved in the interaction with EWS$^{LCD}$ (Fig. 6d). Furthermore, no shifts or peak broadening were observed on the opposite face of the DBD (Fig. 6c). The CSPs and differential broadening observed for the disordered C-terminal tail were deemed non-specific since they are not conserved in other ETS DBDs and since C-terminally truncated ETS domains (FLI1$^{DBD,\Delta\alpha4}$) retain condensate ageing activity (Fig. 4c).
Comparison of the sequences of PU.1 loops 4 and 6 to those of ERG, FLI1 and ETV1 revealed that the total charge varies between +6 to +1 between the DBDs (Fig. 6d). The loops in PU.1 are flexible and enriched in Lys residues and thus have a high net charge of +6, loops 4 and 6 of FLI1 and ERG are identical with a net charge of +3, and ETV1 has a net charge of +1 (Supplementary Fig. 11). Each loop contains at least one highly conserved positively charged residue (Arg or Lys) at 220 and 247 (PU.1 sequence numbering, Fig. 6d). The ThT assays revealed that PU.1DBD induced condensate ageing at the fastest rate, followed by FLI1DBD and ERGDBD, and ETV1DBD induced ageing at the slowest rate (Fig. 5). Therefore, the net charge of loops 4 and 6 is correlated to the rate at which the ETS DBD induces EWSLCD condensate ageing with higher overall charge inducing the fastest ageing. This correlation suggests that the interaction between ETS DBDs and EWSLCD that drives condensate ageing is influenced by electrostatic interactions. However, electrostatic interactions are clearly not the only factor that influences condensate ageing because the EWS5RMRGG2 construct is enriched with positively charged Arg residues, yet this construct does not affect EWSLCD condensate ageing like the ETS DBDs (Fig. 5). Therefore, it is likely that the relative positioning of the two loops in the ETS DBD structure is also an important factor contributing to EWSLCD condensate ageing.

Discussion

Though the roles of EWS are not completely defined, knockout of EWS is postnatal lethal in mice, indicating it functions in homologous recombination, the DNA damage response, and in splicing. The phenotype induced by expression of EWS-FLI1 mimics EWS knockdown phenotype in HeLa and EwS cells leading to hypothesis that EWS-FLI1 exerts a dominant-negative effect on the normal cellular functions of EWS. This work demonstrated that FLI1DBD enhanced the phase separation propensity of EWSLCD, localized specifically to EWSLCD condensates, and accelerated condensate ageing. This effect was conserved for three other ETS DBDs. Boulay et al. recently reported that b-isoxazole mediated precipitation of EWS-FLI1 in EwS cell lines was enhanced relative to WT EWS, supporting the hypothesis that the interaction between EWSLCD and FLI1DBD enhances phase separation. Previous studies in prostate cancer cell lines also identified interactions between EWS and ERG, ETV1, ETV4 and ETV5 and these were proposed to be necessary and sufficient for the tumor phenotype.

Furthermore, co-immunoprecipitation experiments demonstrated a direct interaction between PU.1 and FUS that inhibits the normal functions of FUS in splicing. Therefore, mis-localization of ETS domains either as fusions or due to aberrant regulation negatively impacts the function of EWS and possibly other nucleic acid-binding proteins. The interaction between ETS DBDs and EWSLCD appears to involve residues that are at least partially occluded by DNA binding. As a result, DNA binding
reduces the interactions between ETS DBDs and EWS\textsuperscript{LCD} that modulate phase separation and promote the formation of cross β-structure within EWS\textsuperscript{LCD} condensates (Fig. 7). The in vivo situation is more complicated since other proteins, nucleic acids, and post-translational modifications will modulate the properties of the condensate, thus fibril formation is not a likely endpoint in EwS, rather the dominant negative effect is attributable to enhanced self-association of EWS and likely other, related proteins.

