ANNUAL REVIEWS

Annual Review of Cancer Biology

The Role of Phase-Separated Condensates in Fusion Oncoprotein–Driven Cancers

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Annu. Rev. Cancer Biol. 2023. 7:73–91

First published as a Review in Advance on January 13, 2023

The *Annual Review of Cancer Biology* is online at cancerbio.annualreviews.org

https://doi.org/10.1146/annurev-cancerbio-061421-122050

Keywords

liquid-liquid phase separation, biomolecular condensates, transcription, kinase, signaling, sequence features, intrinsically disordered regions

Abstract

Fusion oncoproteins (FOs) resulting from in-frame chromosomal translocations are associated with many aggressive cancers with poor patient outcomes. Several FOs are now understood to perform their oncogenic functions within biomolecular condensates formed through liquid-liquid phase separation (LLPS). Two classes of phase-separating FOs have emerged, those that form nuclear condensates and alter chromatin biology, including gene expression, and those that form cytoplasmic condensates and promote aberrant signaling, including RAS/MAPK signaling. The amino acid sequences of the FOs within these classes display LLPS-prone intrinsically disordered regions and folded domains that synergistically interact with themselves and other biomolecules to promote condensate formation. This review summarizes the roles of LLPS in the oncogenic functions of these two FO classes, provides examples of FOs that inhibit physiological LLPS in normal cells, and discusses the sequence features commonly associated with LLPS and their enrichment in many FOs.

INTRODUCTION

The role of chromosomal rearrangements in cancer has been appreciated for over 60 years (Nowell & Hungerford 1960), and it is estimated that about 17% of all tumors display oncogenic gene fusions (Gao et al. 2018), with a much higher proportion in certain cancer subtypes. For example, gene fusions are found in over 50% of leukemias (Lobato et al. 2008) and about 30% of soft tissue tumors (Mertens et al. 2016). Importantly, fusion oncoproteins (FOs) encoded by gene fusions are often cancer drivers, accounting for the lower incidence of additional gene mutations in cancers harboring FOs than in those without FOs (Andersson et al. 2015, Crompton et al. 2014). Most FOs function as either transcription factors or protein kinases to deregulate critical cellular processes and transform cells (Salokas et al. 2020). Cancers driven by FOs are often characterized by very poor prognosis and limited therapeutic strategies for patients, highlighting the need for a deeper understanding of FO-driven oncogenic mechanisms to expand curative treatment options (Brien et al. 2019, Perry et al. 2019).

Bioinformatics analysis of the parent proteins of several thousand FOs has provided insights into their aberrant functions. Babu and coworkers analyzed the parents of ~2,700 FOs and showed enrichment in several critical functions, including translation, mRNA splicing, and cell cycle progression (Latysheva et al. 2016). Further, parent proteins of FOs occupy central positions in human protein-protein interaction networks and are likely to connect distinct network clusters (Latysheva et al. 2016). Thus, FOs often join otherwise distinct interaction networks, promoting aberrant functions and altered cellular behavior. Further enhancing their oncogenic potential, FOs often lack regulatory domains of their parent proteins and display altered cellular localization (Dupain et al. 2017, Latysheva et al. 2016). Together, these abnormal features enable FOs to rewire regulation of critical biological functions, including transcription, signaling, and genome maintenance.

Biological functions are performed in distinct cellular subcompartments. The traditional view of compartmentalization solely via lipid membranes has been confuted by mounting evidence that numerous membraneless compartments selectively concentrate biomolecules throughout the cytosol and nucleus to carry out myriad biochemical functions [reviewed by Banani et al. (2017) and Hyman et al. (2014)]. These so-called biomolecular condensates are formed through the process of liquid-liquid phase separation (LLPS), whereby transient, multivalent interactions between specific proteins and nucleic acids drive their demixing from surrounding molecules into a separate, dense phase or compartment. The material properties of biomolecular condensates can range from liquid-like to gel- or solid-like, depending on the strength and kinetics of the multivalent interactions between their constituents. Since 2009 (Brangwynne et al. 2009), dozens of phase-separated biomolecular condensates have been identified, including stress granules and P bodies in the cytosol, as well as Cajal bodies and nucleoli in the nucleus (reviewed in Banani et al. 2017). Importantly, many of the biological functions known to be altered by FOs have been shown to occur within biomolecular condensates.

Seminal work from Young and colleagues showed that activation of gene expression occurs within phase-separated transcriptional condensates (Boija et al. 2018, Hnisz et al. 2017, Sabari et al. 2018). Transcription factors generally display an activation domain and a DNA-binding domain. Activation domains, critical for transcriptional activation, are typically intrinsically disordered regions (IDRs). Weak and transient multivalent interactions between IDRs within transcription factors, cofactors, and even RNA polymerase II drive phase separation to form liquid-like foci, or puncta, along chromatin. Condensation into chromatin-associated puncta activates gene expression by locally concentrating key transcriptional regulators and by altering 3D chromatin architecture to allow for contact between distal regulatory regions. As transcription factors are

enriched among FO parents, the role of phase separation in their function has become an area of interest.

Another cellular process that has recently been linked to LLPS is kinase-mediated cell signaling. Many have noted puncta at the cell membrane and in the cytoplasm when visualizing kinases and their downstream signaling effectors, but the basis for these observations has only recently been elucidated. Su et al. (2016) showed that T cell receptor (TCR) signaling was dependent on co-phase separation with downstream signaling partners. Through both in vitro reconstitution and cellular experiments, they showed that phosphorylation and activation of the TCR resulted in phase separation and clustering of the downstream transmembrane protein LAT (linker for activation of T cells) and its binding partners (Su et al. 2016). These phase-separated condensates enriched positive regulators of TCR signaling and excluded the inhibitory phosphatase CD45, which resulted in enhanced MAPK/ERK activity. More recently, Zhang et al. (2020) showed that a regulatory subunit of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), RIα, also undergoes phase separation, forming cytoplasmic puncta enriched in the second messenger, cAMP, and high PKA activity. Proper control of cAMP levels is critical for normal cell proliferation and survival, and this is dependent on compartmentalization of the regulatory system. When the authors inhibited phase separation by RIα, cAMP compartmentalization was lost and cAMP signaling was deregulated (Zhang et al. 2020). Recent findings from Lin et al. (2022) have increased the number of protein kinases that may function via LLPS to include seven receptor tyrosine kinases (RTKs), including EGFR, HER2, and FGFR2. In this study, FGFR2 was shown to co-condense at the plasma membrane with downstream effector proteins. As was reported for the TCR, the condensates formed by FGFR2 promote RTK signaling by enhancing positive regulatory enzymatic activities (i.e., phosphorylation and phospholipase activity) while inhibiting negative regulatory activities (i.e., phosphatase activity) (Lin et al. 2022). Thus, phase separation is important for normal signaling in cells and many regulators of these pathways are parents of FOs, strengthening the association of phase separation with FOs.

