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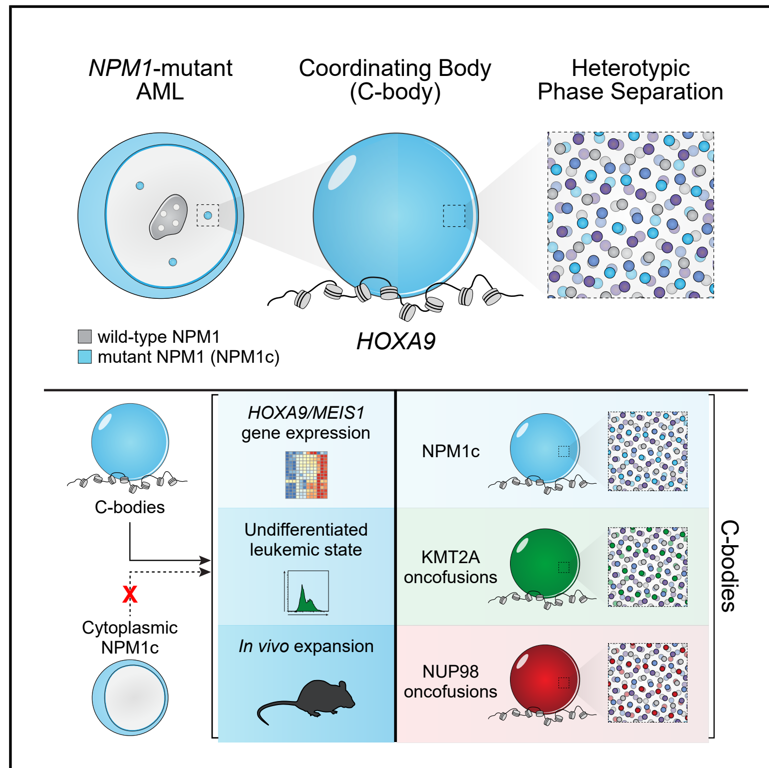
Disparate leukemia mutations converge on nuclear phase-separated condensates

Graphical abstract



Highlights

- Nuclear NPM1c phase separates into coordinating bodies (C-bodies) in *NPM1*-mutant AML
- C-bodies drive punctate co-enrichment of XPO1, NUP98, KMT2A, and MENIN
- C-bodies, and not cytoplasmic NPM1c, are necessary to maintain the leukemic state
- Nucleoporin and KMT2A oncofusion proteins driving multiple leukemias form C-bodies

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In brief

Mutant NPM1 and various leukemia oncofusions form biophysically indistingishable nuclear condensates, termed C-bodies, which orchestrate leukemogenic gene expression. These findings consolidate diverse genetic lesions into a shared pathogenic mechanism in AML.

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Disparate leukemia mutations converge on nuclear phase-separated condensates

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SUMMARY

During cancer development, mutations promote changes in gene expression that cause transformation. Leukemia associated with aberrant *HOXA* expression is driven by translocations of nucleoporin genes or *KMT2A* as well as mutations in *NPM1*. The mechanistic convergence of these disparate mutations remains elusive. Here, we demonstrate that mutant nucleophosmin 1 (NPM1c) forms nuclear condensates in human cell lines, mouse models, and primary patient samples. We show NPM1c phase separation is necessary and sufficient to recruit NUP98 and KMT2A to condensates. Through extensive mutagenesis and pharmacological destabilization of phase separation, we find that NPM1c condensates are necessary for regulating gene expression, promoting *in vivo* leukemic expansion, and maintaining the undifferentiated leukemic state. Finally, we show that nucleoporin and KMT2A fusion proteins form condensates that are biophysically indistinguishable from NPM1c condensates. Together, these data define a new condensate that we term the coordinating body (C-body) and establish C-bodies as a therapeutic vulnerability in leukemia.

INTRODUCTION

Protein networks orchestrate cellular activities encompassing most biological processes. Control of these networks requires spatial organization into subcellular compartments spanning multiple scales, including the separation of cytoplasm and nucleus, membrane-bound organelles like mitochondria, and membrane-less structures—also termed biomolecular condensates—such as nucleoli and super-enhancer condensates.[1] Compartmentalization enables focused protein interactions critical for function. For example, nucleoli direct ribosomal biogenesis and are driven by factors such as nucleophosmin (NPM1).[2–4] Notably, NPM1 mislocalization into the nucleoplasm is a hallmark of cellular stress and indicative of cellular dysfunction,[5,6] demonstrating the close relationship between protein localization and function.

