Phase-separated condensate-aided enrichment of biomolecular interactions for high-throughput drug screening in test tubes

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Modification-dependent and -independent biomolecular interactions, including protein–protein, protein–DNA/RNA, protein–sugar, and protein–lipid interactions, play crucial roles in all cellular processes. Dysregulation of these biomolecular interactions or malfunction of the associated enzymes results in various diseases; therefore, these interactions and enzymes are attractive targets for therapies. High-throughput screening can greatly facilitate the discovery of drugs for these targets. Here, we describe a biomolecular interaction detection method, called phase-separated condensate-aided enrichment of biomolecular interactions in test tubes (CEBIT). The readout of CEBIT is the selective recruitment of biomolecules into phase-separated condensates harboring their cognate binding partners. We tailored CEBIT to detect various biomolecular interactions and activities of biomolecule-modifying enzymes. Using CEBIT-based high-throughput screening assays, we identified known inhibitors of the p53/MDM2 (MDM2) interaction and of the histone methyltransferase, suppressor of variegation 3-9 homolog 1 (SUV39H1), from a compound library. CEBIT is simple and versatile, and is likely to become a powerful tool for drug discovery and basic biomedical research.

Complex networks of modification-dependent and -independent biomolecular interactions lay the foundation of virtually all cellular processes including DNA replication, transcription, protein translation, and signal transduction. Dysregulation of biomolecular interactions causes many diseases, such as neurodegenerative diseases, infection, and cancers (1, 2). Hence, aberrant biomolecular interactions and the associated modifying enzymes, if any, are attractive targets for therapeutic drug discovery (3–5).

High-throughput screening provides a powerful strategy for the discovery of drugs that modulate various biomolecular interactions (6). Many technologies are widely used for high-throughput screening, such as surface plasmon resonance, fluorescence polarization, FRET, and bioluminescence resonance energy transfer. These technologies all have major limitations. Many factors can interfere with the outcomes of surface plasmon resonance, such as ionic strength, DMSO content, charges of immobilized proteins, and nonspecific binding to the biosensor (7–10). Fluorescence polarization requires not only a big difference in molecular mass between the fluorescent probe and the target protein, but also high-affinity probes to achieve a large signal window (9, 11). These drawbacks largely limit its application. FRET and bioluminescence resonance energy transfer have narrow signal windows and high backgrounds because of the limited energy transfer distance (<10 nm) (9, 11, 12). It is vital to develop more robust and easily implementable high-throughput screening technologies for efficient drug discovery.

Cells use numerous membrane-enclosed and membraneless organelles to compartmentalize biochemical reactions. Membraneless organelles, such as P granules (13), nucleoli (14), stress granules (15), and post-synaptic densities (16), are collectively referred to as biomolecular condensates. These condensates are assembled via phase separation driven by multivalent interactions (17, 18). Generally, the constituents of various condensates are divided into two groups: one group is composed of scaffolds, which are essential for the assembly of these condensates and the other group is composed of clients, which are dispensable for the formation of the condensates but can partition into these condensates via direct or indirect binding to the scaffolds (19). Inspired by the scaffold-client model, we explored the possibility of using a similar architecture to implement a system for assaying modification-dependent and -independent biomolecular interactions, which can be used for efficient discovery of drugs targeting these biomolecular interactions and the associated enzymes.

Our system consists of two parts: 1) the scaffold, which drives the formation of phase-separated condensates, and 2) the biomolecular interaction of interest. One interacting partner is fused with the scaffold to generate a composite scaffold and hence is automatically enriched in the condensates, and the other partner (defined as the client) is recruited to the condensates via specific interactions. For visualization, the composite scaffold and the client are labeled with different fluorescent probes such as GFP and mCherry. Thus, the biomolecular interaction of interest is assessed by the enrichment of the mCherry-fused client within the GFP-labeled phase-separated condensates. This strategy is called phase-separated condensate-aided enrichment of biomolecular interactions in test tubes (CEBIT). CEBIT can also be tailored to measure the activities of enzymes modifying biomolecules.

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We extensively validated CEBIT using some representative protein–protein interaction pairs. Subsequently, we tailored CEBIT to probe molecular modifications and the associated enzymes. CEBIT is very amenable for high-throughput screening. Using the MDM2/p53 interaction and the SUV39H1-mediated histone 3 lysine 9 methylation reaction as two test cases, we successfully identified inhibitors of the MDM2/p53 interaction and of SUV39H1 from a commercial compound library. CEBIT is a simple and versatile method, which will have many applications in basic and translational research.

