Nonclassical nuclear localization signals mediate nuclear import of CIRBP

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The specific interaction of importins with nuclear localization signals (NLSs) of cargo proteins not only mediates nuclear import but also, prevents their aberrant phase separation and stress granule recruitment in the cytoplasm. The importin Transportin-1 (TNPO1) plays a key role in the (patho-)physiology of both processes. Here, we report that both TNPO1 and Transportin-3 (TNPO3) recognize two nonclassical NLSs within the cold-inducible RNA-binding protein (CIRBP). Our biophysical investigations show that TNPO1 recognizes an arginine-glycine(-glycine) (RG/RGG)–rich region, whereas TNPO3 recognizes a region rich in arginine-serine-tyrosine (RYS) residues. These interactions regulate nuclear localization, phase separation, and stress granule recruitment of CIRBP in cells. The presence of both RG/RGG and RSY regions in numerous other RNA-binding proteins suggests that the interaction of TNPO1 and TNPO3 with these nonclassical NLSs may regulate the formation of membraneless organelles and subcellular localization of numerous proteins.

CIRBP | Transportin-1 | Transportin-3 | nuclear import | phase separation

Import receptors (importins) mediate active nuclear import of cargo proteins across the nuclear pore complex. Recently, we and others have reported a second function of importins, namely chaperoning and inhibiting liquid–liquid phase separation (LLPS) of aggregation-prone RNA-binding proteins (RBPs): for example, fused in sarcoma (FUS), TAF15, EWSR1, hnRNP1A, and hnRNP2 by the importin Transportin-1 (TNPO1; also known as Karyopherin \( \beta_2 \)) and TDP-43 by importin \( \beta_1 \) (1–4).

Both nuclear import and chaperoning rely on the specific interaction of the importin with a nuclear localization signal (NLS) in its cargo protein (5–7). This interaction and hence, directionality of transport are usually regulated by the small guanosine triphosphate (GTP) hydrolase (GTPase) Ran as binding of Ran to the importin promotes release of the cargo in the nucleus (8).

TNPO1 is known to bind to cargoes containing a so-called proline-tyrosine (PY)–NLS, which has been defined as a sequence with an overall high degree of structural disorder, and a C-terminal R/H/K-X\(_2\)-PY consensus sequence preceded by a hydrophobic or basic region (9). However, TNPO1 has also been reported to bind to and mediate nuclear import of cargoes not containing a PY-NLS, such as ribosomal proteins or several viral proteins (10), suggesting that other regions are able to serve as NLS for TNPO1. For the TNPO1 cargo FUS, for example, we have recently demonstrated that TNPO1 can directly interact with several positively charged arginines in the C-terminal RGG3-PY region in the absence of its PY-NLS and that this interaction is regulated by arginine methylation (11, 12). Such arginine-glycine(-glycine) (RG/RGG) regions are highly abundant in RBPs, are involved in both protein and RNA binding (13), and have recently been identified as key drivers in LLPS (14, 15). Importantly, the interaction of TNPO1 with arginines in RG/RGG regions is also crucial for suppression of LLPS of FUS (1). We hypothesized that RG/RGG-rich regions could serve as a type of NLS for TNPO1 and scrutinized published lists of TNPO1 cargoes (16, 17) for the presence of RG/RGG regions. Indeed, we observed that 94 reported TNPO1 cargoes contain RG/RGG regions (Fig. 14 and Dataset S1). Among these proteins, 61 have both a PY-NLS and an RG/RGG region and could thus follow the same recognition mode as FUS. Among the reported TNPO1 cargo proteins, 33 have an RG/RGG region but no PY-NLS and hence, could be proteins in which the RG/RGG region serves as sole NLS for TNPO1. Thus, we set out to test whether RG/RGG regions could serve as a previously unrecognized class of NLS for TNPO1.

Cold-inducible RNA-binding protein (CIRBP) seems to be a promising candidate as deletion of an RG/RGG-rich region was shown to cause cytoplasmic mislocalization of human CIRBP or its Xenopus laevis ortholog (xCIRBP2), demonstrating the importance of this region for proper nuclear localization (18, 19). Nevertheless, the molecular mechanism of CIRBP nuclear import remains elusive. CIRBP belongs to the family of cold shock proteins and is up-regulated in response to a large variety of cellular stresses, including mild cold shock, ultraviolet irradiation, and other forms of cellular stress. These interactions and nuclear import of CIRBP involve two distinct CIRBP regions, the arginine-glycine(-glycine) (RG/RGG) region and the discovered arginine-serine-tyrosine (RYS)-rich region. We show that these two regions harbor different specificity toward TNPO1 and TNPO3, with the RG/RGG-NLS being specialized in TNPO1 recognition and the RYS-NLS being specialized in TNPO3 recognition.

**Significance**

We uncovered a molecular mechanism by which multiple importins regulate nuclear import, phase separation, and stress granule recruitment of an RNA-binding protein. Our work permitted the identification of two types of nuclear localization signal (NLS) in cold-inducible RNA-binding protein (CIRBP). We show that CIRBP, which lacks any classical NLS, binds both the importin Transportin-1 (TNPO1) and Transportin-3 (TNPO3). These interactions and nuclear import of CIRBP involve two distinct CIRBP regions, the arginine-glycine(-glycine) (RG/RGG) region and the discovered arginine-serine-tyrosine (RYS)-rich region. We show that these two regions harbor different specificity toward TNPO1 and TNPO3, with the RG/RGG-NLS being specialized in TNPO1 recognition and the RYS-NLS being specialized in TNPO3 recognition.

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that serves as NLS for TNPO1 and an arginine-serine-tyrosine identified two so far uncharacterized NLSs: an RG/RGG region of CIRBP. CIRBP lacks a classical NLS or PY-NLS, but we mediate nuclear import and suppress LLPS and SG recruitment.

CIRBP localization and its redistribution under cellular stress are largely unknown. Nevertheless, the mechanisms governing CIRBP chemotherapy (27), making CIRBP a potential target for anti-cancer therapy. Down-regulation of CIRBP is associated with various types of cancers, such as liver, breast, brain, and prostate cancers (26). CIRBP impairs cancer cell survival and enhances response to chemotherapy (27), making CIRBP a potential target for anti-cancer therapy. Nevertheless, the mechanisms governing CIRBP localization and its redistribution under cellular stress are largely unknown.

