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Partitioning of cancer therapeutics in nuclear condensates

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Abstract

The nucleus contains diverse phase-separated condensates that compartmentalize and concentrate biomolecules with distinct physicochemical properties. Here we consider whether condensates concentrate small molecule cancer therapeutics such that their pharmacodynamic properties are altered. We found that antineoplastic drugs become concentrated in specific protein condensates in vitro and that this occurs through physicochemical properties independent of the drug target. This behavior was also observed in tumor cells, where drug partitioning influenced drug activity. Altering the properties of the condensate was found to impact the concentration and activity of drugs. These results suggest that selective partitioning and concentration of small molecules within condensates contributes to drug pharmacodynamics and that further understanding of this phenomenon may facilitate advances in disease therapy.

One Sentence Summary:

Phase-separated condensates concentrate small molecule drugs impacting their on-target activity and pharmacodynamics.

The 5–10 billion protein molecules of cells are compartmentalized into both membrane-and non-membrane-bound organelles (1–3). Many non-membrane-bound organelles are phase-separated biomolecular condensates with distinct physicochemical properties that can absorb and concentrate specific proteins and nucleic acids (4–17). We reasoned that selective condensate partitioning might also occur with small molecule drugs whose targets occur within condensates (Figure 1A), and that the therapeutic index and efficacy of such compounds might therefore relate to their ability to partition into condensates that harbor their target. To test this idea, we focused our study on a collection of nuclear condensates previously reported in cell lines, demonstrated that they all occur in normal human cells and

in tumor cells, and then developed in vitro condensate droplet assays with key components of each of the nuclear condensates to enable testing of small molecules.

Nuclear condensates have been described in diverse cultured cell lines and contain one or more proteins that can serve both as markers of the condensate and as a scaffold for condensate formation in droplet assays in vitro (10–12, 18–32). Specifically, transcriptional condensates are marked by the condensate forming proteins MED1 and BRD4 (10, 12, 19), splicing speckles by SRSF2 (11, 20), heterochromatin by HP1α (21, 22) and nucleoli by FIB1 and NPM1 (23–25) (Figure S1A). To determine whether such condensates can also be observed in the cells of healthy and malignant human tissue, we obtained biopsies of breast ductal epithelium, invasive ductal carcinoma, normal colon, and colon cancer (Figures S1B, S1C). Immunofluorescence revealed nuclear bodies containing these marker proteins in both normal and transformed tissue (Figures 1B, 1C). There was a broad distribution of nuclear body sizes and numbers, as expected for dynamic biomolecular condensates, and no significant differences were observed between benign and malignant tissue (Figures S2A–C). However, tumor cells acquire large super-enhancers (SEs) at driver oncogenes (33) and these can form tumor-specific transcriptional condensates.

We developed an assay to model these nuclear condensates and study the behavior of small molecules within these droplets (Figure 1D). We produced and purified recombinant fluorescently-labeled versions of MED1, BRD4, SRSF2, HP1α, FIB1, and NPM1 (Figure S3), and confirmed the ability of these proteins to form droplets in an in vitro assay (Figures S4A, S4B). To investigate the partitioning behavior of small molecules, we added the dyes Fluorescein (332Da) and Hoechst (452Da), as well as fluorescently-labeled dextrans (4.4 kilodaltons (kDa), to solutions containing each of the six protein condensates. The dyes and dextrans appeared to diffuse through all the condensates without substantial partitioning (Figures 1E, S5, S6A–D). Small molecule drugs are generally smaller than 1 kDa, so these results suggested that small molecules can freely diffuse through these nuclear condensates unless there are factors other than size that influence partitioning.

We next sought to determine whether diverse clinically important drugs with targets that reside in nuclear condensates also exhibit free diffusion across these condensates, or display a different behavior. Cisplatin and mitoxantrone, members of a class of antineoplastic compounds that modify DNA through platination or intercalation, can be modified to have fluorescent properties (cisplatin) (34) or are inherently fluorescent (mitoxantrone). When added to droplet formation buffer with purified MED1, BRD4, SRSF2, HP1α, FIB1, or NPM1, cisplatin was found to be selectively concentrated in MED1 droplets (Figures 2A, S7A), with a partition coefficient of up to 600 (Figures S8A–C). Fluorescent modification of cisplatin did not appear to contribute to this behavior in vitro, as the modified drug could be chased out of the condensate with unmodified cisplatin, and an isomer of cisplatin did not exhibit the same behavior (Figures S7B–D). Mitoxantrone was also concentrated in MED1 condensates, as well as in FIB1 and NPM1 condensates (Figures 2B, S7A, S8A–D). Consistent with these results, mitoxantrone is known to concentrate in the nucleolus where FIB1 and NPM1 reside (35, 36). These results show that in contrast to the dyes tested above, small molecule drugs may concentrate in certain condensates, even in the absence of the drug target.