Results

Establishing robust multivalent scaffolds

First, we sought to establish some multivalent scaffolds that can robustly drive the formation of condensates via phase separation. Numerous studies have demonstrated that interactions between linear and/or dendrimeric (branched) multivalent modular proteins can result in phase separation (18–22). The Saccharomyces cerevisiae protein SmF is known to form a stable tetradecameric (referred to as 14-meric for simplicity hereafter) complex upon expression alone in bacteria (23). We tested whether it was possible to reliably achieve dendrimeric multivalence of various domains/motifs when they were fused to SmF. We created two fusion proteins, one with GFP fused to the C terminus of SmF (SmF-GFP) and the other with the second SH3 domain of human NCK1 fused to the C terminus of SmF-GFP (SmF-GFP-SH3). Size-exclusion chromatography coupled with multiangle light scattering (SEC-MALS) analysis indicated that SmF-GFP also formed a 14-meric complex in solution, and further fusion of a SH3 domain to SmF-GFP did not alter the 14-meric status (Fig. 1a). Therefore, we concluded that SmF is a robust 14-meric protein for multimerizing protein domains/motifs.

Similarly, we investigated the Bacillus subtilis protein Hfq (Bshfq), which is known to form a stable hexameric complex (24). We confirmed that Bshfq could reliably achieve dendrimeric multivalence of fused domains/motifs (Fig. S1).

Multimerized protein–protein interaction pairs undergo phase separation

Next, we investigated whether multimerized protein–protein interaction pairs, created by fusion to SmF, could mediate phase separation. We selected three model interaction pairs: 1) the second SH3 domain of human NCK1 and the proline-rich motif (abbreviated to PRM) of DLGAP2 (18), 2) the third PDZ domain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25); and 3) SUMO3 and the SUMO3 interdomain of human PSD95 and a synthetic PDZ ligand, KKETPV (abbreviated to PV) (25). The resulting proteins are abbreviated as (g/mDOMAIN/MOTIF NAME)14 for simplicity, in which g/m stands for GFP/mCherry and the subscript 14 represents the 14-meric status of the protein complexes. We carried out cross-mixing reactions between (gSH3)14 (gPDZ)14, or (gSUMO3)14 and (mPRM)14, (mPV)14, or (mSIM)14. All three cognate multivalent protein–protein interaction pairs, but not the noninteracting pairs, gave rise to marked phase separation (Fig. 1b). We next carried out phase-separation assays of (gSH3)14 and (mPRM)14 at 1:1 molar ratio over a large module concentration range, 100 nm to 10 μM. Numerous droplets formed at module concentrations as low as 100 nm (Fig. 1c). We further fused all binary combinations of the three modules, SH3, PDZ, and SUMO3, to the C terminus of SmF-GFP to yield six composite scaffolds, in which two different interacting domains are present in two different orders (Fig. 1d). Cross-mixing the six proteins with (mPRM)14, (mPV)14, or (mSIM)14 showed that phase separation occurs as long as and only if a cognate multivalent protein–protein interaction pair is present (Fig. 1d). These results suggest that the presence of a noncognate binding module does not appreciably affect the ability of multivalent cognate protein–protein interaction pairs to drive phase separation. Collectively, these data further revealed the robustness of SmF as a scaffold to multimerize protein–protein interaction pairs for inducing phase separation.

Recruitment of clients into phase-separated condensates via specific biomolecular interactions

As revealed by Banani et al. (19) clients are recruited into scaffold-induced condensates by interacting with free binding sites on the scaffolds. We wondered whether this principle could be used to study biomolecular interactions of interest. To test this, we used (gSH3-PDZ)14 (abbreviation for SmF-GFP-SH3-PDZ, Fig. 2a) and (gPRM)14 to drive phase separation, presumably via the multivalent SH3/PRM interaction, and we simultaneously used the mCherry-fused PDZ ligand, KKETPV (abbreviated to mPV, Fig. 2a), as a client to assess the interaction between PDZ and KKETPV (Fig. 2a). (gSH3-PDZ)14 and (gPRM)14 formed green condensates (Fig. 2a). (gSH3-PDZ)14 and (gPRM)14 formed green condensates (Fig. 2b, lower panels). mPV was recruited into these droplets, as shown by the enriched mCherry signal (Fig. 2b, upper left panel). To test whether the recruitment is reversible, we used a peptide, KKE-TAV, which has a higher affinity for PDZ than KKETPV (25), to compete with mPV. The enrichment of mPV was gradually reduced by increasing concentrations of the competing peptide (Fig. 2b, red), which was confirmed by quantification of the mCherry signal in the green droplets (Fig. 2c). These results indicated that mPV was recruited into the condensates via binding to PDZ.