Self-association of EWS\textsuperscript{LCD} stabilizes EWS-FLI1 binding to GGAA microsatellites, helps recruit RNA Pol II, and is required for transcriptional activation of GGAA-associated genes that drive oncogenesis\textsuperscript{12,19,20}. Chong et al. over expressed exogenous EWS\textsuperscript{LCD} in EwS patient-derived cells to assess the roles of EWS and EWS-FLI1 on transcriptional output of GGAA-responsive genes. Increasing the population of EWS\textsuperscript{LCD} reduced the transcriptional activation by EWS-FLI1 likely through increased LCD-LCD interactions\textsuperscript{40}. The authors proposed that a narrow optimum range of LCD-LCD interactions are required to activate transcription. When considered through the model presented here, the observations of Chong et al., raise an interesting conjecture; could excess EWS\textsuperscript{LCD} act as a molecular sponge and blunt the effects of EWS-FLI1 on transcriptional activation? Therefore, the question remains whether the tunable transcriptional activity of EWS-FLI1 arises from the number of molecules participating in the transcriptional hubs or whether it results from tight regulation of the dynamics of intermolecular interactions at these hubs. The tunable LCD-LCD interactions that appear to regulate the transcriptional output of EWS-FLI1 need to be further investigated in situ in the context of the full-length EWS-FLI1 fusion protein.

EWS\textsuperscript{LCD} will spontaneously form ThT-reactive structures (albeit slowly) and this reaction is much more rapid when catalyzed by ETS DBDs. The critical residues mediating interactions between EWS\textsuperscript{LCD} and PU.1\textsuperscript{DBD} were localized to loops 4 and 6 of the DBD. The overall positive charge of these loops correlated with the rate at which ThT-positive structures formed and therefore positively charged residues, such as lysines, (enriched in PU.1) may contribute to the affinity of ETS DBDs for the EWS\textsuperscript{LCD}. Supporting the role of positively charged residues in mediating the interaction with EWS\textsuperscript{LCD}, lysine residues in the CTD of RNA Pol II were found to be important for its interaction with hydrogels and condensates formed by the FET family proteins, TAF15 and FUS\textsuperscript{38,65}. Despite the observed correlation with positive charge, the conformation of the ETS DBD loops also contribute to EWS\textsuperscript{LCD} aggregation.

While TEM did not conclusively demonstrate the formation of amyloid fibrils, fibril formation by EWS has been observed previously\textsuperscript{45,46}, though unlike typical amyloid fibrils, these are labile to disassembly\textsuperscript{45,66}. Furthermore, hydrogels of the LCD of FET family proteins have been shown to consist of amyloid-like fibrils\textsuperscript{10,11}. There are no high resolution structural models for EWS fibrils, although
several models exist for short segments of FUS\textsuperscript{67,68} and for the entire LCD of FUS\textsuperscript{42}. Assuming EWS\textsuperscript{LCD} forms cross-β structures in a similar way, via the formation of parallel, in-register β-strands, a key structural feature of these fibrils would be “ladders” of identical sidechains stacked at an interval corresponding to the interstrand distance\textsuperscript{69}. These stabilizing ladders could be formed by aromatic tyrosine residues that undergo π-π stacking or by glutamine/asparagine residues that form complimentary hydrogen bonds across the stacked β-strands\textsuperscript{69}. Structural insight remains elusive for how ETS DBDs enhance the rate at which EWS\textsuperscript{LCD} condensates become ThT positive, but tyrosine residues in the EWS\textsuperscript{LCD} are important for functional self-association\textsuperscript{19,70}. Therefore, tyrosine residues in EWS\textsuperscript{LCD} may interact with the positive charges (cation-π) on ETS DBD loops in a conformation conducive for aromatic ladder formation which in turn may serve to catalyze the formation of cross-β structure. This interaction is expected to be extremely transient and thus the increased high-local concentration of the EWS\textsuperscript{LCD} in condensates enhances the rate of cross-β formation, consistent with the observation that the rate of ThT-positivity is enhanced under phase-separating conditions. The CTD of RNA Pol II has been shown to bind to hydrogels comprised of fibrillar polymers formed by the LCD of FET family proteins, and the degree of CTD binding correlates with the degree of transcriptional activation\textsuperscript{11}. It is therefore possible that the interaction between FLI1\textsuperscript{DBD} and EWS\textsuperscript{LCD} in the fusion protein alter the biophysical properties of EWS-FLI1 condensates in a way that enhances recruitment of RNA Pol II via CTD interactions.