PROPERTIES OF PHASE-SEPARATED CONDENSATES

Studies over the past decade have provided insights into the biophysical nature of cellular structures that form through LLPS. While phase separation has been characterized extensively with defined, purified components in vitro, our understanding of this phenomenon in cells has been limited by the complexity of the cellular environment. Some proteins form single-component condensates via LLPS in cells through homotypic interactions (**Figure 1*a*,*b***), while others undergo LLPS to form multicomponent condensates through heterotypic interactions with other proteins or nucleic acids (**Figure 1*a*,*c***) (Riback et al. 2020). Condensates vary from subdiffraction-limited sizes to a few microns (Keber et al. 2021, Shin & Brangwynne 2017). Live cell fluorescence microscopy is commonly used to visualize cellular condensates with at least one fluorescently labeled component. Condensates that form through LLPS exhibit the physical characteristics of liquids, including spherical shape due to surface tension. They also often undergo fusion and fission events, although there are some restrictions on these processes. To fuse, two condensates must come into contact and this can be hindered by competing interactions. For example, high-affinity interactions of transcriptional condensates with DNA can limit condensate interactions and prevent fusion events (Chandra et al. 2021). Another property of liquid-like condensates is free diffusion of their constituent molecules, both within and into and out of the dense phase, which is typically assessed by fluorescence recovery after photobleaching (FRAP) experiments. Fast and complete recovery after photobleaching indicates that the labeled component is mobile (usually on the timescale of seconds or minutes) and is suggestive of liquid behavior.

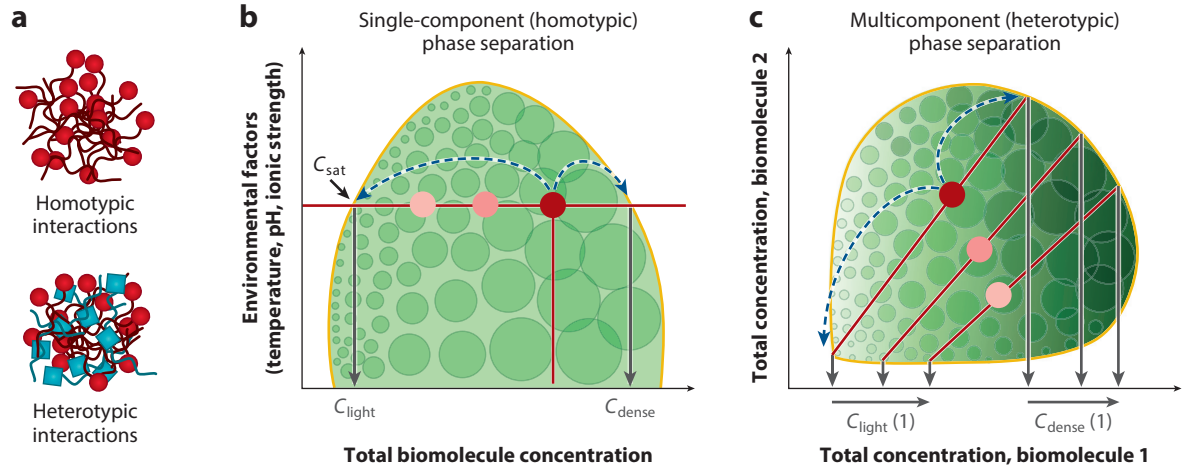

Figure 1

Biomolecules undergo liquid-liquid phase separation (LLPS) via homotypic and heterotypic interactions. (*a*) Homotypic (single-component; *top*) and heterotypic (multicomponent; *bottom*) interactions between condensate-forming proteins. (*b*,*c*) Phase diagrams for (*b*) single-component and (*c*) multicomponent phase separation. The curved yellow line separates the one-phase regime (*white area*; no LLPS) from the two-phase regime (*green area*; LLPS) as a function of varying biomolecule concentration and environmental factors (*b*) or varying concentrations of two biomolecules (*c*). The blue dashed arrows in panel *b* highlight, for a single environmental condition (*red horizontal line*), the light-phase (C_{light}) and dense-phase (C_{dense}) concentrations (*gray arrows*) associated with a given total biomolecule concentration (*red circle* and *vertical red line*). With altered biomolecule concentration (*two light-red circles*), C_{light} (also corresponding to C_{sat}) and C_{dense} remain constant for a single-component system. However, for a multicomponent system (*c*), C_{light} and C_{dense} for each of the two components vary with the slope of the so-called tie lines (*red lines*) and position within the two-component phase diagram. The red circle indicates one pair of total concentrations of two biomolecules, 1 and 2, and the blue dashed arrows indicate the corresponding values for C_{light} and C_{dense} of biomolecule 1. The C_{light} and C_{dense} values associated with the concentrations indicated by the two light-red circles are different. The different shading of the green droplets (*circles*) in panels *b* and *c* reflects the different behavior of C_{light} and C_{dense} values for single- and multicomponent systems, respectively. Panels *b* and *c* adapted with permission from Riback et al. (2020); copyright 2020 the authors.

Phase separation is a concentration-dependent process and is also influenced by environmental factors, including temperature, pH, and ionic strength (**Figure 1*b*,*c***). The concentration at which LLPS occurs for a system is referred to as the saturation concentration (C_{sat}) (**Figure 1*b***). With simple, single-component condensates (termed homotypic phase separation), at a particular environmental condition (red line in **Figure 1*b***), as the total concentration of the phase separating biomolecule increases above C_{sat}, the concentration of the biomolecule within the condensates (termed the dense-phase concentration, C_{dense}; **Figure 1*b***) is constant but the number and size of condensates increase. The biomolecule concentration in the surrounding solution (termed the light-phase concentration, C_{light}; **Figure 1*b***) also remains constant as the total concentration increases. Condensates with multiple components that form through heterotypic LLPS display complex behavior, wherein the light- and dense-phase concentrations of a component (and other components) vary with the total concentration of this component (**Figure 1*c***; the red lines illustrate this behavior) (Riback et al. 2020). This behavior makes multicomponent condensates responsive to changing cellular conditions, for example, as the expression level of one or more condensate components are increased or decreased. The C_{sat} value for a protein condensate component, for either single- or multicomponent phase separation, can be modulated by posttranslational modifications that alter its multivalent interactions. Under certain conditions, phase transitions to form condensates depend upon biomolecule nucleation, which is a process recently shown to be regulated in cells (Shimobayashi et al. 2021). Thus, the process of LLPS

to form condensates can be regulated in cells through control of component concentrations, posttranslational modifications within them, and their nucleation. Finally, the enrichment of biomolecules within condensates through LLPS can promote biological processes, but it can also be inhibitory by sequestering critical process components (Peeples & Rosen 2021).

In this review, we highlight FOs whose oncogenic functions have been shown to involve phase separation. We also discuss the amino acid sequence features of proteins associated with phase separation and their enrichment in FOs, leading us to suggest that many currently uncharacterized FOs function through phase separation–dependent mechanisms.