We selected for further study antineoplastic drugs that target transcriptional regulators expected to be contained within transcriptional condensates in cells. These targets include: a) the estrogen receptor (ER), a transcription factor and nuclear hormone receptor, b) CDK7, a cyclin-dependent kinase that functions in transcription initiation and cell cycle control, and c) BRD4, a bromodomain protein and coactivator involved in oncogene regulation (Figures S9A, S9B). To monitor drug behavior with a confocal fluorescent microscope, we used a fluorescent tamoxifen analog (FLTX1), which targets ER, and modified fluorescent THZ1 and JQ1, which target CDK7 and BRD4, respectively (37, 38). FLTX1 and THZ1 concentrated preferentially in MED1 droplets (Figures 2C, 2D, S7A), and this behavior was not attributable to the fluorescent moiety (Figures S7B, S7D). JQ1 concentration presented a different pattern, being concentrated in MED1, BRD4, and NPM1 droplets (Figures 2E, S7A, S7B). Reinforcing these results, we found that the small molecules that concentrate in MED1 condensates were also concentrated in condensates formed from purified whole Mediator complexes (Figure S10A) and in MED1 condensates formed in an alternative crowding agent (Figure S11A). The targets of these three compounds (ERα, CDK7, and the bromodomains of BRD4) are not present in these in vitro condensates, but are present in the SEs that form condensates with transcription factors and Mediator in vivo (10, 12, 39) (Figures S9A, S9B), suggesting that the ability of some small molecules to concentrate preferentially in the same condensate as their protein target may contribute to the pharmacological properties of these drugs.

To gain additional insight into the nature of interactions governing small molecule enrichment in condensates, we focused on the MED1-IDR condensate. Fluorescence recovery after photobleaching (FRAP) experiments showed that cisplatin molecules are highly mobile in this condensate (Figures S12A, S12B), suggesting that the condensate produces a physiochemical environment that facilitates drug concentration in a state of high dynamic mobility. To gain insights into the chemical features of small molecules that may contribute to selective association with MED1 in condensates, we used a fluorescent boron-dipyrromethene (BODIPY) library of 81 compounds with various combinations of chemical side groups (Figure S13A). Molecules that contained aromatic rings were found to preferentially concentrate in MED1 condensates (Figures S13A–D, S14A). These data suggest that pi-pi or pi-cation interactions are among the physicochemical properties that favor small molecule partitioning into MED1 condensates. Aromatic amino acids participate in pi-system interactions, and are overrepresented in the MED1 IDR relative to the other condensate forming proteins studied (Figure S3B). We generated a MED1 aromatic mutant protein (all 30 aromatic amino acids mutated to alanine) which retained the ability to form droplets in vitro, indicating that the aromatic amino acids are not required for droplet formation (Figure S14B, S14C), but small molecule probes containing aromatic rings and the polar molecule cisplatin no longer partitioned into condensates formed by the MED1 aromatic mutant protein (Figures S14D–F). These results suggest that the aromatic residues of MED1 condensates contribute to the physicochemical properties that selectively concentrate these small molecules.