While fluorescence microscopy revealed, unexpectedly, that HA DNA is mostly excluded from condensates formed by EWS\textsuperscript{LCD}, the in vivo situation involving WT EWS and other proteins is likely to be different. Cation-π interactions between tyrosine residues in the LCD and arginine residues in the RGG motifs likely modulate phase separation of WT EWS, as has been observed for FUS\textsuperscript{71}. Technical considerations including preparation of NMR amenable samples and relative instability of the full-length constructs precluded the use of WT EWS and EWS-FLI1 constructs. EWS-FLI1 constructs with solubility tags are less aggregation prone\textsuperscript{72}, however even these constructs do not form condensates with rapid fluorescence recovery after photobleaching\textsuperscript{20} and the effects of solubility tags confound study of the self-associative properties of the protein. Although DNA colocalization with EWS-FLI1 containing condensates was not observed in this work, it is plausible that in vivo additional binding partners and post-translational modifications of EWS-FLI1 may serve to neutralize the net negative charge that could be expected of a biomolecular condensate formed by EWS-FLI1 and DNA.

EWS-FLI1 drives oncogenesis through dysregulation of genes downstream of GGAA microsatellites and dysregulation of alternative splicing programs, a function dependent on an intact
As part of its oncogenic function, EWS-FLI1 also associates with a multitude of nucleic acid binding proteins, including the remaining copy of EWS\textsuperscript{36}, FUS, and RNA Pol II\textsuperscript{3,21,74}. These interactions remain poorly understood, likely due to their heterogenous and transient nature. The central finding presented here is that ETS DBDs colocalize to and interact transiently yet specifically with the EWS\textsuperscript{LCD}, altering the intermolecular interactions that govern its LLPS and subsequent condensate ageing (Fig. 7). These results provide a mechanism that may explain how EWS-FLI1 interferes with the normal functions of EWS and potentially other crucial nucleic acid binding proteins. The presence of the FLI1\textsuperscript{DBD} (or other ETS DBD) promotes self-association and aggregation of EWS altering the local dynamics and intermolecular interactions crucial for EWS function.

Methods

Protein expression and purification. Protein constructs used are listed in Table 1. All constructs were derived from human sequences, codon optimized for \textit{E. coli} expression, synthesized (GenScript) and cloned into modified pET expression vectors that placed a 6 or 8 x His-tag followed by a tobacco etch virus (TEV) protease cleavage site N-terminal to the protein coding sequence. The F385A mutation in EWS-FLI1 (EWS-FLI1 numbering) and the F362A mutation in FLI1\textsuperscript{DBD} (FLI1 numbering) correspond to the same residue and were introduced to reduce the propensity of FLI1\textsuperscript{DBD} to dimerize\textsuperscript{50}. Full-length MBP-FUS was a gift from N. Fawzi (Addgene plasmid #98651). All plasmids were verified by DNA sequencing. Constructs were expressed and purified from \textit{E. coli} BL21 Star\textsuperscript{TM} (DE3) or BL21 (DE3) pLysS (Invitrogen, MA) cells in Luria Broth or M9 media supplemented with \textsuperscript{15}NH\textsubscript{4}Cl (1 g/L, Sigma) and 0.02% (w/v) yeast extract for \textsuperscript{15}N labelling or \textsuperscript{15}NH\textsubscript{4}Cl and \textsuperscript{13}C\textsubscript{6} D-glucose (3 g/L, Sigma) with 0.02% (w/v) Isogro\textsuperscript{®}\textsuperscript{-13}C, \textsuperscript{15}N (Sigma) for \textsuperscript{15}N, \textsuperscript{13}C labelling. Generally, expression was induced at OD\textsubscript{600} of 0.6 – 0.8 with 1 mM IPTG and continued for 4 hours at 37°C, or overnight at 16°C or 12°C.

All proteins were purified by immobilized metal affinity chromatography or cation exchange chromatography fractionation, followed by cleavage of the His-tag using TEV S219V protease\textsuperscript{75} and a final polishing size-exclusion chromatography (SEC) step. Most ETS DBDs (unless otherwise specified) were purified without cleavage of the His-tag. EWS and FUS constructs were purified in 20 mM CAPS pH 11 as previously described\textsuperscript{57,76}. ETS DBDs and EWS RNA binding domains were purified in either sodium phosphate buffers or Tris buffers at pH 7-8. Protein purity for all constructs was confirmed to be \textgeq 95% by SDS-PAGE. Protein aliquots were stored at -80°C (see Supplementary Material for detailed protocols).
**Fluorescent labelling.** Purified EWS<sup>LCD</sup>, EWS and EWS-FLI1 were fluorescently labelled with DyLight 488, Dylight 650 or fluorescein maleimide 488 (Thermo Scientific) using sortase<sup>77</sup>. The dyes were resolubilized in DMF and used to label the SortC1 peptide, KLPETGG (GenScript). Proteins to be fluorescently labelled were prepared in 0.1 M sodium bicarbonate pH 9.5 with equal concentrations of the dye-labelled peptide and 2.5 µM sortase, and incubated overnight at room temperature protected from light. Free dye, peptide and enzyme was removed by SEC using a HiLoad 16/600 Superdex 75 pg column (Cytiva), labelling was verified by SDS-PAGE, and UV transillumination. Fluorescently labelled protein was stored at -80°C until use. FLI1<sup>DBD C299S S390C</sup> in 20 mM Tris pH 7.3, 2 M GuHCl, 1 mM TCEP was mixed with fluorescein maleimide and incubated overnight at room temperature. Free dye was removed using a 3 kDa cutoff Amicon centrifugal concentrator (Merck, NJ), and the buffer was exchanged to 50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1mM TCEP. Aliquots were stored at -80°C. Fluorescently labeled DNA was purchased from IDT.