FUSION ONCOPROTEINS THAT FORM NUCLEAR CONDENSATES

NUP98 Fusion Oncoproteins

Approximately 10% of pediatric acute myeloid leukemia cases, including most chemotherapy-resistant cases, harbor *NUP98* (nucleoporin 98) gene fusions and express NUP98 FOs (McNeer et al. 2019). The N terminus of NUP98, a component of the nuclear pore complex, fuses to more than 30 C-terminal partner genes, many associated with DNA or histone binding (Michmerhuizen et al. 2020). The N-terminal region of NUP98 displays two IDRs enriched in pairs of phenylalanine and glycine residues (so-called FG motifs), separated by a GLEBS domain involved in binding to the RAE1 protein (Pritchard et al. 1999, Radu et al. 1995). For over two decades, NUP98 FOs were noted to form nuclear puncta (Chandra et al. 2021), but investigations into the mechanism underlying this behavior were not undertaken until recently. NUP98-HOXA9 was the first NUP98 FO identified (Nakamura et al. 1996), and studies into its leukemogenic mechanism provide a foundation for understanding other NUP98 FOs. Recent studies have established that NUP98-HOXA9 undergoes LLPS via multivalent interactions mediated by its FG-rich IDR to form gel-like homotypic condensates in vitro and hundreds of chromatin-associated puncta in the nuclei of cells (Ahn et al. 2021, Chandra et al. 2021, Terlecki-Zaniewicz et al. 2021). In cells, puncta formation is governed by both homotypic interactions (e.g., interactions between NUP98-HOXA9 molecules mediated by multivalent FG motifs) and heterotypic interactions (e.g., interactions mediated by the multivalent FG motifs of NUP98-HOXA9 with other biomolecules). The DNA-binding homeodomain of the C-terminal fusion partner HOXA9 causes NUP98-HOXA9 puncta to localize to specific DNA sites within chromatin. Disruption of DNA binding causes formation of a small number of large, dense puncta, which are disassociated from chromatin (Chandra et al. 2021). Mutating multiple FG motifs in NUP98-HOXA9 to serine or alanine weakens the multivalent interactions that underly LLPS and alters the physical features of puncta (Ahn et al. 2021, Chandra et al. 2021).

NUP98 FOs are known to activate expression of *HOX* cluster genes and transform mouse hematopoietic stem and progenitor cells (mHSPCs). Parallel studies by Ahn et al. (2021) and Chandra et al. (2021) showed that the oncogenicity of NUP98-HOXA9 is dependent upon both its phase separation and DNA-binding properties. Mutant forms of NUP98-HOXA9 defective in either phase separation or DNA binding are impaired in the activation of leukemogenic gene expression and transformation of mHSPCs. These results suggest a mechanistic synergy between LLPS driven by multivalent FG motifs and DNA binding driven by the HOXA9 homeodomain for NUP98-HOXA9 to form aberrant transcriptional condensates at specific HOXA9 chromatin binding sites, promoting leukemic gene expression and transformation of mHSPCs. Chandra et al. (2021) showed that this mechanistic synergy is conserved in other NUP98 FOs, NUP98-KDM5A, NUP98-LNP1, and NUP98-PRRX1, which all form nuclear puncta and transform mHSPCs.

Expression of leukemogenic genes by NUP98 FOs is dependent upon the recruitment of transcriptional and epigenetic regulators to specific genomic sites. Xu et al. (2016) showed that

the MLL and NSL complexes interact with NUP98 FOs and promote leukemogenic gene expression. The CREB-binding protein was shown to interact with the FG motif–rich IDR of NUP98-HOXA9 and contribute to its oncogenicity (Kasper et al. 1999). Moreover, chromatin-bound CRM1, a nuclear transport factor, is crucial for the recruitment of NUP98 FOs to chromatin (Oka et al. 2016). The available data indicate that the multivalent FG motifs common to all NUP98 FOs mediate heterotypic interactions with multiple regulatory partners, including CBP/P300, BRD4, DOT1L, SWI/SNF P-TEFb, and SMARCA5 (Ahn et al. 2021, Jevtic et al. 2022, Terlecki-Zaniewicz et al. 2021). LLPS by NUP98-HOXA9 additionally induces chromatin looping, providing a mechanism for spatial colocalization of otherwise distal DNA enhancer and promoter sites within aberrant transcriptional condensates (Ahn et al. 2021). Finally, using a patient-derived xenograft model, Chandra et al. (2021) showed that the NUP98-KDM5A FO formed nuclear puncta by immunostaining, establishing the relevance of phase separation in NUP98 FO–driven leukemia. Together, the data reviewed above demonstrate that NUP98 FOs undergo LLPS to form aberrant transcriptional condensates that drive leukemogenic gene expression and transform hematopoietic cells. The general features of this LLPS-dependent oncogenic mechanism are illustrated in **Figure 2*a***; these features apply, at least in part, to the other FOs that form nuclear condensates, as discussed below.

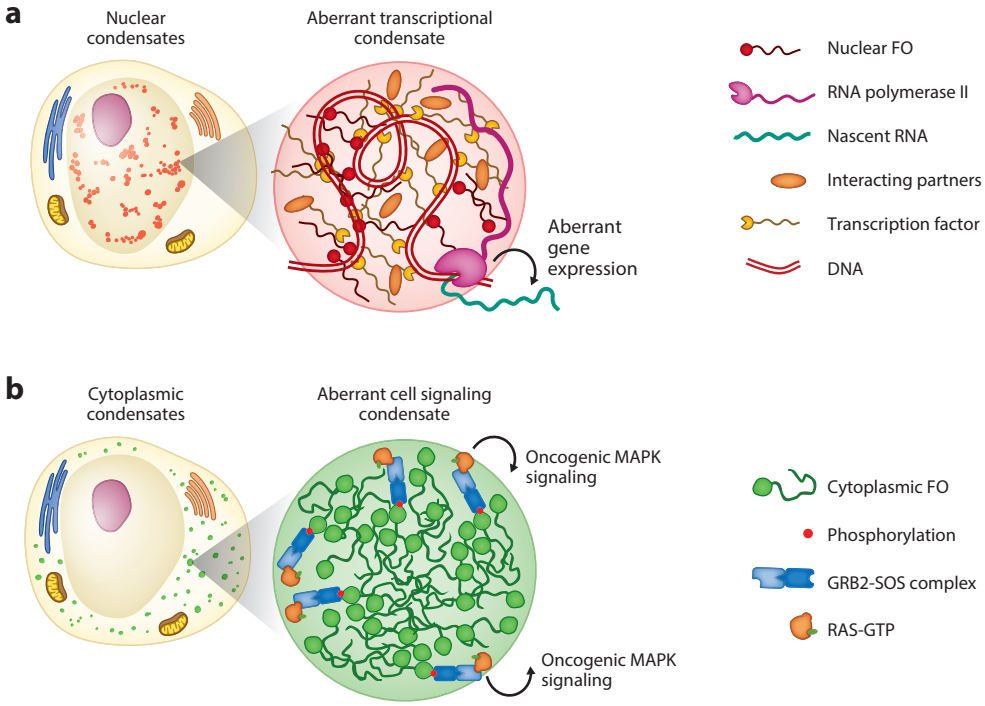

Figure 2

Conceptual representation of fusion oncoprotein (FO) nuclear and cytoplasmic biomolecular condensates. (*a*) Illustration of the aberrant transcriptional condensates formed by nuclear FOs, which often drive aberrant gene expression through co-phase separation with transcriptional regulators, bringing distal chromatin regions together. Examples of these include NUP98 and FET (FUS, EWSR1, TAF15) FOs. (*b*) Illustration of the aberrant cell signaling condensates formed by cytoplasmic FOs, which are active RAS/MAPK signaling centers. They function by concentrating positive regulators of the signaling pathway, while excluding negative regulators. Examples of these include the EML4-ALK and CCDC6-RET FOs.