We anticipated that the ability of small molecules to concentrate in specific condensates would influence target engagement and thus drug pharmacodynamics. To investigate this, we took advantage of the ability of condensates to incorporate DNA (Figure 3A, S15A), and

measured the relative efficiency of DNA platination by cisplatin in MED1 condensates, where cisplatin is concentrated, versus HP1α condensates, where cisplatin freely diffuses (Figure 2A). DNA platination, visualized by size-shift on a bioanalyzer, was more prevalent in MED1 condensates than in HP1α condensates (Figure 3B), consistent with the expectation that elevated concentrations of cisplatin in the MED1 condensates yield enhanced target engagement. If cisplatin becomes concentrated in Mediator condensates in cells, we would expect that DNA colocalized within Mediator condensates would be preferentially platinated. To test this idea, we performed co-immunofluorescence in cisplatin-treated HCT116 colon cancer cells using an antibody that specifically recognizes platinated DNA (Figure S16A) (40) together with antibodies specific for MED1, HP1α, or FIB1. Consistent with cisplatin's preference for MED1 condensates in vitro, we found that platinated DNA frequently colocalized with MED1 condensates, but not with HP1α or FIB1 condensates (Figure 3C). To determine whether the ability of cisplatin to engage DNA is dependent on the presence of a MED1 condensate we treated cells with JQ1, which caused a loss of MED1 condensates (Figure S16B), and observed a concomitant reduction in platinated DNA at the *MYC* oncogene (Figures S16C, S16D). These results are consistent with the idea that concentration of small molecules in specific condensates can influence the efficiency of target engagement.

In cells, the preferential modification of DNA in MED1-containing condensates might be expected to selectively disrupt these condensates with prolonged treatment. To test this, HCT116 colon cancer cells were engineered to express GFP-tagged marker proteins for each of the 6 nuclear condensates (Figures S17A–G, S18A, S18B). When exposed to cisplatin, a selective and progressive reduction in MED1 condensates was observed (Figures 3D, S19A, S19B, S20A). Consistent with this, cisplatin treatment led to a preferential loss of MED1 ChIP-seq signal at SEs (Figures 3E S21A). Furthermore, high throughput sequencing data from platinated-DNA pull-down (41) revealed that cisplatin-modified DNA preferentially occurs at SEs, where MED1 is concentrated (42) (Figure 3F). These results are consistent with reports that cisplatin preferentially modifies transcribed genes (41, 43), and argue that this effect is due to preferential condensate partitioning. Taken together, these results suggest a model where cisplatin preferentially modifies SE DNA, which in turn leads to dissolution of these condensates. Previous studies have shown that diverse tumor cells become highly dependent on SE driven oncogene expression (44–48), which might explain why platinum drugs, which are capable of general DNA modification, are effective therapeutics in diverse cancers (49).

We explored the behavior of another clinically important antineoplastic drug, tamoxifen, to assess whether drug response and resistance are associated with partitioning in condensates (Figure 4A). ERα incorporates into MED1 condensates in an estrogen-dependent manner in vitro (12); droplet assays confirmed this and revealed that the addition of tamoxifen leads to eviction of ERα from the MED1 condensates (Figure 4B). We further investigated the effects of estrogen and tamoxifen on MED1 condensates in breast cancer cells, focusing on the *MYC* oncogene due to its prominent oncogenic role and responsiveness to estrogen (50). MED1 condensates were observed on the *MYC* oncogene in the ER+ breast cancer cell line MCF7 (S9A, S22A–D). DNA FISH with MED1 IF revealed that estrogen enhances formation of MED1 condensates at the *MYC* oncogene and tamoxifen treatment reduces

these (Figures S23A, S23B). Artificial MED1 condensates without ER concentrated FLTX1 at the site of the condensate (Figure S24A), indicating that ER is not required for the partitioning of FLTX1 into MED1 condensates in cells. These results are consistent with the model that ERα interacts with MED1 condensates in an estrogen-dependent, tamoxifen-sensitive manner to drive oncogene expression in breast cancer cells.

The mechanisms that produce drug resistance can provide clues to drug activity in the clinical setting. Endocrine therapy and tamoxifen resistance is an enduring clinical challenge and is associated with multiple mechanisms including ERα mutation and MED1 overexpression (Figure 4A, S25) (51–55). To investigate whether ERα mutations alter ERα behavior in condensates, we produced 4 patient-derived ERα mutant proteins and tested their partitioning in the presence of tamoxifen. In contrast to WT ERα, condensates composed of patient-derived ERα mutants and MED1 were not disrupted upon tamoxifen treatment (Figures 4B, S26A, S26B). The ERα point mutations reduce the affinity for tamoxifen approximately 10-fold (52), indicating that the drug concentration in the droplet is inadequate to evict these ER mutant proteins when this affinity is reduced.