**Thioflavin T assays.** 23 µL samples of ETS DBDs or EWS RNA binding domain constructs (at specified concentrations) and with or without the appropriate DNA oligonucleotide (purchased from IDT, Table 2) were dissolved in 20 mM sodium phosphate buffer pH 7.4, 150 mM NaCl (or 0 mM NaCl), 10 µM ThT and prepared in triplicate in 384-well flat bottom black plates (Greiner Bio-One, NC). Immediately prior to the start of the assay, 2 µL of a 625 µM stock of EWS<sup>LCD</sup> (or EWS-FLI1 or WT EWS) in 20 mM CAPS pH 11 was added and mixed thoroughly by pipetting to initiate phase separation and the plate was sealed (Thermo Scientific). ThT fluorescence was read at 10-minute intervals for 24 hours at 25°C using a Tecan Infinite M200 plate reader (Tecan Trading AG). Raw fluorescence intensities were normalized to a control sample.

**Pelleting assays.** Aged samples (T > 10 hours) from ThT assays were removed from 384-well plates and centrifuged at 21,300 x g for 15 minutes at ambient temperature. The supernatant was removed, and the pellet was resuspended in 8 M urea. The supernatant, pellet, and a sample prior to centrifugation (“total”) were analyzed by SDS-PAGE.

**Microscopy.** Freshly prepared (T ~ 0-1 hour) or aged samples (T ~ 24 hours) were imaged directly in sealed 384-well microwell plates with a BioTek Cytation Gen 5 imaging plate reader (Agilent) using a 20 x objective. Alternatively, sealed, chambered (50-well) coverslips (Grace Biolabs), coated with 1% Pluronic F-127 were imaged with an Olympus FV300 inverted confocal microscope using a 40x oil-immersion objective lens operating at 1% power using the 488 nm laser for transmitted light and the 488 nm and 650 nm lasers for fluorescence imaging. Images were acquired simultaneously in differential interference contrast (DIC) and fluorescent modes. Images were processed using Fiji<sup>78</sup> and fluorescence
intensity ratios were extracted by selecting 16 regions outside of the condensed phase and 16 regions inside the condensed phase across 6–8 image frames per sample.

To measure fluorescence recovery after photobleaching (FRAP), circular regions of interest were irradiated at a laser power of 10% and fluorescence recovery was monitored at 250-millisecond intervals for 30 seconds after bleaching. Image contrast was adjusted globally. The fluorescence intensities for bleached regions were obtained using Fiji. FRAP curves were normalized using a 0–1 scale by setting pre-bleach points to be 1 and the intensity of the first post-bleach point to be 0.

Transmission electron micrographs were acquired on aged samples (T ~ 24 hours) from ThT assay endpoints. Samples were removed from 384-well plates and applied to formvar/carbon coated grids (Electron Microscopy Sciences, PA). Samples were stained with 2% uranyl acetate and imaged using a JEOL 1400 Transmission electron microscope with a XR80 camera (AMT imaging, MA) at a magnification of 50,000 x.