FET Fusion Oncoproteins

Another common FO family involves fusions between FET (FUS, EWSR1, TAF15) RNA-binding proteins and various DNA- or chromatin-binding partners, including CHOP (C/EBP homologous protein) and ETS (E-twenty-six) transcription family proteins. These FOs are recurrent in sarcomas and function as aberrant transcription factors (Kinsey et al. 2006, May et al. 1993). The parent FET family proteins form oligomers and undergo LLPS driven by prion-like IDRs, which are retained in the FET FOs (Kato et al. 2012; Patel et al. 2012, 2015).

While the first FET FO was observed to localize in round, nuclear bodies (NBs) 20 years ago (Thelin-Jarnum et al. 2002), they were only recently linked to LLPS (Boulay et al. 2017). EWS-FLI1 is a driver of Ewing sarcoma, the second most common pediatric bone cancer. This FO is comprised of the prion-like IDR of EWSR1 fused to the ETS DNA-binding domain of FLI1, which binds DNA sequences containing the GGAA motif (Delattre et al. 1992, Guillon et al. 2009). Genomic and transcriptomic analysis in Ewing sarcoma cells established that EWS-FLI1 alters chromatin state, both by generating de novo enhancers at GGAA microsatellite regions and by inactivating conserved enhancers at canonical ETS binding sites by sequestering endogenous ETS proteins, leading to dysregulated gene expression (Gangwal et al. 2008, Patel et al. 2012, Riggi et al. 2014). These large-scale effects on chromatin state require interactions with chromatin remodelers that alter nucleosome occupancy. Boulay et al. (2017) showed that EWS-FLI1 recruits the chromatin remodeler complex SWI/SNF (mammalian switch/sucrose nonfermentable) to GGAA microsatellites, which leads to FO-dependent changes in gene expression. Endogenous EWSR1 also interacts with SWI/SNF, which was dependent on an interaction between the N-terminal, prion-like IDR of EWSR1 and the BRG1 subunit of the SWI/SNF complex. Using biotinylated isoxazole precipitation and sedimentation assays, Boulay et al. (2017) showed that EWS-FLI1 undergoes condensation in vitro and immunostaining experiments showed that the FO forms round nuclear puncta in cancer-relevant cell lines. Combining high-resolution imaging with 3D DNA fluorescence in situ hybridization revealed that the EWS-FLI1 puncta were localized at GGAA microsatellites (Chong et al. 2018). Importantly, mutating multiple tyrosine residues in the N-terminal prion-like IDR of EWS-FLI1 to serine residues abolished puncta formation in cells and condensation in vitro, while also inhibiting FO binding to the SWI/SNF complex and wild-type EWSR1, binding to GGAA microsatellites, and activation of gene expression (Boulay et al. 2017, Chong et al. 2018). However, these activities were rescued by fusing a 37 amino acid fragment of the EWSR1 prion-like domain to the FLI1 fragment of the FO, suggesting that phase separation by the EWSR1 prion-like IDR is essential for EWS-FLI1's oncogenic function (Boulay et al. 2017, Johnson et al. 2017). Importantly, the oncogenic activity of EWS-FLI1 is also dependent on interaction with endogenous EWSR1, which is abrogated by tyrosine-to-serine mutations in the EWS-FLI1 prion-like IDR, suggesting a mechanism of colocalization by co-phase separation (Ahmed et al. 2021).

A second FET FO, FUS-CHOP, also functions as an aberrant transcription factor through phase separation driven by its N-terminal prion-like IDR (Davis et al. 2021, Owen et al. 2021). While a specific amino acid sequence within the prion-like IDR was not required, a threshold IDR length to create multivalence was required for phase separation (Owen et al. 2021). As with NUP98 FOs, the FET FOs drive aberrant gene expression through co-recruitment of additional factors. FUS-CHOP cellular condensates colocalize with critical transcriptional regulators, including BRD4 and the BRG1 subunit of the SWI/SNF complex (Davis et al. 2021, Owen et al. 2021). Sequence analysis has revealed that several additional members of the SWI/SNF complex contain prion-like IDRs and fragments of these IDRs partition into FUS-CHOP condensates in vitro (Davis et al. 2021). These results, in conjunction with studies of EWS-FLI1 discussed

above, suggest that prion-like IDR interactions may drive not only homotypic FET FO phase separation but also heterotypic phase separation with additional prion-like IDR-containing transcriptional regulators. Through this mechanism of condensation and recruitment of key factors, these FOs activate gene expression at specific loci. In vitro single-molecule assays performed with 25× GGAA repeat–containing DNA curtains and purified EWS-FLI1 demonstrated FET FO condensation at the specific DNA repeat sites, followed by recruitment of RNA polymerase II and subsequent transcription of nascent RNA (Zuo et al. 2021). In conclusion, as seen with the NUP98 FOs, the FET FO family drives oncogenesis by undergoing phase separation to form aberrant transcriptional condensates.

SS18-SSX1

Synovial sarcoma is a soft tissue malignancy predominantly characterized by the fusion of the transcriptional coactivator gene *SS18* to one of three genes on the X chromosome, *SSX1*, *SSX2*, or *SSX4*, which are thought to have repressor roles. While there is a distinct transcriptional program induced by the resulting FOs, neither of the parent proteins, SS18 or SSX, contains a known DNA-binding domain, distinguishing the SS18-SSX1 FO from the NUP98 and FET FOs discussed above. SS18 is a member of the canonical SWI/SNF complex and was recently shown to undergo LLPS driven by multiple tyrosine residues in a C-terminal IDR, termed the QPGY domain (Cheng et al. 2022, Kuang et al. 2021). Mutation of the tyrosine residues to alanines abrogated condensate formation and binding to the SWI/SNF complex (Cheng et al. 2022, Kuang et al. 2021).

As seen with endogenous SS18, SS18-SSX1 binds the catalytic subunit of the SWI/SNF complex, BRG1, and abrogating this interaction impairs FO-dependent cell transformation (Nagai et al. 2001). SS18-SSX1 also forms nuclear condensates in cancer-relevant cells, which are dependent on the QPGY domain of the SS18 portion of the FO (Cheng et al. 2022). Importantly, when QPGY domain–dependent phase separation by SS18-SSX1 is preserved, BRG1 colocalizes within the FO-containing condensates. Mutation of either the BRG1 interaction interface (with phase separation preserved) or the phase-separation-promoting QPGY domain in SS18-SSX1 (with BRG1 binding preserved) inhibits fibroblast cell transformation (Cheng et al. 2022). It will be important to elucidate how this FO specifically activates the expression of certain genes, as neither parent protein contains a known DNA-binding domain.