MED1 overexpression is associated with tamoxifen resistance and poor prognosis in breast cancer (51), but it is not clear why overexpression of one subunit of the Mediator complex produces resistance. We considered the possibility that overexpressed MED1 is incorporated into transcriptional condensates, which contain clusters of Mediator molecules (39), thereby expanding their volumes and diluting the available tamoxifen (Figure S27A). We found that the tamoxifen-resistant breast cancer cell line TAMR7 (56), which was derived from the tamoxifen-sensitive cell line MCF7, produces 4-fold elevated levels of MED1 protein (Figure S27B). The volume of MED1-containing condensates is 2-fold larger in these cells (Figures 4C, S27C). When modeled in an in vitro droplet assay, we found that a 4-fold increase in MED1 levels led to a commensurate increase in droplet size (Figures S28A, S28B). Furthermore, we found that 100μM tamoxifen prevented ERα incorporation into MED1 condensates (Figures 4B, 4D), but was much less effective in preventing ERα incorporation into the larger MED1 condensates produced with higher MED1 levels (Figure 4D). To confirm that the levels of tamoxifen in the larger droplets are more dilute, we measured the enrichment of the fluorescent tamoxifen analog FLTX1 in MED1 droplets, and found that the larger condensates have lower concentrations of the drug (Figure 4E). These results were mirrored in cells, where a collection of tethered ERα molecules form a MED1 condensate that is eliminated by tamoxifen, but when MED1 is overexpressed tamoxifen is unable to dissociate the ERα-MED1 condensate (Figure S29A). Similarly, knockdown of MED1 in tamoxifen resistant breast cancer cells sensitizes cells to tamoxifen (51, 55). These results support a model of tamoxifen resistance where MED1 overexpression causes the formation of larger transcriptional condensates, in which tamoxifen is diluted and thereby less effective in dissociating ER from the condensate (Figure 4F).

Our results show that drugs partition selectively into condensates, that this can occur through physicochemical properties that exist independent of their molecular targets, and that cells can develop resistance to drugs through condensate altering mechanisms. This may explain the surprising observation that inhibition of global gene regulators such as BRD4 or CDK7 can have selective effects on oncogenes that have acquired large SEs (46); selective