**Nuclear magnetic resonance spectroscopy.** NMR experiments were conducted on a Bruker Avance NEO spectrometer (Bruker, MA) operating at a proton Larmor frequency of 700.13 MHz at a temperature of 25°C. $^1$H, $^{15}$N-HSQC, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and HCC(CO)NH non-uniformly sampled (15-30% sampling density) data sets were recorded on a 0 of 425 μM sample of $^{15}$N, $^{13}$C PU. 1 168-270 (Table 2, Supplementary Fig. 1) dissolved in 20 mM sodium phosphate buffer pH 6.4, 0.5 mM EDTA, 0.02% sodium azide. Detailed acquisition parameters are included in the Supplementary Material. Spectra were reconstructed using the SMILE algorithm and processed with the nmrPipe suite of programs. Backbone $^1$H, $^{13}$C$_\alpha$, $^{13}$C$_\beta$, $^{13}$C’ and $^{15}$N resonances were assigned using CCPNMR Analysis 3.0 software. Acquisition parameters and a detailed description of the measurement of $R_1$, $R_2$ and heteronuclear NOE is included in the Supplementary Material. Chemical shift perturbations were measured by titrating a sample of 50 μM $^{15}$N labelled PU.1 168-270 (Table 2, Supplementary Fig. 1) in 50 mM sodium phosphate buffer pH 6.5 with aliquots of a 2 mM stock of EWS$^{\text{LCD}}$ in 20 mM CAPS pH 11. The final concentration of the EWS$^{\text{LCD}}$ at each titration point was 12.5, 25, 50, 100 and 150 μM resulting in an ~7% dilution of PU.1 across the titration. $^1$H, $^{15}$N-HSQC spectra were recorded for each titration point processed in Topspin 4.1.1 and analyzed using CCPNMR Analysis 3.0 software. Acquisition parameters and details of CSP calculations are included in the Supplementary Materials.

**Electrophoretic mobility shift assays.** 20 μL samples were loaded into wells of a 20% polyacrylamide gel prepared in 0.5 x TBE buffer. Electrophoresis was performed for 60 minutes at 150 V using 0.5 x
TBE and stained with SYBRsafe (Thermo Fisher, MA, USA) according to the manufacturer’s specifications for 30 minutes before imaging using UV transillumination.

**Circular dichroism.** Circular dichroic spectra were recorded on 10 µM samples of all proteins in 50 mM sodium phosphate buffer pH 6.5 at 25°C in a 2 mm pathlength cuvette using a Jasco 810 spectropolarimeter (Jasco, OK) at a scan speed of 50 nm/min with 0.2 nm increments. Each sample was recorded in triplicate, the data was then averaged and converted to mean residue ellipticity.
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Data Availability: NMRPipe processing scripts are available upon reasonable request, expression plasmids containing the EWS, EWS-FLI1, and the ETS DBD constructs were deposited with Addgene (####). The backbone resonance assignments for the PU.1DBD were deposited in the BMRB (####).

Author contributions: EES made the protein samples, developed the ThT assays, prepared reagents, collected, processed, and interpreted data, wrote manuscript, conceptualized the study, AKRM made protein samples, conducted phase separation assays, processed data, SA made protein samples, conducted phase separation assays, processed data, XX designed expression construct plasmids, purified protein, DSL collected and processed data, wrote manuscript, obtained funding, and conceptualized the study. All authors contributed to editing the manuscript and have read and approved the manuscript for publication.

Competing interests: The authors declare no competing interests.
### Tables

#### Table 1. Recombinant protein constructs of EWS, FUS, EWS-FLI1 and ETS TFs.

| Construct name                | Affinity Tag | MW (kDa) | Margins \(^1\)               |
|-------------------------------|--------------|----------|------------------------------|
| EWS (full-length)             | 8 x His      | 68.5     | EWS 1 – 656                  |
| FUS (full-length)             | His-MBP      | 53.4     | FUS 1 – 526                  |
| EWS-FLI1 (type 2, full-length)| 8 x His      | 54.3     | EWS 1 – 264 + FLI1 220 – 452|
| EWS-FLI1 194-445 F385A        | 8 x His      | 26.0     | EWS 194 – 264 + FLI1 242 – 452|
| EWS\(^{\text{LCD}}\)          | 8 x His      | 27.9     | EWS 2 – 264                  |
| EWS\(^{\text{RRM}}\)          | 8 x His      | 9.9      | EWS 359 – 447                |
| EWS\(^{\text{RRM-RGG2}}\)     | 8 x His      | 16.3     | EWS 359 – 513                |
| FLI1\(^{\text{DBD}}\)         | 8 x His      | 14.6     | FLI1 276 – 399 (F362A)       |
| FLI1\(^{\text{DBD C299S S390C}}\)| 8 x His  | 14.6     | FLI1 276 – 399 (C299S, F362A, S390C) |
| FLI1\(^{\text{DBD R2L2}}\)    | 8 x His      | 14.4     | FLI1 276 – 399 ( R337L, R340L, F362A) |
| FLI1\(^{\text{DBD \Delta a4}}\)| 8 x His  | 10.3     | FLI1 276 – 361                |
| ERG\(^{\text{DBD}}\)          | 8 x His      | 14.5     | ERG 306 – 429 (F392A)        |
| ETV1\(^{\text{DBD}}\)         | 8 x His      | 15.1     | ETV1 332 – 458               |
| PU.1\(^{\text{DBD}}\)         | 8 x His      | 12.3     | PU.1 168 – 270               |

\(^1\) Canonical sequences from Uniprot.