NONO-TFE3

A rare but aggressive subtype of renal cell carcinoma displays a *NONO-TFE3* gene fusion resulting from an inversion in chromosome X (Clark et al. 1997, Xia et al. 2017). TFE3 is a member of the microphthalmia family of transcription factors that regulates genes involved in lysosomal function and metabolism (Martina et al. 2014, Sardiello et al. 2009). NONO is a core component of nuclear paraspeckles, a relatively recently identified membraneless organelle with important roles in gene regulation (Fox et al. 2002, Knott et al. 2016). The coiled-coil oligomerization domain of NONO, which is thought to promote phase separation, is retained in the NONO-TFE3 FO. Importantly, transcription of the NONO-TFE3 FO is regulated by the strong NONO promoter, resulting in overexpression relative to that of endogenous TFE3 (Yin et al. 2019). Additionally, while endogenous TFE3 is subject to mTORC1-dependent nucleocytoplasmic shuttling, NONO-TFE3 accumulates at high levels in the nucleus (Kauffman et al. 2014, Yin et al. 2019). Using renal cell carcinoma cells containing the NONO-TFE3 fusion, Wang et al. (2021) uncovered a positive feedback loop involving the NRF1 transcription factor, which regulates mitochondrial biosynthesis, and the FO. The high levels of NONO-TFE3 observed in patient-derived cell lines were at least partially explained by this feedback mechanism.

The NONO region retained in NONO-TFE3 contains two predicted IDRs (IDR1 and IDR2), one of which is a predicted prion-like domain (IDR1). Given the role of endogenous NONO in paraspeckle formation, Wang et al. (2021) hypothesized that the high levels of NONO-TFE3 might promote phase separation via oligomerization and cause protein stabilization. To test this, they expressed either IDR1 or IDR2 in cancer-relevant cells and found that while mRNA levels were similar under both conditions, protein levels of IDR2 were significantly higher, suggesting that IDR2 might also stabilize NONO-TFE3 (Wang et al. 2021). Ectopic expression of NONO-TFE3 in HEK293T cells resulted in the formation of round, nuclear puncta. A fragment containing just IDR2 formed similar puncta, while an IDR1 fragment did not. Additionally, fusing just IDR2 to TFE3 recapitulated results obtained with the full-length FO, and the puncta exhibited liquid-like properties. This artificial FO also resulted in higher protein levels when compared to TFE3 expression alone, again suggesting that IDR2 stabilizes NONO-TFE3 through a phase-separation mechanism (Wang et al. 2021). To address this stabilization mechanism further, the authors assessed the role of a putative GSK3β phosphorylation site in the TFE3 portion of the FO, which induces proteasomal degradation. Intriguingly, NONO-TFE3 was resistant to GSK3β activation in renal carcinoma cells. Since phase separation is a concentration-dependent process, the authors reduced the driving force for phase separation by using shRNA (short hairpin RNA)-mediated knockdown to reduce the cellular FO concentration. Interestingly, they then found that NONO-TFE3 became sensitive to GSK3β activation, and this sensitivity was reversed using an inhibitor of GSK3β (Wang et al. 2021). This study is the first demonstration of phase-separation-dependent stabilization of an FO.

FUSION ONCOPROTEINS THAT FORM CYTOPLASMIC CONDENSATES

While the majority of FOs shown to undergo phase separation function within nuclei, recent findings have revealed that the predominantly lung cancer–associated ALK and RET RTK FOs function within phase-separated cytoplasmic condensates (Qin et al. 2021, Sampson et al. 2021, Tulpule et al. 2021). RTK FOs typically retain their kinase domains but lose their transmembrane domains, a fact that originally led to questions regarding how they carry out RAS-MAPK signaling, which is canonically dependent on membrane association (Hrustanovic et al. 2015, Richards et al. 2015). Rather than localizing to the plasma membrane, many of these FOs have long been noted to form cytoplasmic foci with unclear function (Fawal et al. 2011, Hrustanovic et al. 2015, Richards et al. 2015). New findings show that EML4-ALK and CCDC6-RET undergo phase separation driven by their N-terminal regions (Qin et al. 2021, Sampson et al. 2021, Tulpule et al. 2021), forming cytoplasmic granules that recruit RAS activating proteins, including GRB2, GAB1, and SOS1. Furthermore, a RAS-GTP reporter colocalized with EML4-ALK cytoplasmic puncta, suggesting that they act as active signaling centers (Tulpule et al. 2021).

The N terminus of EML4-ALK contains a trimerization domain (TD) and a truncated tandem atypical WD (tryptophan-aspartic acid)-propeller in EML4 (TAPE) domain. Cytoplasmic granule formation required the TD and a region of the TAPE domain known as the hydrophobic EML protein (HELP) motif. Importantly, a kinase-deficient EML4-ALK mutant (K589M) also disrupted cytoplasmic granule formation due to loss of valence provided by either FO kinase-dependent GRB2 phosphorylation site binding (Tulpule et al. 2021) or dimerization of the kinase domain (Sampson et al. 2021). Impairing granule formation by disrupting any of these three sources of multivalence resulted in inhibition of RAS/MAPK activation. To support the conclusion that the function of the N-terminal domains is solely to cluster EML4-ALK molecules, Tulpule et al. (2021) used the artificial HOtag (homo-oligomeric tag) method of forced clustering and found that ΔTD or ΔHELP mutants of EML4-ALK no longer abrogated RAS/MAPK signaling.

The authors then tested the generality of phase-separation-dependent activation of RAS/MAPK signaling by generating an intracellular EGFR protein mutant. They found that HOtag-mediated clustering of this cytoplasmic EGFR mutant caused recruitment of RAS-activating proteins and increased RAS/MAPK signaling, similar to EML4-ALK (Tulpule et al. 2021). The authors extended their studies by showing that the CCDC6-RET RTK FO exhibited similar cytoplasmic granule-dependent RAS/MAPK activation. In contrast to EML4-ALK, the CCDC6-RET kinase activity was not required for granule formation, although it was still required for RAS/MAPK signaling. The authors attributed this to CCDC6 having a stronger propensity for phase separation than EML4, thus not requiring the added contribution to multivalence provided by kinase transphosphorylation phospho-docking sites (Tulpule et al. 2021).

Interestingly, the biophysical features of the cytoplasmic condensates formed by these RTK FOs did not exhibit liquid features but rather appeared arrested and solid-like (Sampson et al. 2021, Tulpule et al. 2021). Additionally, while the granules recruit many activators of RAS, many negative regulators of RAS were excluded, providing an environment conducive to productive RAS/MAPK signaling (Tulpule et al. 2021). The cancer relevance of these cytoplasmic RTK granules was strengthened by the finding that genetically engineered mice expressing EML4-ALK also exhibited condensate formation in both murine lung tumors and tumor-derived organoids, providing the first in vivo evidence of EML4-ALK phase separation in cancer (Qin et al. 2021); the general features of this LLPS-dependent oncogenic mechanism are illustrated in **Figure 2*b***. As only two kinase-containing FOs have been shown to drive aberrant cell signaling in the cytoplasm by forming nonmembrane associated condensates, it will be important to determine the generality of this mechanism for this class of FOs in the future.