Here, we show that both TNPO1 and Transportin-3 (TNPO3) mediate nuclear import and suppress LLPS and SG recruitment of CIRBP. CIRBP lacks a classical NLS or PY-NLS, but we identified two so far uncharacterized NLSs: an RG/RGG region that serves as NLS for TNPO1 and an arginine-serine-tyrosine (RSY)—rich region that serves as NLS for TNPO3. NMR spectroscopy and isothermal titration calorimetry (ITC) revealed multiple interactions between CIRBP-TNPO1 and CIRBP-TNPO3 involving both the RG/RGG and RSY regions. We show that the binding sites for the RG/RGG, RSY, and the classical PY-NLS are overlapping on TNPO1, with a long flexible loop in TNPO1 creating an additional and highly specific binding site for the RG/RGG region. Arginine methylation of the CIRBP RG/RGG region via protein arginine methyltransferase 1 (PRMT1) decreases both TNPO1 and TNPO3 binding and consequently, reduces CIRBP nuclear import. These results suggest that nuclear import, LLPS, and SG recruitment of RBPs containing multiple NLSs can be controlled by an intricate interplay of multiple importins and can be further regulated by posttranslational modifications, such as arginine methylation.

Results

The RG/RGG- and RSY-Rich Regions Mediate Nuclear Import of CIRBP.

To investigate the nuclear import mechanism of CIRBP, we made use of a hormone-inducible nuclear transport assay adapted from Love et al. (28). In this assay, the protein of interest is fused to two hormone-binding domains of the glucocorticoid receptor (GCR) and two green fluorescent protein (GFP) moieties. The GCR domain traps the fusion protein in the cytoplasm until addition of a steroid hormone, such as dexamethasone, to cause translocation to the nucleus. We used this assay in conjunction with siRNA knockdown of TNPO1 or TNPO3 to investigate the role of these transportins in CIRBP nuclear import.

Fig. 1. The RG/RGG- and RSY-rich regions in CIRBP mediate TNPO1- and 3-dependent nuclear import in HeLa cells. (A) Venn diagram corresponding to the PROSITE analysis (https://prosite.expasy.org/scansite) of two motifs against the nonredundant protein sequences database filtered for human proteins (taxid:9606) harboring either 1) a di-RG motif (orange) each spaced by zero to five amino acids [R-G-x(0-5)-R-G] or 2) a PY motif (green) preceded by a basic amino acid spaced by zero to nine residues [[RKH]-x(0-9)-P-Y]. These two groups of proteins were compared with the published list of TNPO1 cargoes identified by Kimura et al. (16) and Mackmull et al. (17). (B) Time-dependent nuclear import of GCR2-6-A-CIRBP full length in HeLa Kyoto cells on addition of dexamethasone visualized by live cell imaging. (Scale bar: 20 μm.) (C) Scheme of CIRBP illustrating its domain organization and deletion of individual domains for analysis of their involvement in nuclear import of CIRBP. (D) HeLa Kyoto cells were transiently transfected with constructs coding for GCR2-6-A-CIRBP full length (FL) or CIRBP mutants lacking either the RRM (ΔRRM), the RG/RGG (ΔRG), the RSY (ΔRSY), or both RG/RGG and RSY regions (ΔRG-RSY). A construct coding for only GCR2-GFP2 (Δ-) was used as control for diffusion. Cells were either fixed before (+dexamethasone) or after (-dexamethasone) the addition of 5 μM dexamethasone for 15 min at 37 °C. Nuclei were stained with DAPI, and cells were analyzed by fluorescence microscopy. (Scale bar: 10 μm.) (E) HeLa Kyoto cells transfected with either control small inhibitory RNA (siRNA) or siRNA against TNPO1 or TNPO3, respectively, were transfected with a construct coding for GST-GFP-CIRBP. Cells were fixed, nuclei were counterstained with DAPI, and cells were analyzed by fluorescence microscopy. (Scale bar: 20 μm.)
triggers nuclear import. Fusion with two GCR and two GFP domains increases the molecular mass of CIRBP by ~120 kDa and thus, efficiently reduces nuclear import due to passive diffusion across the nuclear pore complex. In the absence of deamethasone, GCR2-GFP2-CIRBP was predominantly cytoplasmic and rapidly translocated into the nucleus on deamethasone addition (Fig. 1B and Movie S1). In comparison, a GCR2-GFP2 construct lacking an NLS (GCR2-GFP2-stop-M9) did not become enriched in the nucleus relative to the cytoplasm after deamethasone addition but at most, equilibrated between nucleus and cytoplasm due to passive diffusion (SI Appendix, Fig. S1A and Movie S2).

In order to identify the regions of CIRBP that mediate nuclear import, we next examined a series of deletion mutants lacking the N-terminal RNA recognition motif (RRM; CIRBPΔRRM), the RG/RGG-rich region (CIRBPΔRG), the C-terminal RSY-rich region (CIRBPΔRSY), or both the RG/RGG-rich region and the C-terminal RSY-rich region (CIRBPΔRG-RSY) (schematic diagram in Fig. 1C). Deletion of the RRM did not impair nuclear import, whereas deletion of either the RG/RGG or the RSY region strongly impaired nuclear translocation compared with the GCR2-GFP2 control (Fig. 1D; SI Appendix, Fig. S1B shows the quantification). Combined deletion of both the RG/RGG and the RSY regions did not result in stronger impairment of nuclear import than deletion of the RSY region alone, indicating that the RSY region of CIRBP is dominant over the RGG domain in nuclear import, as least in our cellular system. Together, our data demonstrate that both the RG/RGG region and the RSY region are required for efficient nuclear import of CIRBP.

**Nuclear Import of CIRBP Is Mediated by TNPO1 and TNPO3.** We and others have previously demonstrated an interaction of RG/RGG regions in FUS with TNPO1 (2, 11, 12) and therefore, hypothesized that TNPO1 may be responsible for nuclear import of CIRBP via its RG/RGG region. Moreover, since the RSY region is also essential for efficient nuclear import of CIRBP in vitro (13), deletion of the RG/RGG and the RSY regions did not result in stronger impairment of nuclear import than deletion of the RSY region alone, indicating that the RSY region of CIRBP is dominant over the RGG domain in nuclear import, as least in our cellular system. Together, our data demonstrate that both the RG/RGG region and the RSY region are required for efficient nuclear import of CIRBP.

To this end, we silenced TNPO1 or TNPO3 expression in HeLa cells due to passive diffusion ([Fig. 1C](#fig1c)). Deletion of the RRM did not impair nuclear import, whereas deletion of either the RG/RGG or the RSY region strongly impaired nuclear translocation compared with the GCR2-GFP2 control (Fig. 1D; SI Appendix, Fig. S1B shows the quantification). Combined deletion of both the RG/RGG and the RSY regions did not result in stronger impairment of nuclear import than deletion of the RSY region alone, indicating that the RSY region of CIRBP is dominant over the RGG domain in nuclear import, as least in our cellular system. Together, our data demonstrate that both the RG/RGG region and the RSY region are required for efficient nuclear import of CIRBP.