FKBP/FRB is a well-characterized high-affinity interaction that is mediated by rapamycin (26). We wondered whether our system can assay similar interactions. We generated a composite scaffold (gSH3-FKBP)14 (abbreviation for SmF-GFP-SH3-FKBP) and the corresponding client mFRB (abbreviation for mCherry-FRB) (Fig. 2d). (gSH3-FKBP)14 readily underwent phase separation with (gPRM)14; Additionally, in the presence of increasing concentrations of rapamycin, but not the negative control ampicillin, recruitment of mFRB into the green droplets increased in parallel, as indicated by the enhanced mCherry signal (Fig. 2, e and f).

Subsequently we focused on two well-known cancer-related targets, MDM2 and XIAP. MDM2, an endogenous inhibitor of the tumor suppressor protein p53, binds to the N-terminal transactivation domain of p53 and mediates its degradation (27, 28). XIAP is a suppressor of the proapoptotic protein caspase-9. The suppression is alleviated by the binding of Smac to

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(a) SmF-GFP and SmF-GFP-SH3

(b) (gSH3)4, (mPRM)4, (mPV)4, (mSIM)4

(c) (gSH3-PDZ)4, (mPRM-PDZ)4, (gPDZ-SH3)4, (gSUMO3-PDZ)4

(d) (gSH3-PDZ)4, (gPDZ-SH3)4, (gSUMO3-PDZ)4, (gPDZ-SUMO3)4

(gSH3)4, (gPDZ)4, (gSUMO3)4

(gPRM)4, (gPV)4, (gSIM)4

Molecular weight (kDa)

Elution time (min)

559 ± 8 kDa

680 ± 12 kDa

18.0 18.1 18.2 18.3 18.4 18.5 18.6 18.7 18.8 18.9 19.0 19.1 19.2
the Bir3 domain of XIAP (29). Importantly, potent inhibitors of the MDM2/p53 interaction and the Smac/XIAP interaction have been developed (3, 30, 31). We chose the two interactions to assess the feasibility of using CEBIT for drug discovery. The MDM2-binding region of p53 was fused with (gSH3)14 to generate the composite scaffold (gSH3-p53)14 and MDM2 was labeled with mCherry (abbreviated to mMDM2) as the client (Fig. 2g). (gSH3-p53)14 readily underwent phase separation with (gPRM)14 (Fig. 2h, green channels). As the concentration of mMDM2 increased, its enrichment in the green condensates was significantly increased. This enrichment was significantly reduced by two potent MDM2 inhibitors, MI773 and RG7388 (Fig. 2, h and i). Similarly, the XIAP-binding region of Smac was fused to the N terminus of SmF-SH3 to generate (Smac-SH3)14 (abbreviated for Smac-SmF-SH3) as a composite scaffold, and the Bir3 domain of XIAP was tagged by SNAP as the client (Fig. S2a). Phase separation of (Smac-SH3)14 and (gPRM)14 formed green droplets, into which SNAP-surface 546-labeled SNAP-XIAP was recruited (Fig. S2b). This recruitment was blocked by the XIAP inhibitor GDC-0152 (Fig. S2, b and c). Taken together, these data demonstrate that CEBIT is competent for studying various protein–protein interactions by conjugating one member of the target pairs to a phase-separated scaffold and the other to a fluorescent tag as the client.