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In cancer, aberrant protein localization can disrupt processes such as self-renewal or differentiation, activating oncogenic pathways that drive transformation. For example, oncogenic mutations can cause abnormal cytoplasmic export of nuclear-acting proteins such as p53, Rb, and BRCA1.[7] Other mutations, such as PML::RARA fusions, can alter protein localization and prevent condensate formation.[8,9] By contrast, some mutations precipitate condensate formation, stabilizing signaling hubs or transcriptional programs.[10,11] Many groups have reported pathogenic condensates that annex a shared network of regulatory proteins in cancer, including chromatin-associated BRD4-NUT, ENL-driven transcriptional condensates, and NUP98 fusion proteins.[12–16] The emergence of pathogenic condensates as a consequence of protein mislocalization is a compelling model in cancer[17,18]; however, demonstrating the connection between condensate stability and malignant transformation remains a critical challenge.

Condensates have emerged as a ubiquitous biophysical phenomenon that governs aspects of nearly all cellular activities, serving as bioreactors, sensors, signaling hubs, and more.[1,10,19] Nucleoli, PML bodies, and nuclear pores are examples of condensates that act as organizing centers enriching specific proteins to facilitate distinct activities. Condensates are liquid-like phases with physics similar to oil separating from water but with substantially higher complexity.[20–22] Condensates involve both homotypic interactions (occurring between the same biomolecules, e.g., intrinsically disordered regions [IDRs]) and heterotypic interactions (occurring between different biomolecules).[22,23] Moreover, multivalency via oligomerization is a common feature of condensate proteins.[24] For example, NPM1 contains an N-terminal self-pentamerization domain, a large IDR with two alternating acidic and basic tracts, and an RNA-binding domain (RBD). Each of these motifs is implicated in NPM1 phase separation *in vitro*; however, only the pentamerization domain and RBD are critical for driving heterotypic phase transitions that form the nucleolus in cells.[3,22,25,26]

NPM1 is the most frequently mutated gene in adult acute myeloid leukemias (AMLs).[27,28] Essentially all *NPM1* mutations result in a 4-base insertion in one allele of the C-terminal exon, causing a frameshift yielding a novel nuclear export sequence (NES).[29–31] This NES is bound by exportin 1 (XPO1), resulting in cytoplasmic export of mutant NPM1 (NPM1c).[32] Despite nuclear eviction, NPM1c paradoxically drives a characteristic *HOXA/MEIS1* gene expression program essential for leukemia.[33–37] NPM1c loss downregulates gene activation, leading to myeloid differentiation and cell growth arrest.[35] Unlike other AML subtypes that share this gene expression program—such as leukemias driven by nucleoporin (NUP; e.g., *NUP98*) fusions and *KMT2A* rearrangements—NPM1c lacks a clear association with chromatin regulation or even nuclear localization.[34]

Several contradictory models have been proposed to connect NPM1c localization and leukemic transformation, including cytoplasmic export of nuclear proteins, loss of function of wild-type NPM1 (NPM1wt), and direct chromatin binding.[36–39] Whether NPM1c interacts with NPM1wt or is meaningfully present in the nucleus are open questions requiring quantitative assessments. Data linking any proposed mechanism for NPM1c and its role in promoting disease in models of *NPM1*-mutant AML are lacking, slowing the development of therapies.

Furthermore, the connection between *NPM1*-mutant AML and other subtypes of leukemias driven by NUP and KMT2A oncofusions remains unclear. Notably, these leukemias often share therapeutic sensitivities to MENIN inhibitors[40–42] and XPO1 inhibitors,[35,43] suggesting a common mechanism underlying leukemogenesis. A major challenge is to identify a common targetable factor, if any, across multiple disease subtypes.

Here we discover that NPM1c forms nuclear condensates through heterotypic phase separation in *NPM1*-mutant AML. These condensates—termed coordinating bodies (C-bodies)—are necessary and sufficient for recruiting proteins implicated in *HOXA/MEIS1* regulation. We demonstrate that C-bodies are necessary for leukemia cell survival, oncogene expression (*HOXA/MEIS1*), evading differentiation, and promoting *in vivo* expansion. Critically, cytoplasmic NPM1c is insufficient for maintaining disease.