FUSION ONCOPROTEINS THAT FUNCTION THROUGH INHIBITION OF PHASE SEPARATION

DNAJB1-PKA$_{cat}$

Fusion of the DnaJ heat shock protein family member B1 (DnaJB1) with a catalytic subunit of PKA (PKA$_{cat}$) creates the DNAJB1-PKA$_{cat}$ FO, which is a driver of fibrolamellar hepatocellular carcinoma (Graham et al. 2015, Honeyman et al. 2014, Kastenhuber et al. 2017). As discussed above, the regulatory subunit of PKA, RIα, functions through LLPS (Zhang et al. 2020). Phase separation of RIα is essential for proper cAMP compartmentalization and cell signaling (Zhang et al. 2020). Mechanistic insight into the oncogenicity of DnaJB1-PKA$_{cat}$ was provided by the recent finding that overexpression of this FO in HEK293T cells resulted in loss of RIα LLPS (Zhang et al. 2020). Importantly, RIα LLPS was rescued with a kinase-dead mutant of DnaJB1-PKA$_{cat}$. The authors found that kinase-active DnaJB1-PKA$_{cat}$ led to impaired cAMP compartmentalization and aberrant cAMP-dependent signaling (Zhang et al. 2020). While the authors did not explicitly show that DnaJB1-PKA$_{cat}$-mediated loss of RIα LLPS led to disease, they did show that expression of RIα mutants defective in LLPS resulted in increased cell proliferation and transformation, suggesting a mechanism for FO-dependent tumorigenesis (Zhang et al. 2020). This work is the first to demonstrate FO-dependent oncogenesis through loss of phase-separation-dependent compartmentalization of a cellular signaling system.

PML-RARα

Acute promyelocytic leukemia (PML) is characterized by the fusion of the promyelocytic leukemia gene *PML* to that of retinoic acid receptor α (RARα), yielding the PML-RARα FO. PML NBs are membraneless organelles that contribute to tumor suppression by promoting antiproliferation functions. While suspected for some time, recent data support the hypothesis that PML NBs form

via LLPS (Shao et al. 2022). Expression of PML-RARα results in disruption of PML NB structures, but the mechanism of this behavior has only recently been viewed in the context of LLPS.

Shao et al. (2022) screened for compounds that restored PML NBs in cells expressing PML-RARα, with neddylation inhibitors identified as the top hits. RARα experiences neddylation on lysine residues 227 and 360, and blocking this with either lysine-to-arginine point mutations or small-molecule inhibitors targeting the NEDD8-activating enzyme E1 resulted in the small PML-RARα microspeckles fusing to form larger foci that were similar in appearance to PML NBs (Shao et al. 2022). These reformed NBs also recruited several canonical PML NB partners, suggesting that NB function was restored. The authors hypothesized that neddylation inhibits phase separation by PML-RARα by increasing its association with sites within DNA. Indeed, ChIP-qPCR (chromatin immunoprecipitation–quantitative polymerase chain reaction) experiments revealed that neddylation-deficient PML-RARα mutants showed no association with DNA, while the unmutated FO bound to the expected *RARB* promoter. In agreement with this, mRNA levels from RARα target genes were diminished when mHSPCs were transduced with wild-type PML-RARα, but this was reversed with the neddylation-deficient mutant, supporting the conclusion that the repressive function of RARα was impaired by the lack of DNA binding. Importantly, loss of PML-RARα neddylation resulted in failure to transform mHSPCs and blunted leukemogenesis in vivo (Shao et al. 2022). Thus, PML-RARα presents a second example of LLPS being required for normal cell function and FO-mediated disruption of this phenomenon underlying disease.

AMINO ACID SEQUENCE FEATURES ASSOCIATED WITH PHASE SEPARATION

While cellular condensates are assemblies containing tens to hundreds of biomolecules, their formation is typically driven by multivalent interactions mediated by just a few components. These drivers, also termed scaffolds, create molecular networks within the condensates that can recruit other biomolecules as clients (Ditlev et al. 2018). Many of the phase-separating FOs discussed above display IDRs enriched in particular types of amino acids (e.g., phenylalanine and glycine in NUP98 FOs; tyrosine in FET FOs). IDRs that exhibit biased composition through enrichment of amino acids that promote inter-residue interactions often promote LLPS (Borcherds et al. 2021), consistent with observations that many FOs form aberrant biomolecular condensates through LLPS. The biased sequence features of IDRs create multivalence for weak, transient interactions that underlie LLPS. FOs shown to form condensates, in addition to IDRs, also contain folded protein domains that mediate, for example, oligomerization; additional protein-protein interactions; binding to DNA, RNA, or chromatin; and enzymatic activity. The IDRs and folded domains within FOs, together, govern their phase separation behavior, as discussed in the sections above.

We have adopted existing computational sequence analysis tools to identify sequence features associated with LLPS within FOs, including low sequence complexity [Shannon entropy (Adami 2004)], IDRs [IUPred2A (Meszaros et al. 2018)], enrichment in amino acids prone to pi-pi and pi-cation interactions [PScore (pi-pi and pi-cation interaction propensity score) (Vernon et al. 2018)], prion-like domains [PLAAC (Prion-Like Amino Acid Composition) (Lancaster et al. 2014)], alternating tracts of negatively and positively charged residues [CIDER (Classification Of Intrinsically Disordered Ensemble Relationships) (Holehouse et al. 2017)], and general biased amino acid enrichment. We also use available tools to identify conserved protein domains [National Center for Biotechnology Information's CDD (Conserved Domain Database) (Lu et al. 2020)] and secondary structures [Porter5 (Torrisi et al. 2019)]. We created a computational pipeline termed SAK [Swiss Army Knife (Chandra et al. 2021)] that generates graphical reports of the output from these various tools, which enable sequence features associated with LLPS to be manually identified for FOs. These features are illustrated for EWS-FLI1 (**Figure 3**); for example, the SAK output predicts