A high-throughput screening assay targeting the MDM2/p53 interaction

Next, we asked whether CEBIT can be used in high-throughput screening for drug discovery. We tested the effects of compounds in a commercial library on the MDM2/p53 interaction with the above established assay (Fig. 2, g–i). The library contains 2148 compounds, including five known inhibitors of MDM2/p53, Nutlin-3, Nutlin-3a, Nutlin-3b, YH239-EE, and MI773. The activity of each compound was determined by assessing the enrichment of mMDM2 in the p53-containing droplets. Most compounds did not inhibit the recruitment of mMDM2. However, all five known MDM2 inhibitors (Fig. 3a, 5 red dots) significantly reduced the mCherry signal and were the most effective inhibitors among all the compounds tested. Treatment with the five known inhibitors resulted in mCherry signals well over 4 S.D. below the mean, which indicates that the screening process is effective. In addition, we found another compound, VER (abbreviation of VER155008, Fig. 3a, green dot), which also attenuated mMDM2 enrichment, suggesting that it is a potential novel MDM2/p53 inhibitor. Fluorescence images confirmed that these inhibitors decreased the enrichment of mMDM2 in the phase-separated droplets (Fig. 3b).

The Z factor is a critical quality control parameter for high-throughput screening assays and it was 0.52 for our screen (Fig. 3a), which suggests that our assay is suitable for high-throughput screening (32). An improved Z value (Z’ = 0.72) was obtained when we evaluated the enrichment of MDM2–SNAP–labeled with SNAP-Surface 546 in the droplets with a microplate reader after removal of the surrounding bulk solution (Fig. S3).

Detailed dose-response analysis confirmed the inhibitory activities of these screening hits (Fig. 3c and Fig. S4). Next, a competitive fluorescence polarization (FP) assay using a high affinity fluorescent probe (33) was performed to confirm the inhibitory effect of the newly identified compound VER on the MDM2/p53 interaction. Two known MDM2 inhibitors, MI773 and Nutlin-3b, were used as controls. The control inhibitors caused an apparent decrease in polarization as their concentration increased, but VER failed to do so (Fig. 3d). The observation that CEBIT, but not fluorescence polarization, detected the inhibitory effect of VER on the MDM2/p53 interaction suggested that the former technique is more sensitive than the latter in this experimental setting. Collectively, these data revealed that CEBIT has the potential for drug discovery via high-throughput screening.

Tailoring CEBIT to probe biomolecular modifications

Regulated chemical modifications, including methylation, acetylation, phosphorylation, of various biomacromolecules, such as proteins, RNAs, and DNAs, are extremely important for all aspects of biology. Aberrant modifications and the associated enzymes are attractive therapeutic targets. However, efficient high-throughput screening assays that are generally applicable to the discovery of drugs modulating these enzymes are still lacking. These modifications can often be recognized by specific effector proteins via their reader domains. We postulated that CEBIT can be adopted for examining these modifications and hence the activities of the associated modifying enzymes. Our strategy is to confine specific reader domains in phase-separated condensates via fusion to the scaffold and use the modification-dependent recruitment of cognate substrates as the readout for the enzymatic activities of interest.

Here we used methylation of biomacromolecules (proteins, RNAs, and DNAs) as examples to test our idea. Protein lysine methylation plays important roles in various biological pathways. For example, methylation of histone H3 lysine 9 (H3K9me) is a hallmark of heterochromatin in cells. We wonder whether this modification can be detected with CEBIT. To this end, we fused the human H1P1β chromo domain (CD), a well-known reader domain of H3K9me (34–36), to (gSH3)14, resulting in a composite scaffold (gSH3-CD)14 (Fig. 4a). We also fused H3K9 peptides (carrying different levels of lysine methylation) with the synthetic PDZ ligand, KKETPV, to create H3K9-KKETPV (abbreviated to H3K9-PV). Our strategy was to create condensates via phase separation (gSH3-CD)14 and (gPRM)14, then to trace the partitioning...
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(a) Diagram showing the interaction of SH3, PRM, SnF, SH3-PDZ, and mPV.

(b) Graph showing the effect of KKETAV on mCherry intensity.

(c) Diagram illustrating the effect of KKETAV on mCherry intensity.

(d) Diagram showing the interaction of SH3, PRM, SnF, FKBP, mFRB, and rapamycin.

(e) Graph showing the effect of rapamycin on mCherry intensity.

(f) Graph showing the relative intensity of rapamycin as a function of concentration.

(g) Diagram showing the interaction of SH3, PRM, SnF, SH3-p53, and mMDM2.

(h) Graph showing the effect of mMDM2 on cell growth.