partitioning of inhibitors like JQ1 and THZ1 into SE condensates will preferentially disrupt transcription at those loci. These results also have implications for future development of efficacious disease therapeutics; effective target-engagement will depend on measurable factors such as drug partitioning in condensates (Figures S30A–D). Condensate assays of the type described here may thus help optimize condensate partitioning, target engagement, and the therapeutic index of small molecule drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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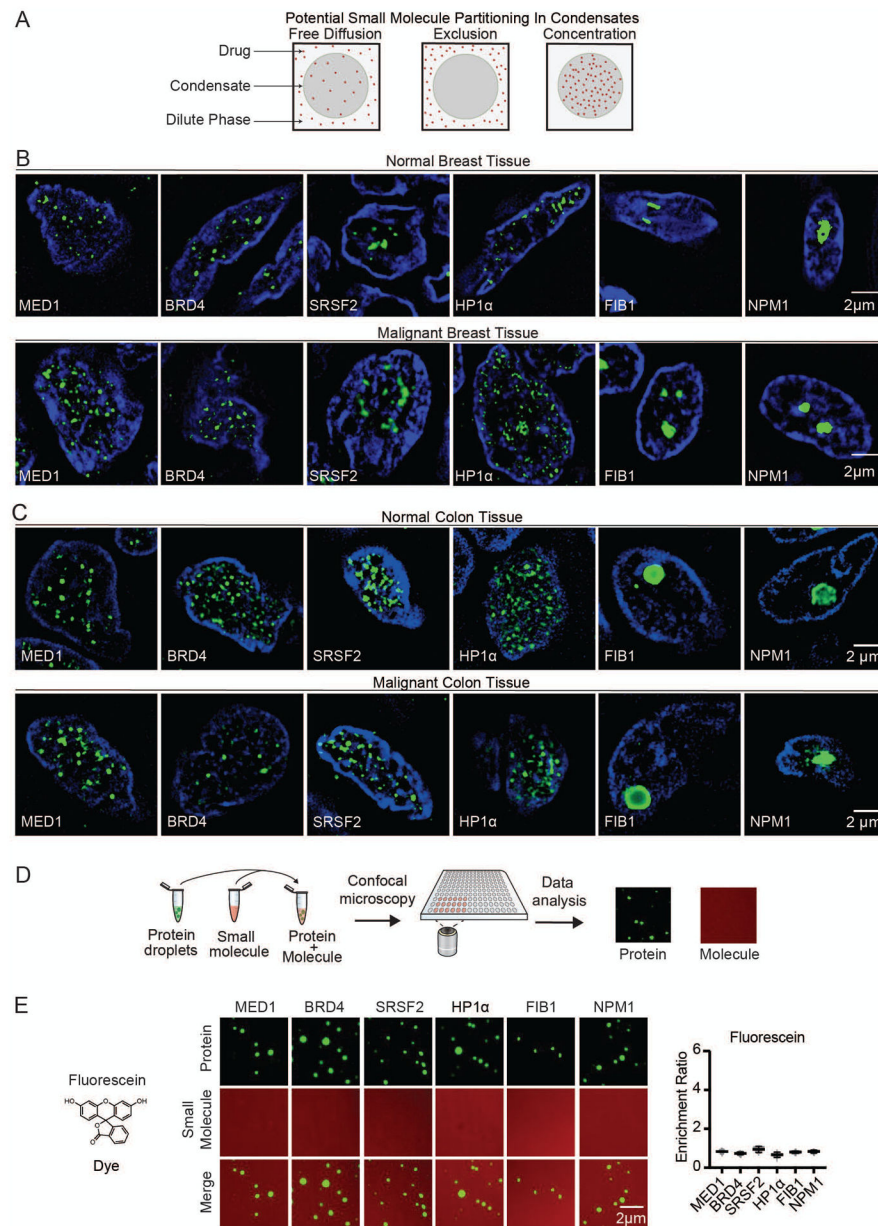
Fig. 1.
Nuclear condensates in human tissue and in vitro. (A) Model illustrating potential behaviors of small molecules in nuclear condensates. (B–C) Immunofluorescence