Looking toward leukemia subtypes sharing *HOXA/MEIS1* gene expression, we discover that NUP and KMT2A fusion proteins phase separate, recruiting C-body-associated proteins. Oncofusion-driven C-bodies are biophysically indistinguishable from NPM1c-driven C-bodies. Together, these data provide unprecedented clarity into the role of NPM1c in AML and suggest that C-bodies may be a unifying feature of multiple leukemia subtypes. Our work highlights important biophysical principles underlying how distinct oncogenic condensates can be driven by small genetic events. Finally, we provide an experimental framework to determine whether multiple reported pathogenic mutations form a common targetable mesoscale structure.

RESULTS

NPM1c forms nuclear condensates that are distinct from nucleoli

To examine the role of NPM1c in the cytoplasm or nucleus, we first sought to quantify the localization of NPM1c and NPM1wt. Using CRISPR, we introduced fluorescent tags into the terminal exon of both *NPM1* alleles in OCI-AML3 cells, a model of *NPM1*-mutant AML. NPM1c and NPM1wt were predominantly cytoplasmic and nucleolar, respectively, consistent with seminal works (Figures 1A and 1B).[27,35] Strikingly, we observed bright nuclear puncta in which NPM1c—but not NPM1wt—was highly enriched. NPM1c also decorated the nuclear lamina. Subcellular localization was independent of the specific tag (Figures S1A–S1C), and puncta were confirmed with immunostaining using antibodies targeting NPM1c (Figure S1D). These nuclear puncta, herein referred to as NPM1c condensates, are distinct from other common nuclear bodies (Figure S1F).

We next determined whether NPM1c condensates are observed in other models of *NPM1*-mutant AML. Using the same knockin strategy, we identified condensates in IMS-M2 cells—an *NPM1*-mutant cell line—and in a patient-derived xenograft (PDX2) model of *NPM1/FLT3*-mutant AML (Figures 1C and 1D).[35] In contrast to an NPM1$^{wt/wt}$ primary AML sample, immunostaining (STAR Methods) in *NPM1*-mutant samples showed NPM1c condensates (Figures 1E and S1E). These observations suggest that NPM1c condensates are a universal feature of *NPM1*-mutant leukemia.