(i) Graph showing the relative intensity of DMSO, MITT3, and RG7388 as a function of mMDM2 concentration.
of H3K9-PV into the chromo-domain-containing droplets by the further recruitment of mCherry-labeled PDZ (Fig. 4a). We first used monomethylated H3K9-PV peptide (H3K9(me1)-PV) to test our design. As indicated, (gSH3-CD)14 and (gPRM)14 formed green droplets; however, a very weak mCherry signal was observed in the presence of monomeric mCherry-labeled PDZ, mPDZ (Fig. 4b). To enhance the signal, mPDZ was multimerized by fusing with the hexameric protein Bsh6q, resulting in (mPDZ)6 (Fig. S1 and Fig. 4a). Compared with mPDZ, (mPDZ)6 significantly enhanced the mCherry signal within the green droplets (Fig. 4b), which was confirmed by quantification (Fig. 4c). Hence, (mPDZ)6 was used for subsequent H3K9 methylation-related studies.

Next, we used mixtures of H3K9(me0)-PV and H3K9(me1)-PV with increasing ratios of the latter to roughly mimic the methylation reaction process. As the proportion of H3K9 (me1)-PV increased, the mCherry signal in the green droplets increased in parallel (Fig. 4, d and e). We next analyzed the effect of the methylation status of the H3K9-PV peptide (from me0 to me3) at various concentrations and found that higher levels of methylation resulted in stronger mCherry enrichment (Fig. 4, f and g), which was consistent with the binding preference of the HP1α chromo domain (37, 38).

We subsequently studied the methylation of DNA and RNA with CEBIT. The recognition of methylated CpG by the MBD domain of human MBD1 (which binds mC on DNA) and of N-m5A RNA by the YTH domain of human YTHDC1 was taken as examples. SmF is part of a RNA-protein complex involved in splicing. To avoid the nonspecific nucleic acid binding of SmF, we used another set of phase-separation scaffolds of linear topology developed by Rosen and co-workers (19), polySUMO/polySIM. The clients were ROX-labeled DNA or RNA oligonucleotides carrying two MBD- or YTH-recognition motifs, of which 0, 1, or 2 were methylated (designated DNA(0 me)RNA(0 me), DNA(1 me)RNA(1 me), and DNA(2 me)/RNA(2 me) (Fig. 5a)). Their corresponding recognition domains, MBD and YTH, were fused to GFP-labeled tandem pentameric SUMO3, g(SUMO3)5, as the composite scaffolds, which assembled biomolecular condensates with a hexameric SUMO3 interacting motif, SIM6 (Fig. 5a) via phase separation. We reasoned that the methylation status of DNA and RNA could be assessed by their partition in the MBD- or YTH-enriched condensates, respectively. g(SUMO3)5-MBD and SIM6 formed green droplets via multivalent SUMO3/SIM interaction. Both DNA(1 me) and DNA(2 me) were appreciably enriched within these droplets, and the latter was more strongly enriched than the former. This observation was further confirmed by quantification of ROX intensity in the droplets (Fig. 5b). Additionally, when mixtures of DNA(0 me) and DNA(2 me) with various ratios but at a constant total concentration were used, the enrichment of ROX signal in MBD-containing droplets was proportional to the ratio of DNA(2 me) (Fig. 5c). For RNA, identical experiments were performed and similar results were achieved (Fig. 5, d and e). Collectively, these results showed that CEBIT can effectively probe the methylation reactions of biomacromolecules.

**CEBIT-based high-throughput screening for SUV39H1 inhibitors**

SUV39H1 is a critical methyltransferase that catalyzes the transfer of methyl groups to H3K9 (39). We took SUV39H1 as an example to assess the capacity of CEBIT to screen for drugs that modulate various enzymes. The process of determining the activity of SUV39H1 consists of two steps. In the first step, the enzymatic reaction was carried out: the substrate peptide H3K9(me0)-PV was methylated with enzyme without or with different small molecules for a certain time before quenching. In the second step, the quenched reaction solutions were added to CD-enriched condensates as established in Fig. 4 for CEBIT-mediated detection (Fig. 6a). The substrate was H3K9(me0)-PV, and SUV39H1 activity was assessed by enrichment of mCherry signal of (mPDZ)6 in the droplets. Indeed, the mCherry signal was enhanced as the concentration of SUV39H1 increased in the *in vitro* methylation reaction (Fig. 6b). Prolonging the reaction time also increased the mCherry signal (Fig. 6c). Methylation of H3K9 (me0)-PV by SUV39H1 was largely abolished by a potent SUV39H1 inhibitor, chaetocin, and a feedback inhibitor for generic methyltransferase reactions, S-adenosine homocysteine (SAH). The IC50 values were 1.6 and 56 μM for chaetocin and SAH, respectively (Fig. 6, d and e).