**TNPO1 and TNPO3 Suppress Phase Separation of CIRBP In Vitro and Its Recruitment to Stress Granules in Cells.** It has been previously shown that import receptors, in addition to mediating nuclear import, also suppress phase separation of RBPs containing prion-like low-complexity domains and prevent their recruitment into SGs (1–4). As CIRBP contains an extended disordered region including the RG/RGG and RSY regions ([SI Appendix, Fig. S2A](#app2A)), we tested its ability to phase separate using purified recombinant full-length CIRBP. Indeed, in a turbidity assay, which uses the optical density (OD) of a protein solution as a measure of phase separation, the turbidity of full-length CIRBP increased in a concentration-dependent manner beyond 15 μM (Fig. 2A). Considering that CIRBP is an RBP and that both the RRM domain and the RG/RGG region are involved in RNA recognition (35–37), we next tested the effect of RNA on CIRBP phase separation. Here, titration of (UG)12 RNA to a fixed CIRBP concentration (30 μM) led to a progressive turbidity increase at low RNA concentrations, whereas higher amounts of RNA had a suppressive effect on CIRBP phase separation (Fig. 2B). This is in accordance with a previous study showing that the RNA:protein ratio regulates the phase separation behavior of prion-like RBP in the above-described manner (38). Importantly, addition of increasing amounts of either TNPO1 or TNPO3 to full-length CIRBP in the presence of RNA led to a concentration-dependent decrease in turbidity, demonstrating that both TNPO1 and TNPO3 have the ability to inhibit phase separation of CIRBP (Fig. 2C). In order to confirm these findings, we monitored CIRBP phase separation by differential interference contrast (DIC) microscopy. In the presence of substoichiometric amounts of RNA, CIRBP immediately formed small condensates that increased in size over time, most likely due to fusion of condensates, indicating their “liquid-like” behavior (Fig. 2D). In line with the turbidity assay, both TNPO1 and TNPO3 were able to suppress CIRBP condensate formation.

CIRBP has previously been shown to be recruited into SGs in response to various cellular stresses (18, 19). In order to confirm that the chaperoning activity of both TNPO1 and TNPO3 for CIRBP in the cellular context, we made use of our previously established SG recruitment assay in semipermeabilized cells (1, 39). Addition of recombinant GFP-tagged CIRBP to these semipermeabilized cells resulted in its accumulation in GTase-activating protein-binding protein (G3BP1)-positive SGs (Fig. 2E). Importantly, concomitant addition of either TNPO1 or TNPO3 suppressed the SG association of MBP-CIRBP (Fig. 2E; Fig. 2F shows the quantification) in a concentration-dependent manner (SI Appendix, Fig. S2 B and C). This result demonstrates that both importins not only suppress phase separation of CIRBP in vitro but also, reduce its association with SGs in cells. Taken together, both TNPO1 and TNPO3 exert all known functions of importins toward CIRBP and hence, are bona fide import receptor for CIRBP.

The RG/RGG Region of CIRBP Contributes to Phase Separation and Is Required for SG Recruitment. Recently, we and others have shown that RG/RGG regions in FUS are essential for LLPS in vitro (1, 4, 40, 41). Both the RG/RGG and C-terminal RSY regions of CIRBP are strongly predicted to be disordered (SI Appendix, Fig. S2A), suggesting that these regions may be responsible for phase separation and SG recruitment of CIRBP. We first investigated the contribution of these two regions for SG recruitment of CIRBP in intact cells. In order to analyze the tendency of CIRBP deletion mutants to localize to SGs irrespective of their different nuclear import capabilities, we used the tool of our cytologically anchored GCR2-GFP2-CIRBP reporter. In the absence of steroidal hormones, the reporter protein remained in the cytoplasm and was efficiently recruited into T-cell intracellular antigen 1 (TIA-1)—positive SGs on MG132 treatment (Fig. 2 G and H shows the quantification). In contrast, deletion of the RG/RGG region abolished SG recruitment of the reporter nearly completely, while a CIRBP reporter lacking the RSY region still localized to SGs. In order to investigate whether the region that drives SG recruitment in cells is also the region that drives phase separation in vitro, we examined the ability of individual recombinant CIRBP regions CIRBPΔRRM, CIRBPΔRG-RGG and CIRBPΔRSY to undergo phase separation in vitro. In contrast to full-length CIRBP, none of the domains alone were able to phase separate in the concentration range tested by turbidity assay or DIC microscopy (up to 100 or 30 μM, respectively) (SI Appendix, Fig. S2 D and E). However, successive addition of RNA strongly promoted phase separation of CIRBPΔRG-RGG in a concentration-dependent manner in the turbidity assay (SI Appendix, Fig. S2F). In line with this, DIC microscopy showed that...
CIRBP<sub>RGG</sub> formed liquid-like condensates in the presence of RNA similar to the full-length protein (SI Appendix, Fig. S2E), suggesting that the interaction of the RG/RGG region with RNA contributes to phase separation of the full-length protein. In both assays, addition of either TNPO1 or TNPO3 was able to suppress the RNA-induced phase separation of CIRBP<sup>RGG</sup> (SI Appendix, Fig. S2 E and G). When we analyzed the RNA-driven phase separation of CIRBP<sup>RGG</sup> using NMR spectroscopy, addition of unlabeled (UG)<sub>12</sub> RNA to a solution of <sup>15</sup>N-labeled CIRBP<sup>RGG</sup> led to disappearance of <sup>1</sup>H-<sup>15</sup>N cross-peaks (SI Appendix, Fig. S2H) and decreased signal intensity in the corresponding one-dimensional (1D)-<sup>1</sup>H NMR spectra (Fig. 2 I and J). This is in agreement with the formation of high-molecular mass CIRBP<sup>RGG</sup> RNA condensates, which leads to extensive broadening of NMR signals due to the increased rotational tumbling time of CIRBP<sup>RGG</sup> within the condensates. Nevertheless, we cannot exclude that intermediate timescale chemical exchange processes contribute to the observed line broadening. RNA was bound by CIRBP<sup>RGG</sup> condensates as NMR signals of unlabeled RNA were absent in the 1D-<sup>1</sup>H NMR spectra (Fig. 2 I and J). Addition of increasing amounts of TNPO1 or TNPO3 to the CIRBP<sup>RGG</sup> RNA sample caused reappearance of the RNA-<sup>1</sup>H NMR signals and CIRBP<sup>RGG</sup> <sup>1</sup>H-<sup>1</sup>5N cross-peaks (Fig. 2 I and J and SI Appendix, Fig. S2H). This indicates that both TNPO1 binding and TNPO3 binding displace the RNA from the RG/RGG region of CIRBP and hence, lead to dissolution of the condensates.