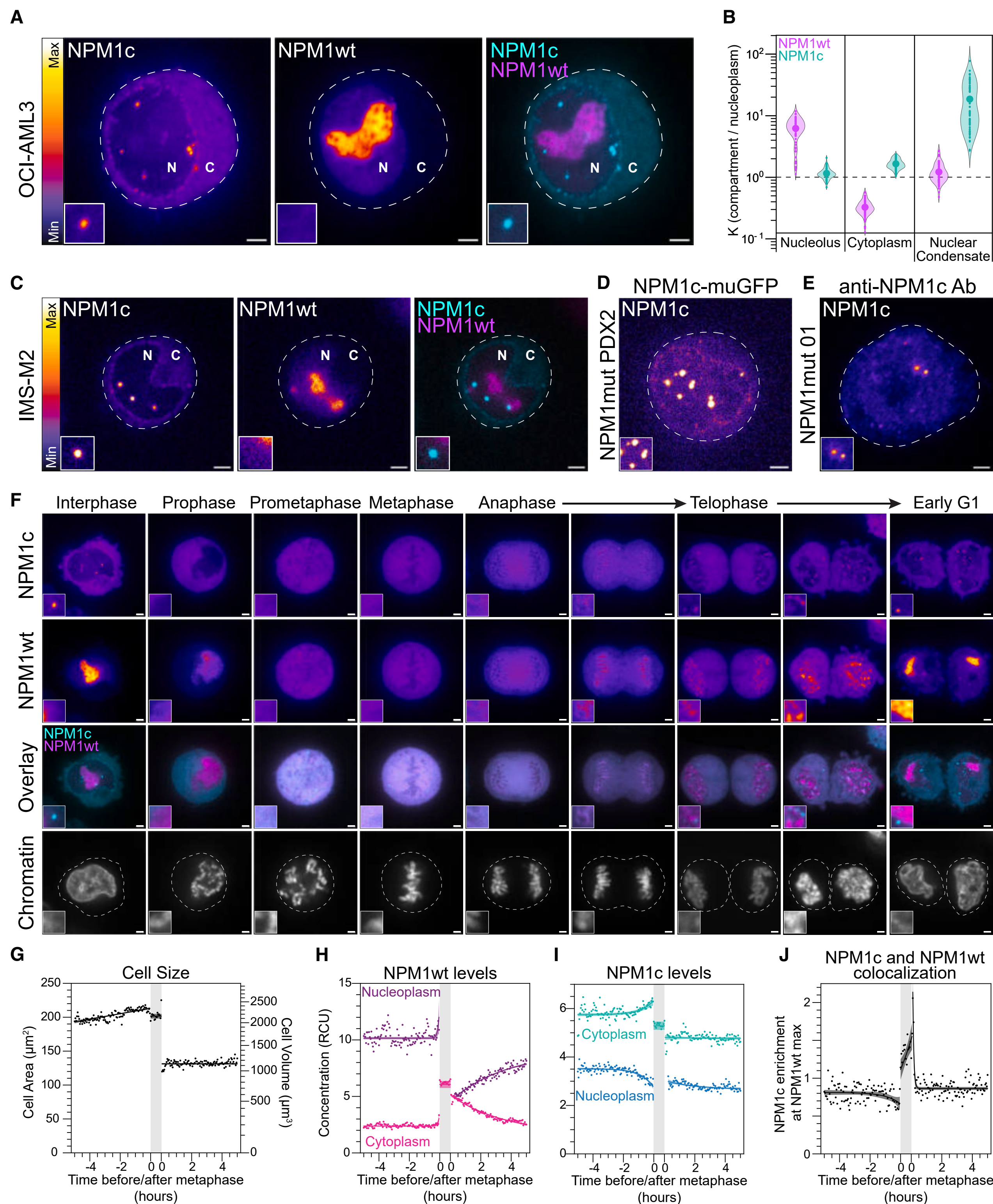

Figure 1. NPM1c resides in nuclear condensates established during cell division

(A) Live OCI-AML3 cells with CRISPR-edited endogenous C-terminal-tagged NPM1c (muGFP) and NPM1wt (mCherry).

(B) Partition coefficients (K) within listed compartments relative to nucleoplasm (n = 100).

(C) Live IMS-M2 cells CRISPR-edited as (A).

(D) Live CRISPR-edited *NPM1*-mutant patient-derived xenograft (PDX2) cells with NPM1c (muGFP).

(legend continued on next page)

To quantify subcellular localization of NPM1c and NPM1wt, we utilized established microscopy methods measuring the strength of preferential recruitment or "transfer" between compartments, quantified as a ratio of levels known as the partition coefficient (K^{tr}).[22,44] NPM1wt strongly partitioned into nucleoli (6.2 ± 0.3) (Figure 1B). NPM1c was only moderately enriched in the cytoplasm compared with the nucleoplasm (1.66 ± 0.03) but showed substantially higher partitioning (18 ± 2) into condensates (Figure 1B), equating to a concentration of roughly 100 μM (STAR Methods). These data reveal NPM1c enrichment is heavily biased toward a previously unidentified nuclear condensate rather than the cytoplasm.

NPM1c condensates are established during cell division

The stark separation of NPM1c and NPM1wt proteins into distinct condensates is unexpected given their shared oligomerization domain mediating self-pentamerization. We therefore asked how self-sorting occurs following cell division.

We performed live imaging throughout the cell cycle. NPM1wt recapitulated well-characterized behavior during mitosis,[45,46] including nucleolar breakdown during prophase, localization to the mitotic sheath in anaphase, and formation of pre-nucleolar bodies (PNBs) in telophase (Figure 1F). NPM1c condensate and nuclear lamina enrichment were lost during prophase, consistent with most nuclear bodies.[47] During metaphase and anaphase, NPM1c strongly colocalized with NPM1wt, including at the mitotic sheath. During telophase, NPM1c condensates and NPM1wt PNBs reformed rapidly, separating NPM1c and NPM1wt (Figure 1F).