We next explored the potential of CEBIT for the discovery of SUV39H1 inhibitors via high-throughput screening. We screened the Selleck-2148 compound library in which chaetocin was added as a positive control. Treatment with chaetocin resulted in the lowest mCherry signal in the phase-separated droplets.
condensates (Fig. 6, red dot, and g). A few compounds from the library also caused low mCherry signals (Fig. 6, f and g). We selected three of them, deltarasin, β-lapachone, and NH125 (Fig. 6f, green dots) for further testing. Their inhibition of SUV39H1 activity was assessed by detailed dose-response analysis in vitro and their IC50 values were within the single-digit micromolar range (Fig. 6h). These results demonstrated that CEBIT worked effectively for in vitro high-throughput screening-based discovery of drugs that modulate SUV39H1 activity. This suggests that CEBIT will be generally applicable to other methyltransferase enzymes as well as enzymes for other molecular modifications, such as phosphorylation, acetylation, and ubiquitination.

**Discussion**

In this study, we developed a method for assaying biomolecular interactions called CEBIT. CEBIT is amenable to determining diverse modification-dependent and -independent biomolecular interactions and associated enzymes.
in vitro, and hence discovering therapeutic drugs to modulate these interactions and enzymatic activities via high-throughput screening. CEBIT is distinct from existing methods for the detection of in vitro biomolecular interactions in two respects: 1) it is based on, and hence is analogous to, the general architecture of the prevalent membraneless organelles in cells; and 2) the actual readout is based on the recruitment of nanometer-sized molecules into micrometer-sized condensates.

Many membraneless organelles are assembled via phase separation (13–16) and the complex compositions of these assemblies are generally divided into two groups: scaffold and client (19). This architecture revealed two distinct strategies to study various biomolecular interactions. In principle, the interaction
pairs of interest can serve as either the scaffold or the client. In the former setup, both partners of the interaction are multimerized via fusion to homooligomeric proteins and the appearance of phase-separated puncta serves as a readout for the interaction (20, 22, 40). This strategy has been adopted by two recently published methods, Fluoppi (20) and SPARK (22). In the latter setup, the recruitment of a client into condensates enriched for its binding partner serves as a readout for the interaction, as revealed by our method, CEBIT. The former strategy has some limitations. Phase separation of multimerized interaction pairs is controlled by several factors, such as valence, concentration, stoichiometry, and the binding affinity between the interacting partners.
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1. Methylation
2. Detection
3. Imaging

H3K9-PV → H3K9(me)-PV → CEBiT based assay

**b**

SUV39H1 (nM) vs. Relative Intensity (%)

- 0, 12.5, 25, 50, 100, 200, 400 nM
- Graph showing relative intensity as a function of SUV39H1 concentration

- Images of cells with different SUV39H1 concentrations

**c**

Reaction Time (min) vs. Relative Intensity (%)

- 0, 10, 20, 30, 40, 50, 60 min
- Graph showing relative intensity as a function of reaction time

- Images of cells with different reaction times

**d**

Chaetocin (µM) vs. mCherry Intensity (a.u.)

- 0.47, 1.88, 7.5, 15 µM
- Graph showing mCherry intensity as a function of chaetocin concentration

- Images of cells with different chaetocin concentrations

**e**

SAH (mM) vs. mCherry Intensity (a.u.)

- Log[SAH] (µM) vs. mCherry Intensity (a.u.)
- Graph showing mCherry intensity as a function of SAH concentration

- Images of cells with different SAH concentrations

**f**

Compounds (#) vs. mCherry Intensity (a.u.)