Taken together, we show that the CIRBP RG/RGG region is required for SG localization in cells. Moreover, it undergoes phase separation in vitro in the presence of RNA, which is suppressed by TNPO1 and TNPO3 via RNA displacement. As both TNPO1 and TNPO3 are able to import CIRBP and suppress its phase separation and SG recruitment, we sought to...
characterize the binding of these two import receptors to CIRBP on a molecular level in more detail.

**Distinct CIRBP Regions Are Directly Recognized by TNPO1 and TNPO3.** In order to obtain residue-resolved information for the interaction of CIRBP with TNPO1 and TNPO3, we performed NMR spectroscopy. The $^1$H-$^15$N heteronuclear single quantum coherence (HSQC) NMR spectrum of isotope-labeled full-length CIRBP in isolation showed NMR signals characteristic for both folded and intrinsically disordered regions (Fig. 3A and B). Stepwise addition of unlabeled TNPO1 or TNPO3 resulted in a progressive disappearance of the CIRBP $^1$H-$^15$N HSQC cross-peaks characteristic for disordered residues (Fig. 3A and B), which indicates binding of both TNPO1 and TNPO3 to the corresponding residues. The identified complex shows extensive line broadening and disappearance of CIRBP NMR signals at a low stoichiometry of TNPO1 and TNPO3, which are characteristic for a complex with a high nanomolar to micromolar dissociation constant ($K_d$). In line with this, ITC-derived dissociation constants ($K_d$) are $741 \pm 106$ and $289 \pm 36$ nM for CIRBP binding to TNPO1 and TNPO3, respectively (Table 1 and SI Appendix, Fig. S3A).

To further define the CIRBP regions involved in TNPO1 binding, we examined interaction of individual recombinant regions (CIRBP$^{RRM}$, CIRBP$^{RGG}$, and CIRBP$^{RSY}$) with recombinant full-length TNPO1 and TNPO3 in vitro by NMR spectroscopy. In line with the cellular import assay (Fig. 1D), addition of TNPO1 or TNPO3 to a solution of isotope-labeled CIRBP$^{RRM}$ had only a minor effect on the $^1$H-$^15$N HSQC spectrum, showing that neither TNPO1 nor TNPO3 binds to the RRM alone (SI Appendix, Fig. S3D). In contrast, both TNPO1 and TNPO3 bind to CIRBP$^{RGG}$ and CIRBP$^{RSY}$ as shown by disappearance of CIRBP $^1$H-$^15$N HSQC cross-peaks on addition of unlabeled TNPO1 or TNPO3 (Fig. 3C and D). Close inspection of the affected $^1$H-$^15$N CIRBP$^{RGG}$ cross-peaks revealed that the RG/RGG region of CIRBP (CIRBP$^{RGG}$) interacts with both TNPO1 and TNPO3 (Fig. 3C). Similarly, close inspection of $^1$H-$^15$N CIRBP$^{RSY}$ NMR cross-peaks affected by addition of TNPO1 and TNPO3 revealed that the N-terminal part of the CIRBP$^{RSY}$ region interacts with TNPO3, whereas both the N-terminal and C-terminal parts bind to TNPO1 (Fig. 3D). Extensive line broadening is observed across both RG/RGG and RSY CIRBP regions on addition of TNPO1 or TNPO3, suggesting dynamic exchange involving multiple elements across these disordered regions.

Importantly, the interaction of TNPO1 and TNPO3 with CIRBP$^{RGG}$ and CIRBP$^{RSY}$ can be displaced by RanGTP as stepwise addition of RanGTP to a solution of isotope-labeled CIRBP$^{RGG}$ or CIRBP$^{RSY}$ in complex with TNPO1 or TNPO3 leads to progressive signal recovery of the CIRBP $^1$H-$^15$N HSQC cross-peaks, indicating competition between the CIRBP-NLSs and RanGTP for TNPO1 and TNPO3 binding (SI Appendix, Fig. S3B).
Table 1. Thermodynamic parameters of ITC titrations

| Cell     | Syringe          | \(K_a\) (nM) | \(\Delta H\) (kcal mol\(^{-1}\)) | \(\Delta S\) (cal mol\(^{-1}\)K) |
|----------|------------------|--------------|---------------------------------|-----------------------------------|
| TNPO1    | CIRBP            | 741 ± 106    | −21 ± 0.66                      | −42.7                             |
| TNPO1    | CIRBP\(^{RGG}\)  | 75 ± 13      | −11.3 ± 0.16                    | −5.3                              |
| TNPO1    | meCIRBP\(^{RGG}\)| No detectable|                                 |                                   |
| TNPO1    | CIRBP\(^{RSY}\)  | 7,800 ± 545  | −10 ± 0.77                      | −12.2                             |
| TNPO1    | FUS\(^{PY}\)     | 48 ± 6       | −21.9 ± 0.15                    | −40                               |
| TNPO1    | CIRBP\(^{RSY}\)  | 364 ± 15     | −22.3 ± 0.18                    | −45.3                             |
| TNPO1    | CIRBP\(^{RSY}\)  | 8,075 ± 556  | −19 ± 0.55                      | −43.9                             |
| TNPO1    | FUS\(^{PY}\)     | 62 ± 4       | −20.6 ± 0.1                     | −36                               |
| TNPO1/FUS\(^{PY}\) | CIRBP\(^{RGG}\) | 413 ± 41    | −13.4 ± 0.21                    | −15.8                             |
| TNPO3    | CIRBP\(^{RGG}\)  | 391 ± 77     | −12.4 ± 0.36                    | −12.4                             |
| TNPO3    | CIRBP\(^{RSY}\)  | 584 ± 77     | −27 ± 0.56                      | −62                               |
| TNPO3    | CIRBP\(^{RSY}\)  | 114 ± 5      | −34.9 ± 0.14                    | −91.6                             |
| TNPO3    | CIRBP            | 289 ± 36     | −58.6 ± 0.93                    | −167                              |
| TNPO3    | meCIRBP\(^{RGG}\)| 3,300 ± 320  | −23 ± 1.4                       | −48.6                             |
| TNPO3    | meCIRBP          | 1,002 ± 100  | −63 ± 1.4                       | −184                              |

The reported errors correspond to the SD of the fit. All of the stoichiometry associated with the complex formation was equal to one.

Fig. S3 G and H). Moreover, even though both TNPO1 and TNPO3 can interact with the CIRBP-RG/RGG region as well as the RSY region yet with different affinities, the FUS PY-NLS specifically bound only to TNPO1 but not TNPO3 as shown by NMR (SI Appendix, Fig. S3J).