To assess dynamics, we followed individual cells undergoing mitosis, imaging every 3 min. We observed an increase in cell size and volume as cells approached metaphase, a predictable halving after division, and corresponding changes in the number of condensates per cell (Figures 1G and S1G). NPM1wt redistributed across compartments during the last 6 ± 2 min of prophase due to nucleolar breakdown (Figure 1H). NPM1c focal enrichment was lost during the 50 ± 7 min of prophase, increasing cytoplasmic levels, suggesting NPM1c-XPO1 complexes released from foci are exported to the cytoplasm (Figure 1I).

NPM1wt and NPM1c rapidly segregate during early telophase (Figure 1J). After cytokinesis, NPM1wt levels are approximately equal in the nucleoplasm and cytoplasm, consistent with behavior in U2OS cells (Figures S1H and S1J). Strikingly, NPM1wt in OCI-AML3 cells requires 3 h (200 ± 25 min) to establish its basal interphase level in the nucleoplasm, compared with only a half hour (25 ± 1 min) in U2OS cells (Figures 1H and S1J). Expression of NPM1c in U2OS delays import of cytoplasmic NPM1wt (Figures S1H–S1K). By contrast, NPM1c levels in the cytoplasm did not significantly change following cytokinesis (Figure 1I). These results indicate that the presence of NPM1c substantially delays NPM1wt nuclear import.

Together, these data indicate two distinct sorting mechanisms. During telophase, NPM1c and NPM1wt rapidly segregate into nuclear condensates and PNBs, respectively. Shortly thereafter, NPM1c appears trapped in nuclear condensates with little change in nucleoplasmic and cytoplasmic levels. By contrast, NPM1wt slowly transits from the cytoplasm into the nucleus. We anticipate this is due to the presence of mixed wt/mutant pentamers, which are rapidly exported via XPO1 binding. Over time, NPM1wt is able to complete its transit to the nucleolus without NPM1c.

NPM1c condensates recruit factors implicated in gene regulation

The rapid reformation of NPM1c condensates distinct from PNBs indicates that interactions not native to NPM1wt likely drive condensate formation. During cell division, coordination of NUPs and XPO1 is necessary for nuclear envelope breakdown and formation,[48,49] coinciding with NPM1c condensate formation and dissolution. Therefore, we considered whether NUPs and XPO1 are associated with NPM1c condensates.

Immunostaining in OCI-AML3 and IMS-M2 cells expressing endogenous GFP-labeled NPM1c (*NPM1*$^{WT/Degron}$)[35] identified XPO1 and NUP98 (a core nuclear pore complex protein) in nuclear condensates and at the nuclear periphery (Figures 2A and S2A). To quantify colocalization with NPM1c condensates, we calculated the radial distribution function (RDF) as previously described.[50] XPO1 and NUP98 show maximal spatial enrichment at the center of NPM1c condensates (i.e., zero displacement; Figure 2B). By contrast, COILIN staining for Cajal bodies—a well-known nuclear body—showed depletion from NPM1c condensates (Figures 2A and 2B).

Recent reports further suggest that histone methyltransferase KMT2A and its adaptor protein MENIN facilitate gene expression with NPM1c at *HOXA/MEIS1* chromatin.[36,37] We asked whether the KMT2A-MENIN complex is enriched within NPM1c condensates. Similar to XPO1 and NUP98, KMT2A showed spatial enrichment in NPM1c condensates (Figures 2A and 2B). By contrast, MENIN localizes to NPM1c condensates and other nuclear puncta, consistent with its multifunctional roles[51] (Figures 2A and 2B). Inversely, all NPM1c condensates exhibited spatial enrichment of MENIN (Figure 2B). We also observed

(E) Immunostained *NPM1*-mutant primary AML patient sample with anti-NPM1c antibody (STAR Methods).
(F) Live dual-labeled OCI-AML3 cells imaged with chromatin dye (SPY650-DNA). Representative images of cell cycle stages.
(G–J) Quantification of dividing cells imaged every 3 min for ~10 h ($n = 10$). Metaphase (shaded gray) is the average time between prometaphase and anaphase.
(G) Cell area (μm^2) and approximate volume (μm^3). Mean fit confidence intervals are shown.
(H and I) (H) NPM1wt and (I) NPM1c concentration in relative concentration units (RCUs). Time points reflecting prometaphase through anaphase were annotated as cytoplasm.
(J) NPM1c enrichment in regions with high NPM1wt abundance, defined as the brightest 0.15 μm^2 box for each cell. All images are shown in Fire look up table or LUT (see color scales in A and C) except for colocalization (cyan and magenta) and chromatin (grayscale). The min/max of images was adjusted to optimally visualize condensates and cytoplasm. Dashed lines outline cytoplasmic membranes. N = nucleus. C = cytoplasm. Scale bars, 2 μm. Insets are magnified 2 μm square regions.
See also Figure S1.