- Graph showing mCherry intensity distribution across different compounds

- Images of cells treated with different compounds

**g**

DMSO, Chaetocin, Deltaarsin, Betla-Lapachone, NH125

Images of cells treated with different compounds
molecules of interest. If any of the factors is below the threshold for phase separation, no puncta can be observed. Therefore, this strategy is less efficient. In contrast, with CEBIT, the selection of scaffolds to drive the formation of phase-separated condensates is flexible, and hence robust ones are chosen. Additionally and importantly, multimerization cannot be easily achieved for many interaction components due to the insolubility of target proteins when fused into a homo-oligomeric protein. However, with CEBIT, we can choose to deploy the components of an interaction of interest in a monovalent fashion by using linear multivalent scaffolds (such as polySUMO-polySIM (19)). For at least these reasons, CEBIT is more reliable to study various biomolecular interactions.

Surface plasmon resonance, fluorescence polarization, and FRET are some extensively used technologies for high-throughput drug screening in vitro. Surface plasmon resonance is sensitive and versatile for detection of diverse biomolecular interactions. However, many factors can interfere with the outcomes of surface plasmon resonance. Our work suggested that CEBIT is robust for studying diverse interactions and tolerates the factors that interfere with surface plasmon resonance.

The readout of CEBIT is the enrichment of a fluorophore-labeled client into condensates enriched with its binding partner; thus, the readout does not rely on the difference in molecular mass between interaction pairs, which is required by fluorescence polarization. Compared with FRET, CEBIT exhibits a good signal window for studying diverse biomolecular interactions. It is, nevertheless, conceivable that fluorescence polarization, FRET, and bioluminescence resonance energy transfer can be incorporated in CEBIT as alternative readouts.

Many biomolecular interactions are weak. However, characterizing weak interactions is challenging, especially for high-throughput assays. With CEBIT, the signal can further be enhanced by increasing the valence of the client so that weak interactions are readily assessed.

There is a major drawback of CEBIT in that it relies heavily on high-content imaging for signal detection, which is time-consuming and costly. With this issue in mind, we subsequently developed a detection protocol utilizing microplate readers, which makes the detection efficiency of CEBIT comparable with other high-throughput screening methods and substantially improves the Z score. For example, in our screen targeting the MDM2/p53 interaction, the Z score increased from 0.52 using high-content imaging to 0.72 using the new protocol. Another limitation is that the proteins (including both scaffolds and clients) must be purified. Some multimeric proteins cannot be expressed and purified. In addition, CEBIT is less applicable to transmembrane proteins, which are difficult to purify. Taking these caveats into account, we developed another version of this method that works well in cells (41).

It is also conceivable that certain clients can be nonspecifically recruited to condensates and hence produce noise. For example, our SmF-based condensate has intrinsic nucleic acid-binding potential and hence we could not use it for assessing RNA and DNA methylation reactions. To circumvent this issue, we adopted phase-separated condensates derived from a system developed by Rosen and co-workers (19), which uses tandem SUMO3 and tandem SUMO3 interaction motifs. These condensates showed little nonspecific recruitment of nucleic acid.

Nevertheless, CEBIT is highly versatile and has great scope for adaptation and improvement as far as its applicability and efficiency are concerned. It is conceivable that by fusing streptavidin to the phase separation scaffolds, CEBIT can be used to detect any biomolecular interactions in which one component of the interaction pair can be biotinylated.1 Besides in vitro studies, this method can also be applied to study diverse biomolecular interactions in vivo (41) and identify regulators of these biomolecular interactions via high-throughput screening in cells. Furthermore, it may be possible to use CEBIT to analyze the interactions between membrane receptors and their ligands by creating membrane-attached receptor-enriched condensates. Other important applications will likely be developed.

Materials and methods

Reagents

All modified DNAs and RNAs were provided by Zixi Biotech Company (Beijing, China). Peptides used in this study were chemically synthesized by GL Biochem Ltd. (Shanghai, China). The fluorescent probe SNAP-Surface Alexa Fluor 546 (number S9132S) was from New England Biotech. The chemical library Selleck-2148 for high-throughput screening and compounds for the dose-response assay were provided by the drug facility of Tsinghua University. Other small molecules were purchased from Selleck.