ITC analysis demonstrated that TNPO1 and TNPO3 have different preferences for the RG/RGG and RSY regions. CIRBP\(^{RGG}\) binds TNPO1 and TNPO3 with \(K_a\) values of 75 ± 13 and 589 ± 70 nM, respectively (Table 1 and SI Appendix, Fig. S3 B and C), while CIRBP\(^{RSY}\) binds TNPO1 and TNPO3 with \(K_a\) values of 7,800 ± 545 and 114 ± 5 nM, respectively (Table 1 and SI Appendix, Fig. S3 B and C). The high affinity of CIRBP\(^{RSY}\) for TNPO3 was striking given that it contains a much lower number of tyrosines or all arginines into alanines (CIRBP RSA, 7,800 nM, respectively (Table 1 and SI Appendix, Fig. S3). The absence of CIRBP\(^{RSY}\) or meCIRBP\(^{RGG}\) completely abrogated both TNPO1 and TNPO3 binding to their respective ligands. To summarize, we show that TNPO1 and TNPO3 bind both the RG/RGG and RSY regions of CIRBP-RGG and CIRBP-RSY, respectively.

To further validate that both CIRBP\(^{RGG}\) and FUS\(^{PY-NLS}\) compete for binding to TNPO3, we carried out ITC competition experiments. In line with the NMR experiments, binding affinity of CIRBP\(^{RGG}\) to TNPO1 was reduced in the presence of FUS\(^{PY-NLS}\) with an associated \(K_a\) of 391 ± 77 nM compared with 75 ± 13 nM in the absence of FUS\(^{PY-NLS}\) (Table 1 and SI Appendix, Figs. S3B and S4A). Importantly, a number of RBPs, such as FUS, possess urgent binding motifs engaged in static interactions. This loop has been removed previously (47).

Interestingly, TNPO1 possesses a 30-amino acid-long acidic loop enriched in glutamic acid and aspartic acid residues, which could be involved in binding to basic residues in the CIRBP\(^{RGG}\) region via electrostatic interactions. This loop has been removed previously (47). We recorded \(^{1}H_{−}\)\(^{15}N\) HSQC spectra of full-length TNPO1. Here, only flexible regions can be seen due to the high molecular mass of TNPO1. All visible \(^{1}H_{−}\)\(^{15}N\) HSQC cross-peaks of TNPO1 correspond to residues located within the TNPO1 loop region as identified by NMR resonance assignment (Fig. 4B). Next, we examined binding of CIRBP\(^{RGG}\) and FUS\(^{PY-NLS}\) to full-length TNPO1. Addition of CIRBP\(^{RGG}\) to a solution of CIRBP-RGG binds TNPO1 and TNPO3 with 

\(K_a\) values of 413 ± 41 nM compared with 48 ± 6 nM in the absence of CIRBP\(^{RGG}\) (Table 1 and SI Appendix, Figs. S3B and S4A).
specifically affected by CIRBPRGG or FUSPY binding are surrounded in red, and the peaks affected by both CIRBPRGG and FUSPY are surrounded in black. The affinity of CIRBPRGG for TNPO1 with an associated with our NMR data, deletion of the acidic loop decreased the absence and presence of the disordered acidic loop. In line plot, with the TNPO1loop amino acids strongly affected (CSP 1H-15N HSQC cross-peaks related to the TNPO1 loop time, whereas addition of FUSPY-NLS showed no detectable perturbations of the 1H-15N HSQC cross-peaks corresponding to the TNPO1 loop induced progressive chemical shift perturbations of 1H-15N-labeled TNPO1loop (Fig. 4A) (SI Appendix, Fig. S4C). Conversely, neither FUSPY-NLS nor CIRBPRGG binds to TNPO1loop (SI Appendix, Fig. S4B). To determine the contribution of the TNPO1 loop region to the overall affinity of TNPO1 for its cargo, we compared the affinity of CIRBPRGG and FUSPY-NLS for TNPO1 in the absence and presence of the disordered acidic loop. In line with our NMR data, deletion of the acidic loop decreased the affinity of CIRBPRGG for TNPO1 with an associated $K_d$ of 364 ± 15 nM, whereas the deletion had no impact on binding of FUSPY-NLS and CIRBPRGG (Table 1 and SI Appendix, Fig. S4D).

When in complex with TNPO1, it is possible that multiple NLSs occupy different or similar binding sites. Three-dimensional (3D) structures of human TNPO1 in complex with PY-NLSs of different RBPs show that the PY-NLSs bind at two sites on TNPO1 involving PY-specific and hydrophobic/basic-rich regions, respectively (Fig. 4D) (46). Using NMR spectroscopy and ITC, we show that the RG/RGG region of CIRBP contacts two key sites on TNPO1, a site within the folded region of TNPO1 and competing with FUSPY-NLS binding and a unique, nonoverlapping site within the disordered negatively charged loop of TNPO1. Based on the structure of TNPO1 bound to the FUSPY-NLS and the electrostatic potential of the importin, we propose that RG/RGG regions bind to the acidic groove of TNPO1, where it competes with the positively charged N-terminal part of PY-NLS (Fig. 4D). In the previously solved RanGTP-bound TNPO1 structure, the TNPO1 disordered loop points toward the acidic groove (47), suggesting that it could stabilize the interaction with the RG/RGG region by creating an extended negatively charged patch.
two-way ANOVA on TNPO1 and TNPO3 binding using NMR spectroscopy of recombinant PRMT1 and tested the effect of arginine methylation on TNPO1 and TNPO3 binding. Recently, we have shown that asymmetric dimethylation of arginines in the RGG3 region of FUS by PRMT1 reduces its affinity for TNPO1 and hereby, regulates FUS nuclear import (11, 48). Interestingly, in *Xenopus* xCIRBP2 has previously been demonstrated to be methylated by PRMT1 in vitro, and overexpression of PRMT1 results in increased cytosolic localization of GFP-xCIRBP2 (18), although the molecular mechanisms underlying this relocalization remain elusive. In line with these data, we found that both PRMT1 silencing and inhibition of arginine methylation by adenosine diphosphate (AdOx) led to enhanced nuclear localization of GST-GFP—tagged CIRBP, which was mostly cytoplasmic in HeLa S3 cells under control conditions (Fig. 5A). One possible mechanism could be that arginine methylation weakens TNPO1 and/or TNPO3 binding and consequently, decreases nuclear import of CIRBP. To test this hypothesis, we carried out in vitro arginine methylation of full-length CIRBP and CIRBP<sub>ARGG</sub> using purified recombinant PRMT1 and tested the effect of arginine methylation on TNPO1 and TNPO3 binding using NMR spectroscopy and ITC. Successful methylation of several arginines, including several previously published residues (49), was confirmed by NMR using <sup>1</sup>H-<sup>15</sup>N HSQC experiments (SI Appendix, Fig. SS4). Interestingly, the CIRBP methylation sites are located adjacent to the main TNPO1- and TNPO3-interacting residues of CIRBP. When we tested binding of TNPO1 or TNPO3 with in vitro methylated CIRBP<sub>ARGG</sub> or full-length CIRBP by NMR and ITC, we detected no binding to TNPO1 and reduced binding to TNPO3 (Fig. 5B, Table 1, and SI Appendix, Fig. S5 B and C). In line with this, unmethylated recombinant CIRBP pulled down more TNPO1 and TNPO3 from HeLa cell lysates than methylated CIRBP did in a biochemical pull-down experiment (SI Appendix, Fig. SSD).