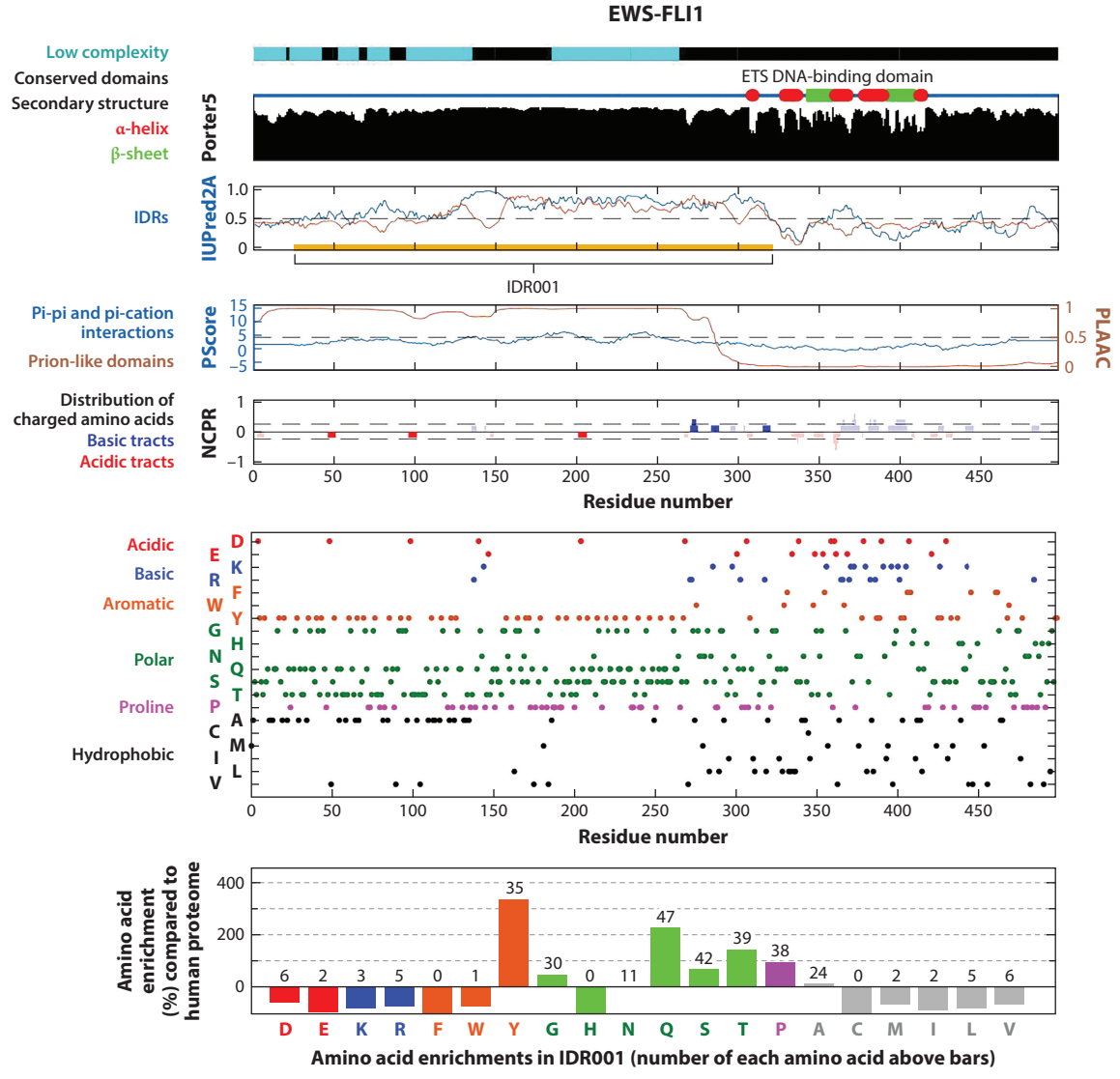

Figure 3

SAK sequence analysis results for EWS-FLI1 reveal LLPS-prone sequence features. The SAK output for the EWS-FLI1 amino acid sequence illustrates features associated with LLPS, including enrichment of regions of low sequence complexity (*cyan*), predicted disorder (IDRs, *blue trace*; scores > 0.45), enrichment in pi-pi and pi-cation interactions (*blue trace*; PScore values > 4), the presence of prion-like domains (*brown trace*; PLAAC values > 0.5), and the occurrence and enrichment of individual amino acids within the IDR001 region within the EWS-FLI1 sequence (note the enrichment of Y, Q, T, and P residues). The enrichment of amino acids is relative to the human proteome. The conserved domain, an ETS DNA-binding domain, corresponds to a region with predicted secondary structure (α-helix, *red*; β-sheet, *green*). This sequence does not exhibit prominent acidic or basic tracts. Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; IDRs, intrinsically disordered regions; K, lysine; L, leucine; LLPS, liquid-liquid phase separation; M, methionine; N, asparagine; NCPR, net charge per residue; P, proline; PLAAC, Prion-Like Amino Acid Composition; PScore, pi-pi and pi-cation interaction propensity score; Q, glutamine; R, arginine; S, serine; SAK, Swiss Army Knife; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

a long, N-terminal IDR (IDR001 in **Figure 3**) with low sequence complexity, which also scores highly for pi-pi/pi-cation interactions and prion-like domain features. Inspection of the occurrence and enrichment of amino acids within this IDR, revealed by the SAK output, shows an enrichment greater than 100% for tyrosine, glutamine, threonine, and proline residues (**Figure 3**, bottom panel), which are known to contribute to multivalence that underlies LLPS by FET family proteins (Wang et al. 2018). Supporting their importance for phase separation, the mutation of 37 of the tyrosine residues within the N-terminal IDR to serine residues abolishes EWS-FLI1 condensate formation (Ahmed et al. 2021, Boulay et al. 2017). Furthermore, the mutated FO was unable to bind GGAA microsatellite repeats and failed to activate gene expression at these sites (Boulay et al. 2017). The EWS-FLI1 FO also contains an ETS DNA-binding domain, which is also identified by the prediction of secondary structure and low disorder scores, as highlighted in the SAK output (**Figure 3**). The sequence features of the other FOs discussed in this review associated with LLPS can similarly be identified using SAK reports (see **Supplemental Figure 1**). Analysis of these reports for novel FOs, or other proteins in general, that are suspected of undergoing LLPS allows researchers to generate hypotheses regarding the sequence features underlying this behavior. Our inspection of SAK reports for many FOs not discussed in this review suggests that LLPS is a common feature of these cancer-driving proteins (H.K. Shirnekhi, B. Chandra & R.W. Kriwacki, unpublished results).

Supplemental Material >

LIMITATIONS, CHALLENGES, AND FUTURE DIRECTIONS

The findings reviewed above clearly establish the relevance of LLPS in FO-driven cancers, with several FOs that form nuclear condensates demonstrated to cause altered chromatin states and aberrant gene expression and others that form cytoplasmic condensates shown to promote altered signaling and cell proliferation. Many of the FOs that form condensates display IDRs with amino acid sequence features that are hallmarks of LLPS. The process of gene fusion often juxtaposes LLPS-prone IDRs with folded domains of known function within the resulting FOs. For example, many FOs that form nuclear condensates display globular domains that bind specifically to DNA or nucleosomes. These types of domains localize FOs to chromatin and enable the LLPS-prone IDRs to drive chromatin compaction and recruitment of endogenous transcriptional or epigenetic regulatory factors through heterotypic LLPS. Together, these changes in chromatin state lead to altered gene expression, cell transformation, and oncogenesis. While LLPS-prone IDRs can be identified in the sequences of FOs that form cytoplasmic condensates, they also contain folded protein domains that are required for LLPS and oncogenic function (Tulpule et al. 2021). Based upon these albeit limited observations, we speculate that future studies of larger numbers of FOs will reveal varying degrees of synergy between LLPS-prone IDRs and folded domains, giving rise to a diverse array of aberrant biological functions that transform cells and promote oncogenesis.