#### Table 2. DNA oligonucleotides used for binding studies with ETS DBDs

| Construct name | Sequence                                      |
|----------------|-----------------------------------------------|
| HA DNA         | TTTACCGGAAGTGTGTTT                            |
| 10 x GGAA DNA  | CGCGGATCCGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAAGGAAGGAAGGAAGGAAGGAAGGAAGGAACTGCAGTTTT |
| Scrambled DNA  | TTGAGAGAGAGAGAGATT                            |
Figure 1. Biomolecular condensation of EWS and EWS-FLI1. a The domain architecture of EWS, FLI1 and the EWS-FLI1 fusion protein. The breakpoint for the fusion protein is indicated by a black triangle for EWS and FLI1. b Phase diagrams for FUS, EWS and EWS-FLI1 as a function of protein concentration and NaCl concentration. Phase separation was assessed using turbidity measurements at 600 nm. c Fluorescence recovery after photobleaching of EWS\textsuperscript{LCD} within condensates containing 25 µM EWS\textsuperscript{LCD} with 9 µM WT EWS (blue), 5µM EWS-FLI1 (red) or 25 µM EWS\textsuperscript{LCD} (black). Scale bars indicate 25 µm. FRAP data are presented as mean values ± SEM.
Figure 2. FLI1\textsuperscript{DBD} alters the phase separation propensity of EWS\textsuperscript{LCD}. a EWS\textsuperscript{LCD} phase separation at 25 µM in 100 mM NaCl assessed by turbidity at 340 nm (left panel) and bright-field microscopy (right panels) alone or in the presence of equimolar concentrations of FLI1\textsuperscript{DBD}, EWS\textsuperscript{RRM} or EWS\textsuperscript{RRM-RGG2}. Turbidity data are presented as the average of triplicates ± SEM. Scale bars indicate 25 µm. b ThT assay of 5 µM FLI1\textsuperscript{DBD} alone, 50 µM EWS\textsuperscript{LCD} alone, and with 5 µM FLI1\textsuperscript{DBD} in non-phase-separating conditions (no NaCl), (left panel) and bright-field images (right panels). Scale bars indicate 50 µm. c ThT assay (left panel) of 50 µM EWS\textsuperscript{LCD} alone or with 2 and 5 µM FLI1\textsuperscript{DBD} under phase-separating conditions (150 mM NaCl). Transmission electron micrographs (right panels) of aged samples (T ~ 24 hours) after ThT assays. Scale bars indicate 1 µm. ThT data are presented as mean values ± SEM.
Figure 3. DNA inhibits the effect of FLI1DBD on EWSLCD condensates. Condensates were formed under phase separating conditions (50 µM EWSLCD, 150 mM NaCl). a ThT assay of 50 µM EWSLCD incubated with 5 µM FLI1DBD (red) and 1 µM (green), 3 µM (blue), and 5 µM (magenta) HA DNA (Table 2). b ThT assay of 50 µM EWSLCD alone (black), with 5 µM FLI1DBD (red), or with 5 µM FLI1DBD and 5 µM HA DNA (blue), or GGAA10 DNA (magenta) or scrambled DNA (green) (Table 2). c Pelleting assay of aged samples (T ~ 24 hours) of 50 µM EWSLCD incubated alone, with 5 µM FLI1DBD, with 5µM of FLI1DBD and HA DNA, or with 5 µM HA DNA. A control sample of 5 µM FLI1DBD is also shown. Lanes correspond to the total (T) sample prior to centrifugation, and the supernatant (S) or pellet (P) after centrifugation. d Fluorescence and DIC microscopy of 50 µM EWSLCD (1% EWSLCD650) alone (left panels) or with 5 µM FLI1DBD (10% FLI1DBD488) (middle panels). EWSLCD (1% EWSLCD488) condensates with 5 µM unlabeled FLI1DBD and 5µM HA DNA (10% HA DNA650). Scale bars represent 10 µm. e Fluorescence microscopy of 50 µM EWSLCD (1% EWSLCD650) with 5 µM FLI1DBD (10% FLI1DBD488) and increasing concentrations of HA DNA. Quantification of the 488 nm-fluorescence intensity ratio I_{in}/I_{out} measured in and outside of the condensates. Data are presented as the average of 16 replicates ± SEM. Scale bars indicate 10 µm. f FRAP of 50 µM EWSLCD (1% EWSLCD650) condensates alone, with 5µM FLI1DBD, or 5 µM FLI1DBD and 50 µM HA DNA. FRAP recovery profiles for 30 seconds post-bleach (right panel) and corresponding images (left panel). Scale bars represent 2 µm. ThT and FRAP data are presented as mean values ± SEM.
Figure 4. FLI1^{DBD} helices α3 and α4 do not contribute to the ageing of EWS^{LCD} condensates. a Cartoon representation of the structure of FLI1^{DBD} indicating the DNA recognition helix (α3), two highly conserved arginine residues that contact DNA (Supplementary Fig. 5), and C-terminal helix α4 (PDB 5e8g). Condensates were formed in the presence of 150 mM NaCl. b ThT assay of 50 µM EWS^{LCD} alone (black), or with 5 µM FLI1^{DBD} (red), 5 µM FLI1^{DBD} and 5 µM HA DNA (green), 5 µM FLI1^{DBD R2L2} mutant (blue), or 5 µM FLI1^{DBD R2L2} and 5 µM HA DNA (magenta). c ThT assay of 50 µM EWS^{LCD} alone (black), or with 5 µM FLI1^{DBD} (red) or 5 µM FLI1^{DBD Δα4} truncation mutant (orange). ThT data are presented as mean values ± SEM.
Figure 5. Ageing effect on EWS\textsuperscript{LCD} condensates is conserved for other ETS DBDs. ThT assays (left panels) and bright-field microscopy of aged samples at the end of the ThT assays (T ~ 24 hours, right panels) of 50 µM EWS\textsuperscript{LCD} incubated alone or with 5 µM of EWS\textsuperscript{RRM}, EWS\textsuperscript{RRM-RGG2}, or PU.1\textsuperscript{DBD}, FLI1\textsuperscript{DBD}, ERG\textsuperscript{DBD}, and ETV1\textsuperscript{DBD} as indicated. All samples were prepared with 150 mM NaCl. Dashed lines estimate half the maximum signal. Scale bars indicate 50 µm. ThT data are presented as mean values ± SEM.
Figure 6. Residues in the DBD wings of PU.1 are involved in the interaction with EWS\textsuperscript{LCD}.  