Summarizing, we show that PRMT1-mediated arginine methylation of the CIRBP RG/RRG region regulates nuclear import of CIRBP by reducing its binding affinity to both TNPO1 and TNPO3. This implies that arginine methylation of RG/RRG-rich NLSs may generally regulate importin binding and hence, nuclear import of cargoes containing this class of NLS.

**Discussion**

A large number of RBPs contain RG/RRG- or RS-rich regions and combinations thereof (Fig. 6A and Dataset S2). Here, we report two types of NLS and demonstrate that they function in regulating nuclear import, phases separation, and SG recruitment of CIRBP (Fig. 6B). We show that CIRBP, which lacks a PY-NLS or RS-NLS, binds both TNPO1 and TNPO3 in a RanGTP-dependent manner (Fig. 3). This interaction involves two distinct CIRBP regions, the RG/RRG region and the RSY region. We show that these two regions harbor different specificity toward TNPO1 and TNPO3, the RG/RRG NLS being specialized in TNPO1 recognition and the RSY-NLS being specialized in TNPO3 recognition.

TNPO1 Recognizes RG/RRG Regions via Multiple Interactions. The molecular determinants involved in TNPO1-mediated cargo recognition via the PY-NLS have been studied extensively (2, 50–52). In contrast, little is known about the role of RG/RRG regions in TNPO1 recognition. Among the reported TNPO1 cargoes, we identified 61 proteins harboring a combination of a PY-NLS and an RG/RRG region (Fig. 4A and Dataset S1). Here, we show that a flexible, acidic loop in TNPO1 allows specific binding to the RG/RRG region, whereas the inner acidic groove of TNPO1 is involved in both RG/RRG and PY-NLS recognition (Fig. 4). Several models or a combination thereof could explain the role of the flexible TNPO1 acidic loop in cargo binding: the “fly-casting” effect or an increase in the number of collisions leading to productive binding (53). In the two-step fly-casting binding mechanism (54, 55), the flexible TNPO1 acidic loop screens, with a high capture radius, for binding partners and binds the positively charged RG/RRG regions even at a low concentration of the labeled, NMR-visible, partner colored black (unbound), orange (TNPO1-bound), or cyan (TNPO3-bound), respectively.

**Fig. 5.** PRMT1-mediated arginine methylation of the CIRBP RG/RRG region weakens TNPO1 and TNPO3 binding. (A) HeLa cells, either transfected with a control siRNA or siRNA against PRMT1 or treated with AdOx, were transfected with a construct coding for GST-GFP-CIRBP. Nuclei were counterstained with Hoechst 33342, and cells were analyzed by fluorescence microscopy. A quantification of the subcellular localization of the individual reporter proteins (n > C, n = C, and n < C) is shown. Values represent the mean of five independent experiments ± SEM. (Scale bar: 20 μm.) **P < 0.01 by two-way ANOVA + Bonferroni multiple comparison test; ***P < 0.001 by two-way ANOVA + Bonferroni multiple comparison test. (B) 1<sup>H</sup>-<sup>15</sup>N HSQC spectrum of <sup>15</sup>N-labeled PRMT1-mediated methylated CIRBP<sub>ARGG</sub> at 50 μM (without black) or with addition of one equivalent of TNPO1 (orange; Left) or TNPO3 (cyan; Right). A representative cartoon is associated with each NMR spectrum with the unlabeled, NMR-invisible, protein colored gray and the labeled, NMR-visible, protein colored gray and the labeled, NMR-visible, partner colored black (unbound), orange (TNPO1-bound), or cyan (TNPO3-bound), respectively.
relatively large distance from the structured core of TNPO1. In the second step, the PY motif gets recruited, binds, and folds inside the inner groove of TNPO1. Through this mechanism, the binding rate can be enhanced over the rate of binding to a fully folded protein. However, the bound state could still undergo conformational exchange both within the PY-NLS site as well as within the RG/RGG regions, with the RG/RGG region being more likely to undergo conformational exchange in the bound state. Although fly casting is difficult to prove or disprove experimentally, it is further supported by the observation that electrostatic attraction, as in this case between the positively charged RG/RGG motif and the negatively charged flexible TNPO1 loop, can enhanced binding rates (55). On the contrary, the slower translational diffusion of TNPO1 with the disordered loop could, in part, offset the kinetic advantage of an increased capture radius (53, 56). Alternatively, the TNPO1 disordered loop could speed up association with RG/RGG and PY-NLS-containing cargo proteins by a reduction of the free energy barrier between initial and final complex due to an increase in the number of collisions leading to productive binding (53, 56). As in the fly-casting process, electrostatic interactions might play an important role in this process by reducing the redissociation rate after initial encounter. Moreover, electrostatic interactions might increase the probability of native-like topologies in the collision complexes (53, 57). In contrast to the fly-casting mechanism, the flexible TNPO1 loop would help maintain conformational restraints and steric hindrance (53, 58) and “guide” the PY-NLS to the structured core of TNPO1, with the help of the RG/RGG region.

Our study provides a mechanistic explanation for the reported involvement of RG/RGG regions in nuclear import of several RBPs, including hnRNPA2 (59), TAF15 (60), SERBP1 (61), PABPN1 (62), and Sam68 (63). As CIRBP, SERBP1 does not possess a PY-NLS or any other known NLS, suggesting that the RG/RGG region in SERBP1 may be responsible for nuclear import. The interaction between TNPO1 and RG/RGG regions can be of high affinity, in line with our observation that the RG/RGG region of CIRBP binds TNPO1 with high affinity (Table 1 and SI Appendix, Fig. S3B), over 100 times stronger than the C-terminal RG/RGG region of FUS (11). It is tempting to speculate that TNPO1-mediated nuclear import could generally be used for the large class of RG/RGG-containing proteins (13).