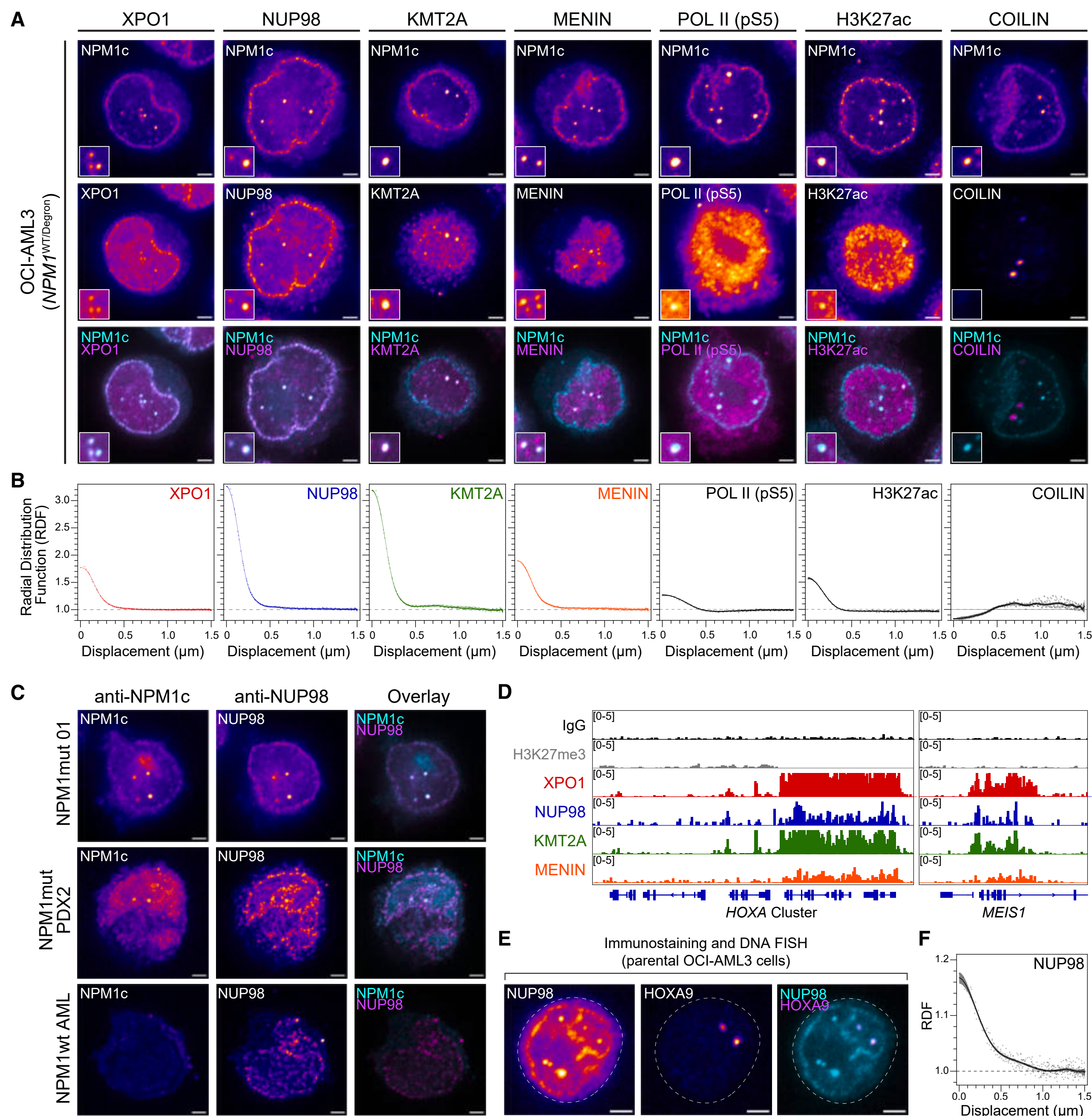

Figure 2. NPM1c condensates recruit chromatin regulatory proteins essential for leukemia

(A) Immunostaining of *NPM1*$^{WT/Degron}$ OCI-AML3 cells expressing NPM1c-GFP with indicated antibodies.