a) Chemical shift perturbations and b) signal broadening of \textsuperscript{15}N-labelled PU.1\textsuperscript{DBD} residues, upon titration with a 3:1 molar ratio of EWS\textsuperscript{LCD}. Red horizontal dashed lines indicate the standard deviation of the CSP or intensity ratio. Residues with CSPs (intensity differences) greater than 1 standard deviation are plotted in orange. Overlapped and ambiguously assigned residues are indicated by black asterisks and two C-terminal proline residues are denoted by red asterisks in (a) and (b). 

c) Residues with CSP (broadening) greater (less) than the standard deviation in (a) and (b) were mapped to the AlphaFold structure of the human PU.1\textsuperscript{DBD} (168-257). 

d) Sequence alignment of loops 4 and 6 that were identified to interact with the EWS\textsuperscript{LCD}. The net charge summed across the two loops is indicated, conserved positive charges in each loop are bolded (Supplementary Fig. 5).
Figure 7. A model for the effect of ETS DBDs on biomolecular condensation by EWS\textsuperscript{LCD}. EWS\textsuperscript{LCD} exists in an equilibrium between monomers, liquid-like condensates, gel-like condensates, and amyloid fibrils. Colocalization of ETS DBDs to EWS\textsuperscript{LCD} condensates, enhances the propensity for EWS\textsuperscript{LCD} to phase separate and increases the rate at which the condensates age, forming ThT-positive structures. DNA-binding by free ETS DBDs inhibits colocalization to EWS\textsuperscript{LCD} condensates and thereby reduces the effect ETS DBDs exert on EWS\textsuperscript{LCD} condensates.
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