**TNPO3 Recognizes an RSY-Rich NLS with High Affinity.** The molecular determinants involved in TNPO3-mediated cargo recognition are poorly understood. TNPO3 has been proposed to recognize phosphorylated RS-rich regions in RBPs (classical RS-NLS) (29). Recently, Jing et al. (64) showed that, while an (RS)$_4$ repeat peptide does not interact with TNPO3, its phosphorylated version binds to TNPO3 with micromolar affinity. Here, we present quantitative affinity data of TNPO3 for its native cargo CIRBP and show that its RSY-rich region binds with higher affinity at a low-nanomolar $K_d$ in the absence of phosphorylation. This is comparable with the affinity reported to be necessary for nuclear import of cargo in cells (9, 11, 65). This finding furthermore suggests that unmodified RS-like regions could serve as NLS for TNPO3 in absence of any posttranslational modifications, extending our knowledge of the RS/RS-like NLS consensus sequence. In addition to the arginine residues, the tyrosine residues in this region are essential for TNPO3 binding (SI Appendix, Fig. S3 E and F). Basic Local Alignment Tool for proteins analysis of the CIRBP RSY-NLS against the human protein database identified several similar regions present in RS domain-containing proteins involved in RNA metabolism (SI Appendix, Fig. S6). Notably, such RSY-rich regions are present in SRSF4 and SRSF6 and are located in the flexible linker between the two RRM domains. In addition to the putative RSY-rich NLS, SRSF4 and SRSF6 contain a classical RS-NLS for TNPO3 in their C-terminal tails (SI Appendix, Fig. S6). In contrast, the RSY-rich region of TRA2A is located next to its RS-2 domain (SI Appendix, Fig. S6). RSY-rich regions can be found in several other proteins (SI Appendix, Fig. S6). The lack of a specific and conserved amino acid consensus sequence in the superposition of these regions suggests that the RSY NLS is degenerate and a dynamic binding region. Nevertheless, the detailed relationship of transportin binding/specificity and distribution of R, S, Y and R, G in RSY-rich and RG/RGG-rich regions remains to be determined. Similar to as in CIRBP, the presence of a putative high-affinity TNPO3 binding region (i.e., RSY region) could mediate phosphorylation-independent nuclear import of these RBPs.

Given that we identified two distinct regions (RG/RGG rich and RSY rich) in CIRBP to be involved in its nuclear import, we carried out a bioinformatics analysis focusing on the co-occurrence of RG/RGG and RS/RS-like regions. Strikingly, other than CIRBP, numerous other proteins harbor a combination of RG/RGG and RS/RS-like regions (e.g., LENG8, RBMX, SCAF15, SRSF5) (Fig. 6A and Dataset S2). We propose that nuclear import of these proteins may be mediated by both TNPO1 and TNPO3. We envision that the presence of multiple TNPO1 and/or TNPO3 binding regions together with posttranslational modifications of these regions, such as arginine methylation and phosphorylation, could fine tune nuclear import, phase separation, and SG recruitment of numerous RBPs and in turn, regulate their (patho-)physiological function.

**TNPO1 and TNPO3 Suppress CIRBP Phase Separation and Stress Granule Recruitment.** We found that TNPO3 has “chaperone-like” activity toward CIRBP and inhibits its phase separation and SG recruitment (Fig. 2), like the recently discovered chaperone TNPO1 (1–3). As both TNPO1 and TNPO3 suppress phase separation of CIRBP and its localization in SGs, they might be involved in regulating CIRBP function in cell adaption to stress, possibly allowing release of CIRBP-bound RNA targets and making them available for the translation machinery. Other than CIRBP, several proteins that contain an NLS for TNPO3 localize to cellular condensates, including ASF/SF2 (66) and SRSF3 (67, 68) in humans and several serine/arginine-rich proteins in Caenorhabditis elegans (69), suggesting a general role of TNPO3 in chaperoning its cargo RBPs. Numerous studies have underscored a critical role of RBPs in neurodegenerative diseases (70–72). Indeed, several proteins that undergo LLPS are found mutated in such disorders, including TDP-43, FUS, hnRNPA1, and Tau, and disease-linked mutations promote aberrant phase transitions and SG recruitment of the mutant proteins (1, 70, 73–77). Therefore, a deeper understanding of the molecular mechanisms regulating phase separation and SG dynamics is a critical step toward the development of therapeutic approaches. Our data revealing regulators of phase separation and SG recruitment will contribute to this end.

**CIRBP Nuclear Import Is Regulated by Arginine Methylation of the RG/RGG Region.** RG/RGG regions are often subject to arginine methylation (13), which can regulate transportin–cargo interaction as previously shown for FUS (11, 48). Here, we show that the RG/RGG region of CIRBP is methylated by PRMT1 and that arginine methylation interferes with both TNPO1 and TNPO3 binding (Fig. 5). Depending on the methylation status of the CIRBP RG/RGG region and the relative affinity of each NLS for TNPO1 and TNPO3, we propose two different import scenarios: 1) if the RG/RGG region of CIRBP is unmethylated, active nuclear import of CIRBP is mediated by both the RG/RGG-NLS (TNPO1) and the RSY-NLS (TNPO3), and 2) if the RG/RGG region of CIRBP is methylated, active nuclear import of CIRBP is mediated solely by the RSY-NLS (TNPO3) (Fig. 6B). Although detailed studies are needed in the future, first indications of the roles of arginine methylation in regulating nuclear import

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1. Bourgeois et al. PNAS April 14, 2020, vol. 117, no. 15, 8511

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and disease pathophysiology have been published by us and others. In the case of the RBPs CIRBP (Fig. 5), FUS (11), and SERBP1 (61), pharmacological inhibition of arginine methylation enhances nuclear localization the corresponding RBP. Alteration in arginine methylation of RG/RGG-containing proteins is associated with a plethora of neurodegenerative diseases and cancers (11, 13, 48, 63–78). In line with this, PRMT1 mRNA levels are increased in several cancer tissues and tumors (81–83). Moreover, PRMT1 has been shown to regulate epithelial–mesenchymal transition, cancer cell migration, and invasion, which are hallmarks of cancer progression and metastasis (84). Anomalous PRMT1 expression/distribution might alter cell localization and function of the RG/RGG proteome. Detailed understanding of the molecular mechanisms of RG/RGG-mediated nuclear import is, therefore, essential for the development of therapeutic approaches that may benefit patients with amyotrophic lateral sclerosis or frontotemporal dementia (FUS), brain ischemia (CIRBP), or neurodegenerative disease and cancer in general. Given that the RG/RGG region is highly abundant in the human proteome (13), we anticipate that many more diseases linked to misregulation of RG/RGG regions will appear in the upcoming years.

Materials and Methods

Plasmids. Constructs for mammalian cell expression of CIRBP or for Escherichia coli expression of human CIRBP, TNPO1, TNPO3, PRMT1, and FUS have been obtained as described in SI Appendix.

Protein Expression and Purification. For expression of recombinant ZZ-His6 proteins, the different bacterial expression pETM11-ZZ-His6 vectors were transformed into E. coli BL21-DE3 Star strain and purified using Ni-NTA agarose beads (Qiagen), and the ZZ-His6 tag was cleaved with Tobacco etch virus (TEV) protease treatment. A final size exclusion chromatography purification step was performed in the buffer of interest. Detailed information and protocols are in SI Appendix.

cDNA and siRNA Transfection. Complementary DNA (cDNA) and siRNA transfection for analysis of cellular localization in HeLa Kyoto cells and Hela S3 cells is described in detail in SI Appendix.