of scaffold proteins of various nuclear condensates in tissue biopsies from benign and malignant human breast (B), and benign and malignant colon tissue (C), in nuclei stained with Hoechst, imaged at 100× on a fluorescent confocal microscope (see also Figures S1, S2). (D) Schematic of in vitro droplet formation assay to measure small molecule partitioning into nuclear condensates. (E) In vitro droplet assay showing the behavior of fluorescein dye in the presence of six protein condensates formed in 125mM NaCl and 10% PEG, with 10μM protein and 5μM fluorescein, imaged at 150× on a confocal fluorescent microscope (see also

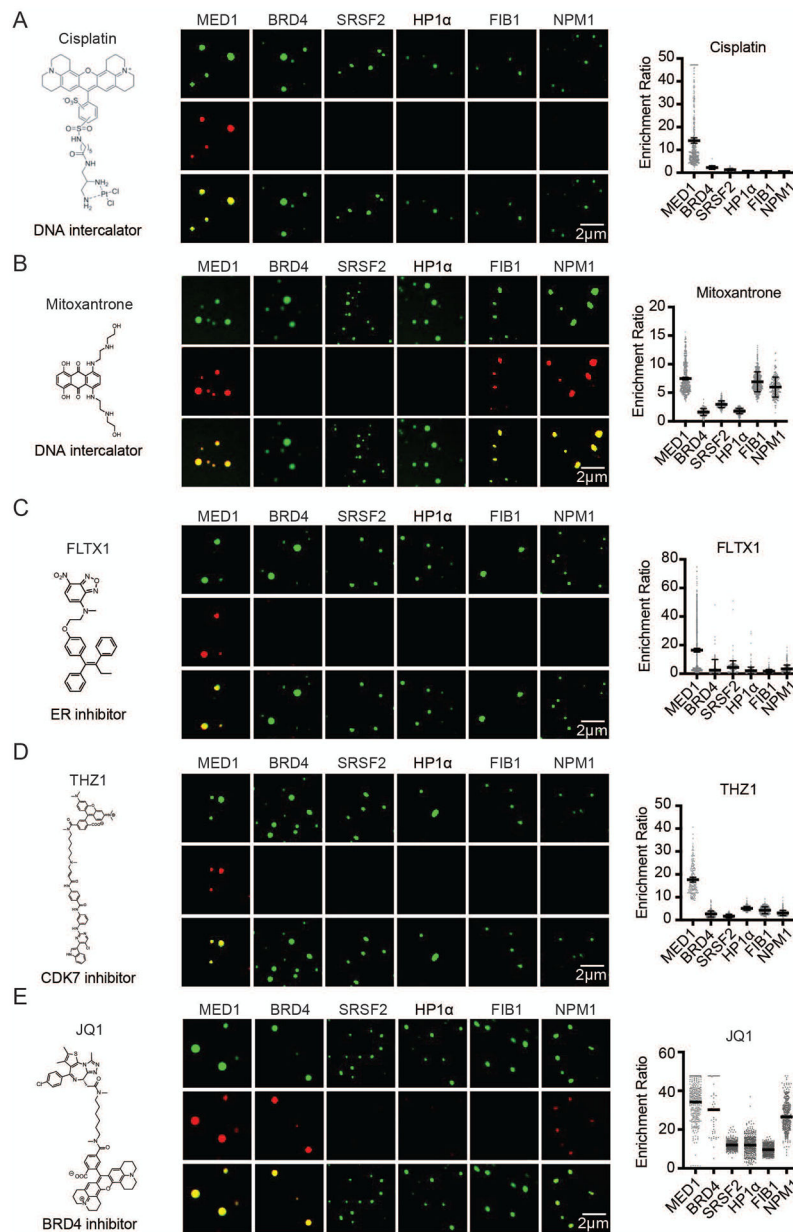Figures S3–S6). Quantification of enrichment of the drug is shown to the right, error bars represent SEM.