Our understanding of how biomolecular condensates form and function is rapidly expanding, and we now understand that the concepts central to this field apply to the behavior of numerous FOs in cancer cells. However, despite this progress, there are several limitations and challenges that have hindered progress, as we discuss below.

One challenge is to rigorously establish that the process of LLPS is responsible for the formation of condensates by FOs in cells. Liquids display fission, fusion, dripping, and wetting behavior (Brangwynne et al. 2009), but these can be challenging to monitor due to limitations of fluorescence microscopy. For example, condensates formed by FOs in cells are often very small and close to the diffraction resolution limit of light, making it challenging to observe these liquid behaviors using traditional fluorescence microscopy. Advances in super-resolution microscopy of live cells using, for example, lattice light sheet microscopy hold promise for visualizing the

liquid behavior of FO condensates. Furthermore, several of the nuclear FOs discussed above interact strongly with chromatin, which limits the movement of the resulting condensates and opportunities to observe liquid behavior. With NUP98-HOXA9, abrogation of interactions with DNA by mutating residues in the DNA-binding domain has led to the formation of large, chromatin-dissociated condensates that, due to their liberation from slowly fluctuating chromatin, were observed to fuse, supporting the hypothesis that they form through LLPS (Chandra et al. 2021). Another property of liquids is surface tension, which causes liquid droplets to be spherical. However, mechanisms of biomolecular assembly other than LLPS can give rise to spherical structures in cells, limiting conclusions that can be drawn regarding the mechanism of formation based upon the observation of round or spherical structures in cells. Molecules within liquids exhibit diffusion, and as discussed above, FRAP of fluorescently labeled components of condensates is commonly used to monitor biomolecular diffusion. The observation of diffusion, in the absence of other observations of liquid behavior, is not a rigorous criterion to support the conclusion that condensates have liquid properties and are formed through LLPS. In addition, the observation of limited FRAP for a condensate component is not necessarily an indication that the condensate did not form through LLPS because what are originally liquid-like condensates can ripen into gel- or solid-like structures over time (Mathieu et al. 2020). FRAP experiments on small condensates are problematic because only large regions can be bleached, complicating measurements of diffusion coefficients, which require bleaching of small regions within condensates. Condensate movement in cells, which is most pronounced for small condensates, also complicates the analysis of fluorescence recovery over time, although condensate tracking image analysis algorithms can address this issue. Increasingly, advanced fluorescence microscopy methods for monitoring biomolecular diffusion, including fluorescence correlation spectroscopy (Mitrea et al. 2018, Wei et al. 2017) and single-molecule tracking (Chong et al. 2018), are being applied to biomolecular condensates, but not yet routinely to those formed by FOs.

The expression of FOs in cancer-relevant cells is often low, which can limit assessments of condensate liquidity (Chandra et al. 2021). Overexpression studies of FOs in nonprimary cells can be performed, and the use of transient transfection leads to populations of cells expressing fluorescently tagged FOs at widely different levels, enabling the cellular concentration dependence of their LLPS behavior to be studied (Baggett et al. 2022). Care must be taken, however, to demonstrate the relevance of these types of findings to cancer-relevant cell model systems (Chandra et al. 2021). Endogenous FOs can be monitored with immunofluorescence when an antibody is available to detect one of the two parent regions of an FO (Chandra et al. 2021). However, cell fixation can alter biomolecular condensate morphology (Irgen-Gioro et al. 2022); therefore, live cell imaging is preferred. In cases where patient-derived cancer cells harboring an FO are available, genome engineering methods can in principle be used to introduce, for example, GFP into the fused gene locus. The NUP98 FOs were shown to form nuclear condensates through both homotypic and heterotypic interactions, and this is likely to be true of other FOs. Therefore, it is important to determine the constituents of FO condensates (Terlecki-Zaniewicz et al. 2021) and their contributions to condensate formation. In vitro reconstitution of condensates reflecting the constituents of stress granules and nucleoli was recently demonstrated, and these methods can be applied in the future to identify the biomolecular constituents of FO-driven condensates (Freibaum et al. 2021). However, both in vitro biochemical and cellular studies of FO condensate components will be needed to understand whether FOs are scaffolds that drive condensate formation (Ditlev et al. 2018).

While evidence for the formation of cellular condensates via LLPS is available for several FOs, it is possible that FO condensates may form through alternative mechanisms. The Tjian group has shown through single-molecule tracking studies that while herpes simplex virus can enrich RNA

polymerase II within membraneless compartments, these structures are not formed via LLPS (McSwiggen et al. 2019a,b). Further, they have also argued that the transcriptional condensates formed by EWS-FLI1 are not formed via LLPS (Chong et al. 2018). As LLPS is not the sole mechanism of concentrating biomolecules, it is important to quantitatively analyze the properties of membraneless condensates within cells and consider alternative mechanisms of their formation.

Therapeutic targeting of FOs has had limited success due to poor understanding of the complex functional mechanisms at play. The last five years have seen the establishment of several FOs that function through a phase-separation-dependent mechanism. This review highlights that features of proteins that undergo LLPS are enriched in parents of FOs, which leads us to speculate that function through LLPS is common to many FOs. As FO-driven malignancies are often among the most aggressive, this offers new perspectives on targeting these cancers. Using our SAK pipeline, an FO that functions via LLPS can be manually analyzed to identify sequence features that are associated with phase separation, including IDRs and their amino acid enrichments. By applying mutagenesis techniques, such as multisite mutagenesis to modulate multivalence, researchers can identify the residues critical for condensation by an FO, providing target regions for therapeutics. Additionally, recent work has established that different drugs selectively partition into certain condensates and are excluded from others (Klein et al. 2020). A better understanding of the properties of biomolecular condensates formed by FOs and the types of chemical moieties that partition into them will inform future drug design. Additionally, while some FOs function through their own LLPS, others function via inhibiting the LLPS of other proteins. In such cases, targeting the implicated protein-protein interaction may be effective. An example of this is provided by the dennedylation inhibitors that nullify PML-RARα (Shao et al. 2022). As posttranslational modifications are known to influence phase separation, this strategy of targeting may be useful for other FOs as well. As cellular FO condensates are multicomponent systems, future work should assess the specific heterotypic protein-protein interactions that are crucial for their function and pursue their targeting by either small-molecule-based drugs or advanced protein degradation techniques such as PROTACs (proteolysis-targeting chimeras).

DISCLOSURE STATEMENT

R.W.K. has received compensation unrelated to this work for advisory board membership of Dewpoint Therapeutics, for consulting with GLG Consulting, and for a patent licensing relationship with New Equilibrium Biosciences.

ACKNOWLEDGMENTS

This work is supported by a National Cancer Institute (NCI) Cancer Center Support Grant (P30 CA021765), NCI grant R01 CA246125 (to R.W.K.), NCI FusOnC2 grant U54 CA243124 (to R.W.K.), a Neoma Boadway Fellowship from St. Jude Children's Research Hospital (to B.C.), and the American Lebanese Syrian Associated Charities.

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