Cell Culture. HeLa S3 cells were seeded on 1.5-h high-precision glass cover slips (Marienfeld-Superior) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (1.25 μg/mL; all Gibco) in a humidified incubator (37 °C, 5% CO₂, 95% air). HeLa Kyoto cells were grown in DMEM high-glucose Glutamax (Invitrogen) supplemented with 10% FBS and 2 μg/mL gentamicin. Note that for fluorescence microscopy the reporter assay was performed on semi-permeabilized cells. The first layer was pre-coated with 0.22 μM magnetic beads (M-Bio, Santa Clara, CA) and then 200 μg/mL wheat germ agglutinin (WGA) on ice. Cells were then incubated for 30 min at room temperature with 200 nM MBP-CIRBP-ECD in the absence or presence of increasing concentrations of His-TNPO1 or His-TNPO3, respectively, in KPB. After several washes, cells were fixed, and SGs were visualized by immunostaining of G3BP1 and confocal microscopy (SI Appendix).

Image Processing and Statistics. All microscopy images were processed using Fiji/ImageJ software, applying linear enhancement for brightness and contrast. Statistical analyses were performed in GraphPad Prism 5.

Pull-Down Assay. Ni-NTA beads (Qiagen) were equilibrated in wash buffer (50 mM Na2HPO4/NaH2PO4, pH 7.5, 150 mM NaCl, 20 mM imidazole, 4 mM β-mercaptoethanol [βME]) and blocked in wash buffer supplemented with 0.3 mg/mL bovine serum albumin (BSA). HeLa cells were lysed in lysis buffer (50 mM Na2HPO4/NaH2PO4, pH 7.5, 150 mM NaCl, 20 mM imidazole, 4 mM βME, 1x protease inhibitor). Supernatant was preincubated with half of previously blocked Ni-NTA beads for 1 h at 4 °C. Precleared cell lysate was equally distributed to 50 μL BSA-blocked Ni-NTA beads and supplemented with 200 μg unmethylated and methylated CIRBP full-length protein. Reactions in a final volume of 1.5 mL were incubated for 2 h at 4 °C on a rotary shaker. Beads were washed three times in wash buffer, resuspended in 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) sample buffer, and boiled for 5 min. Eluted proteins were separated by SDS/PAGE (10 to 20%) and detected by western blot using specific antibodies for TNPO1 (clone D4S2; Novocea), TNPO3 (abcam; ab64533), and His6 (abcam; ab18184). Note that, due to the small size of recombinant CIRBP, CIRBP could not be detected by standard western blotting techniques. Instead, two layers of 0.22 μm of nitrocellulose membrane were used. TNPO1 and TNPO3 were detected on the first layer, CIRBP was detected on the second layer, and band intensities were analyzed with ImageJ software and normalized to signal intensity of unmethylated CIRBP full length. Statistical analyses were performed in GraphPad Prism 5.

ITC. All proteins samples were equilibrated in the same buffer containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, and 2 mM Tris(carboxyethyl)phosphine. ITC measurements were taken using a MicroCal VP-ITC instrument (Microcal) with 36 rounds of 8-μL injections at 25 °C. Integration of peaks corresponding to each injection, subtraction of the contribution of protein dilution, and correction for the baseline were performed using the Origin-based 7.0 software provided by the manufacturer. Curve fitting was done with a standard one-site model and gives the equilibrium binding constant (K_s) and enthalpy of the complex formation (ΔH).

NMR.

Binding assay. All proteins samples were equilibrated in the same buffer containing 50 mM Tris HCl, pH 6.7, 150 mM NaCl, and 2 mM Tris(carboxyethyl)phosphine (TCEP). NMR experiments were performed at 25 °C on Bruker DRX-400 and 900-MHz spectrometers equipped with a triple-resonance cryoprobe. Other than the 1H-15N HSQC spectrum, the following 3D spectra were acquired for assignment of the CIRBP and TNPO1 reference samples (CIRBP^G60, CIRBP^R51, TNPO1, and TNPO3^G60): HNCO, HN(CA)CO, HN(CA)CB, CBCA(CO)NH, (H,C,C,O)NH-TOCSY, HN(CA)NH, and H(N,C,NH)NHNH between 400 and 700 μM 1H-13C-labeled proteins and 10% deuterium oxide. Spectra were processed using NMRPipe (85).

Phase separation assay. All proteins (12 x 10^6 repeats) samples were prepared in 20 mM Na2HPO4/NaH2PO4, pH 7.5, 75 mM NaCl, 2.5% glycerol, 1 mM DTT, and 10% H2O added for the lock signal. NMR experiments were performed at 25 °C on Bruker 600-MHz spectrometer. Spectra were processed using Topspin 3.5 and Mnova 11.

Turbidity Assay. All proteins RNA (12 x 10^6 repeats) samples were prepared in 20 mM Na2HPO4/NaH2PO4, pH 7.5, 75 mM NaCl, 2.5% glycerol, and 1 mM DTT. Turbidity measurements were conducted at 620 nm in 96-well plates with 90-μL samples using a BioTek Power Wave HT plate reader (BioTek). All experiments were performed in triplicates.

Differential Interference Contrast Microscopy. All proteins RNA (12 x 10^6 repeats) samples were prepared in 20 mM Na2HPO4/NaH2PO4, pH 7.5, 75 mM NaCl, 2.5% glycerol, and 1 mM DTT. The 25-μL sample was placed on a 30-mm No. 1 round glass coverslip and mounted on an Observer D1 microscope with 100x/1.45 oil immersion objective (Zeiss). Protein droplets were viewed using HAL 100 halogen lamp, and images were captured with an OrcaD2 camera (Hamamatsu) using VisiView 4.0.0.13 software (Visitron Systems).
In Vitro Methylation Assay. Untaggred CIRBPrgg and full-length CIRBP recombinant proteins and His6-PRMT1 were equilibrated in methylation buffer containing 50 mM Na-phosphate, pH 8, 150 mM NaCl, and 2 mM Tris(2-carboxyethyl)phosphine; 50 μM CIRBPrgg or full-length CIRBP was incubated with 7 μM His6-PRMT1 in the presence of 2 mM S-Adenosylmethionine (New England Biolabs) for 16 h at room temperature. Untagged methylated CIRBPrgg (mCIRBPrgg) and full-length CIRBP (mCIRBP) were then isolated from PRMT1 performing a second affinity purification using Ni-NTA beads, further equilibrated in the buffer of interest, and analyzed using NMR (SI Appendix).

Data Availability Statement. All data discussed in this study are included in the text and SI Appendix.

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