Fig. 2.
The partitioning behavior of small molecule drugs in nuclear condensates in a droplet assay. Six nuclear condensates formed in 125mM NaCl and 10% PEG, with 10μM protein treated with either (A) 5μM Cisplatin-TMR, (B) 50 μM Mitoxantrone, (C) 100μM FLTX1, (D) 5μM THZ1-TMR, or (D) 1μM JQ1-ROX imaged at 150× on a confocal fluorescent microscope (see also Figures S7–S11). Quantification of enrichment of the drug within droplets is shown to the right of each panel, error bars represent SEM (see also S12–S14).
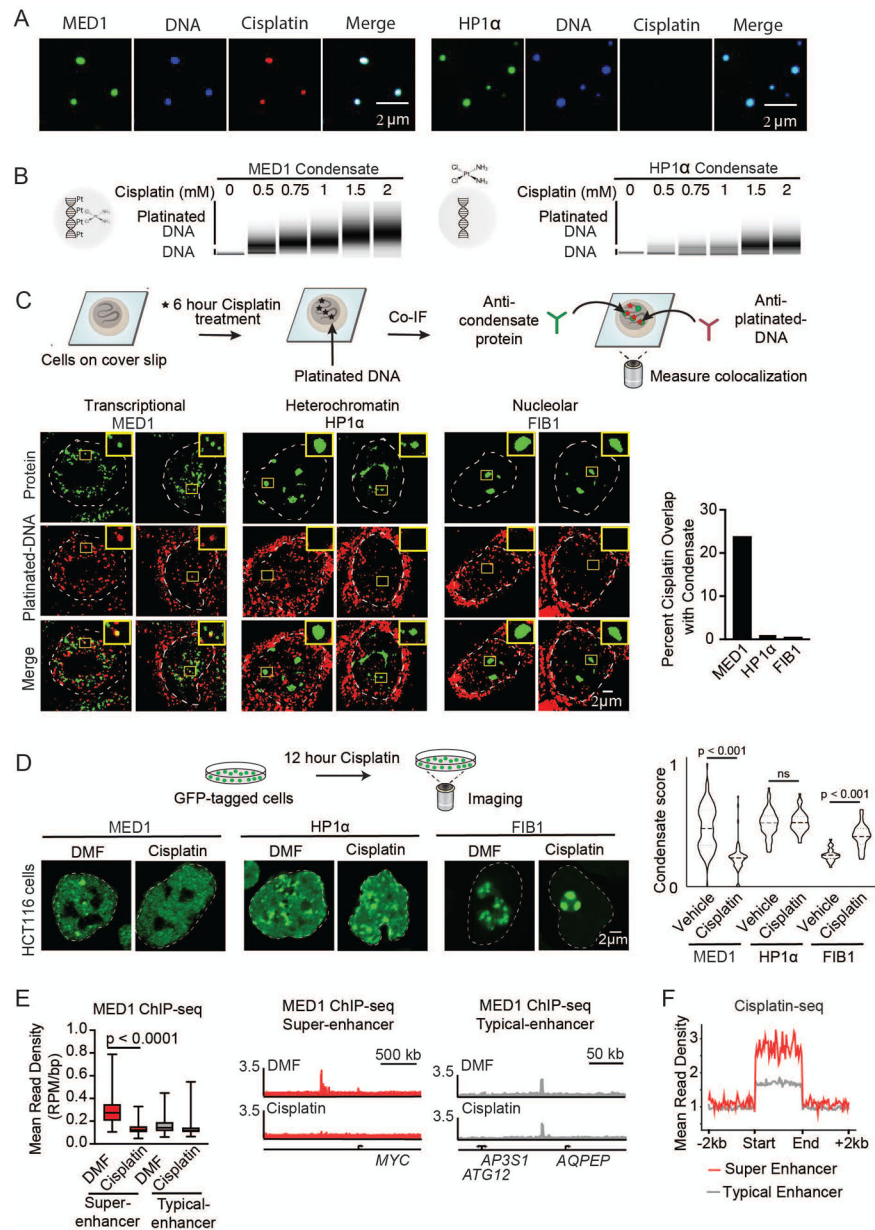
Fig. 3.