(B) Radial distribution function (RDF) centered around NPM1c condensates (*n* = 50 cells per RDF).

(C) Immunostaining of *NPM1*-mutant and wild-type NPM1 primary AML patient samples and a PDX2 model of *NPM1*-mutant AML using indicated antibodies.

(D) CUT&RUN data from *NPM1*$^{WT/Degron}$ OCI-AML3 cells measuring chromatin-enrichment of indicated proteins at relevant genetic loci (1 replicate of *HOXA* gene cluster and *MEIS1* locus shown, *n* = 2 replicates per antibody). Immunoglobulin G (IgG) and H3K27me3 are displayed as negative and positive controls, respectively.

(E) Combined NUP98 immunostaining and *HOXA9* DNA FISH.

(F) RDF of NUP98 antibody staining centered around *HOXA9* loci (*n* = 68 loci). All images are shown in Fire LUT except colocalization (cyan and magenta). Scale bars, 2 μm. Insets are magnified 2 μm square regions. Shaded regions are mean fit confidence intervals.

See also Figures S2 and S3.

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co-enrichment of NPM1c and NUP98 in primary AML patient samples (Figure 2C). These data suggest that NUP98, XPO1, KMT2A, and MENIN are colocalized within NPM1c condensates.

Accordingly, we asked whether these condensate-associated proteins are enriched at *HOXA/MEIS1* genes. We performed CUT&RUN in *NPM1*$^{WT/Degron}$ OCI-AML3 cells to identify active chromatin regions bound by these proteins. Consistent with reports,[36,37] we observed XPO1, KMT2A, and MENIN at the *HOXA* cluster and *MEIS1*. We also identified NUP98 at these same loci (Figure 2D). Our data showed significant overlap with previously annotated NPM1c-bound genes from chromatin immunoprecipitation sequencing (CHIP-seq)[36] (Figures S3A and S3B).

We questioned whether NPM1c condensates could be found at active chromatin regions. Immunostaining for active RNA polymerase II (RNA POL II pS5) and histone marks associated with active enhancers (H3K27ac) revealed substantial enrichment in NPM1c condensates (Figures 2A and 2B). To examine whether condensates are at *HOXA9*, we performed DNA fluorescence *in situ* hybridization (FISH) with immunostaining of NUP98 to identify C-bodies as the anti-NPM1c antibody also marks nucleoli. DNA FISH revealed that condensates are frequently associated with *HOXA9* chromatin (Figures 2E, 2F, and S3C). Together, these data suggest a strong correspondence between NPM1c condensates, the proteins enriched within them, and the genetic loci essential for driving *NPM1*-mutant AML.

NPM1c is necessary and sufficient for condensate formation and protein recruitment

To determine whether NPM1c is necessary for nuclear focal enrichment of XPO1, NUP98, KMT2A, and MENIN, we utilized an inducible degron model to degrade NPM1c (*NPM1*$^{WT/Degron}$ cells).[35] After inducing degradation with dTAG-13, NPM1c abundance decreased throughout the cell, and markers of myeloid differentiation increased (Figures 3A, 3B, S3D, S3F, and S3G). Immunostaining and quantification of the brightest focus per cell (0.15 μm^2 box) after NPM1c degradation revealed a clear lack of punctate enrichment for all proteins except MENIN (Figures 3C–3H).

To test for sufficiency, we expressed NPM1c in an NPM1wt context (U2OS cells) and observed *de novo* nuclear condensate formation (Figures S2D and S2E), and this occurred without artificial genetic elements, such as LacO arrays.[37] In fact, NPM1c is sufficient for *de novo* condensation in HL-60 acute promyelocytic leukemia cells and human primary CD34$^+$ cells (Figures S2B and S2C). Therefore, NPM1c is sufficient for condensate formation in hematologic and non-hematologic contexts. As observed in OCI-AML3 cells, NPM1c condensation was required for focal enrichment of XPO1, NUP98, KMT2A, and MENIN in U2OS cells (Figures S2D and S2E). Together, these data indicate that NPM1c is both necessary and sufficient to coordinate the recruitment of XPO1, NUP98, KMT2A, and MENIN into previously unreported nuclear condensates, hereafter referred to as C-bodies.