Small molecule concentration within condensates influences drug activity. (A) In vitro droplet assay of MED1 and HP1α condensates formed in 125mM NaCl and 10% PEG, 5nM of 450bp DNA, 10μM MED1, and 5μM cisplatin-TR, imaged at 150× on a confocal fluorescent microscope (see also Figure S15). (B) Bioanalyzer tracings of DNA contained within either MED1 or HP1α droplets exposed to the indicated concentration of cisplatin. (C) (Top) Schematic of an assay to determine the location of platinated DNA relative to various nuclear condensates. (Bottom) Co-immunofluorescence of platinated DNA and the indicated protein in HCT116 cells treated with 50μM cisplatin for 6 hours. Imaged at 100× on a confocal fluorescent microscope. Quantification of overlap shown to the right. (D) (Top) Schematic of a live cell condensate dissolution assay. (Bottom) HCT116 cells bearing

endogenously mEGFP-tagged MED1, HP1α, or FIB1 treated with 50μM cisplatin for 12 hours. Quantification of MED1, HP1α, or FIB1 condensate score is shown to the right. (E) MED1 ChIP-seq in HCT116 cells treated with vehicle or 50μM cisplatin for 6 hours. (Left) Plotted are mean read density of MED1 at super-enhancers and typical-enhancers (error bars show min and max) and (Right) gene tracks of MED1 ChIP-Seq at the *MYC* super-enhancer and AQPEP typical-enhancer. (F) Metaplot of cisplatin-DNA-Seq in cisplatin treated Hela cells comparing super-enhancers and typical enhancers (41) (see also Figures S16–S21).
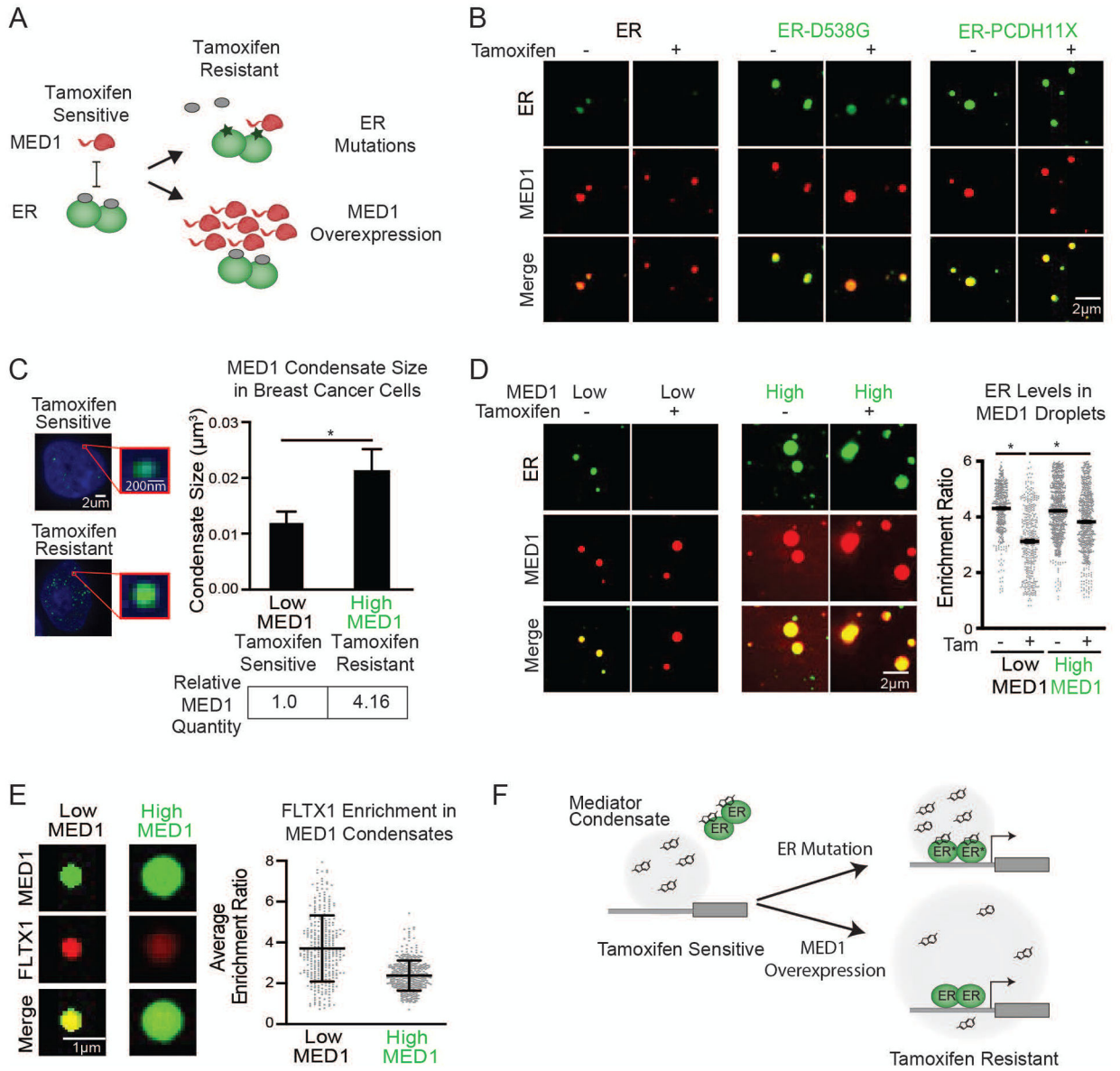
Fig. 4.

Tamoxifen action and resistance in MED1 condensates. (A) Schematic showing tamoxifen resistance by ER mutation and MED1 overexpression in breast cancer. (B) In vitro droplets assay of the indicated form of GFP-tagged ER in the presence of estrogen, $+/- 100\mu$M tamoxifen. Droplets are formed in 125mM NaCl and 10% PEG with 10μM each protein and 100μM estrogen. (C) (Left) Immunofluorescence of MED1 in tamoxifen sensitive (MCF7) and resistant (TAMR7) ER+ breast cancer cell lines imaged at 100× on a confocal fluorescent microscope. (Top right) Quantification of MED1 condensate size in breast cancer cells. (Bottom right) Relative quantities of MED1 in the indicated breast cancer cell line by western blot, error bars show SEM. (D) In vitro droplets assays of ER in the presence of 100μM estrogen, $+/- 100\mu$M tamoxifen with either 5μM (Low) or 20μM (High) MED1. Droplets are formed with 5μM ER in 125mM NaCl and 10% PEG, imaged at 150× on a confocal fluorescent microscope, error bars are SEM. (E) In vitro droplet assay with either

5μM (Low) or 20μM (High) MED1 with 100μM FLTX1 in 125mM NaCl and 10% PEG, error bars are SD. (F) Models for tamoxifen resistance due to altered drug affinity (via ER mutation) or concentration (via MED1 overexpression) (see also Figures S22–S30).