Multiple protein interactions are necessary for condensate stability, composition, and chromatin binding

To ask whether specific protein-protein interactions drive C-body formation, we utilized pharmacological strategies blocking XPO1-NPM1c and MENIN-KMT2A interactions.[35,42,52,53] XPO1 and MENIN inhibitors alter chromatin binding, decrease leukemogenic transcription, and demonstrate clinical activity in *NPM1*-mutant AML patients.[41,54]

Consistent with previous work, treatment with XPO1-inhibitors (Eltanexor and Selinexor) depleted NPM1c from the cytoplasm, dissolved C-bodies, and enhanced nucleolar localization of NPM1c (Figures 3A, 3B, and S4A–S4D).[35,53] We observed decreased *HOXA/MEIS1* expression and myeloid differentiation (Figures S3E–S3G). Immunostaining revealed loss of nuclear focal enrichment of XPO1, NUP98, and KMT2A (Figures 3C–3E, 3G, 3H, and S4A–S4D). MENIN puncta persisted but were not enriched with NPM1c (Figures 3F–3H and S4A–S4D). These data suggest that XPO1-NPM1c interactions are necessary for C-body formation.

Treatment with MENIN inhibitors, VTP50469 and MI-503, did not substantially impact NPM1c localization (Figures 3A, 3B, and S4A–S4D). Immunostaining revealed XPO1, NUP98, and KMT2A persisted in C-bodies (Figures 3C–3E, 3G–3I, and S4A–S4D). By contrast, MENIN was depleted from C-bodies, despite remaining in other nuclear puncta (Figures 3F–3I and S4A–S4E). Loss of MENIN C-body enrichment indicates that KMT2A and MENIN interact within C-bodies, but their interaction is not necessary for C-body formation. MENIN depletion from C-bodies corresponded to decreased *HOXA/MEIS1* expression and increased myeloid differentiation (Figures S3E–S3G), suggesting that C-body composition is essential for driving gene expression.

Our data show that degrading NPM1c, inhibition of XPO1, or disruption of the MENIN-KMT2A interaction can alter C-body stability and composition. Consistent with previous reports,[36,37,55,56] we observed decreased XPO1, KMT2A, and MENIN at *HOXA/MEIS1* chromatin after dTAG-13, Eltanexor, or VTP50469 treatment (Figure S3H). We also report here that NUP98 recapitulates this pattern across treatments (Figure S3H). Together, our data suggest that C-bodies facilitate chromatin binding of regulatory proteins in *NPM1*-mutant AML.

Pharmacological C-body disruption hinders *in vivo* models of *NPM1*-mutant AML

To elucidate the role of C-bodies in leukemia, we turned to *in vivo* and *ex vivo* models of *NPM1*-mutant AML. Using an inducible mouse model of *NPM1*-mutant pre-leukemia,[57] we observed that NPM1c is necessary for C-body formation in hematopoietic stem and progenitor cells (HSPCs), indicated by focal co-enrichment of NUP98 and MENIN upon induction (Figure 4A). To probe C-bodies in bona fide leukemia, we developed a rapid AML model utilizing *NRAS*^{G12D}-GFP retroviral overexpression in *Dnmt3a*-mutant;*Npm1*-mutant murine HSPCs in line with other strategies[58] (Figures 4B and S6A; STAR Methods). We isolated c-Kit+ progenitors from recipient mice and treated with MENIN inhibitors *ex vivo*. Treatment resulted in loss of MENIN from NUP98-marked C-bodies after 24 h, transcriptional downregulation of C-body-associated genes (including *Hoxa9*, *Hoxa10*, and *Meis1*) after 72 h, and evidence of monocytic differentiation after 6 days (Figures 4C–4E). These data demonstrate that a mouse model of *NPM1*-mutant leukemia generates C-bodies whose composition is critical for AML features.