Figure 6. Discovery of SUV39H1 inhibitors via CEBIT-based high-throughput screening. a, workflow of the CEBIT-based assay to determine the activity of methyltransferase SUV39H1. The substrate H3K9-PV was in vitro methylated by SUV39H1, the reaction solution was then subjected to the CEBIT-based assay established in Fig. 4a. Enrichment of methylated substrates in the chromo domain-containing condensates was measured by imaging after a period of incubation. b, in vitro enrichment of H3K9(me0)-PV peptide substrate (8 μM) by various amounts of SUV39H1 was conducted and the reaction products were subjected to the methylation assay system shown in Fig. 4a. Normalized mCherry signal in the phase-separated green droplets was plotted against SUV39H1 concentration (left panel). Representative fluorescence images are shown (right panel), c, in vitro methylation of H3K9(me0)-PV (8 μM) by SUV39H1 (0.4 μM) was conducted and then the reaction products were assessed. The time course of SUV39H1 activity was evaluated by plotting normalized mCherry signal against reaction time. Fluorescence images are presented. d and e, analysis of the in vitro methylation of H3K9(me0)-PV (8 μM) by SUV39H1 (0.4 μM) in the presence of various doses of the SUV39H1 inhibitors chaetocin (d) and SAH (e). The reaction products were assessed with the indicated assay, and mCherry intensity was plotted against inhibitor concentration. f and g, high-throughput screening for discovery of SUV39H1 inhibitors. f, for each compound in the library, enrichment of the SUV39H1-methylated peptide substrate, H3K9me-PV, in the phase-separated green droplets was assessed according to the intensity of the mCherry signal. Screening hits are indicated by colored dots. The red dot represents chaetocin (#1) and the three green dots represent deltarasin (#1578), β-lapachone (#1661), and NH125 (#1803). g, fluorescence images to show the inhibitory effect of these compounds on SUV39H1. h, dose-response analysis of screening hits to confirm their inhibition on SUV39H1 activity. mCherry signal intensities in phase-separated condensates were evaluated. All scale bars, 10 μm.

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Plasmid construction

All plasmids used in this study were constructed following standard molecular cloning techniques. The genes of interest were amplified by PCR and subsequently inserted into the expression vectors. Insertion of these genes was confirmed via sequencing by Ruibo Biotech Company (Beijing, China). All the SmF-derived composite scaffold proteins were created by modifying SmF-EGFP or SmF-mCherry, which were generated by sequentially inserting SmF and EGFP or mCherry into pRSF-Duet1 (Invitrogen) with an N-terminal His6 tag, except (Smac-SH3)14, a PCR fragment of SmF-SH3 was created by sequentially inserting His6, MBP, and SUMO tag. To generate (Smac-SH3)14 as the composite scaffold, and the other was fused to SmF-EGFP/mCherry to create multimeric composite scaffold proteins such as SmF-EGFP-SH3 (abbreviated to (gSH3)14) and SmF-mCherry-PRM (abbreviated to (mPRM)14).

To generate (Smac-SH3)14 and (SH3-p53)14, two more backbone constructs were first generated in pRSF-Duet1: pHMS1 was created by sequentially inserting His6, MBP, SUMO tag (cleaved by ULP1 protease), and SmF; and pHMS2 was created by inserting His6, MBP, and SUMO-Smac. To construct (SH3-p53)14, a PCR fragment of SH3-p53 was ligated to pHMS1. To generate (Smac-SH3)14, a PCR fragment of SmF-SH3 was inserted into pHMS2.

In this study, (gSH3)14 and (gPRM)14 were the most extensively used scaffolds. To study protein–protein interactions of interest, one member of the interaction pair was fused to (gSH3)14 as the composite scaffold, and the other was fused with an N-terminal mCherry tag as the client. To multimerize mCherry-PDZ, a hexameric protein BsHfq was fused at the N terminus.

Another set of scaffold proteins used in this study, poly-SUMO and polySIM, were modified from constructs that were generous gifts from Professor MK Rosen (University of Texas, Southwestern, USA). PCR fragments of (SUMO3)5 and SIM6 were inserted into pRSF-Duet1 fused with an N-terminal His6 tag. GFP was further fused to the C terminus of (SUMO3)5, resulting in g(SUMO3)5. To detect nucleic acid methylation, the indicated DNA or RNA-binding domains (MBD for methylated DNA and YTH for methylated RNA) were fused to g(SUMO3)5 to generate the composite scaffold proteins.

A full-length cDNA encoding the methyltransferase SUV39H1 was amplified by PCR and fused with an N-terminal His6-MBP tag in pET-Duet1 (Invitrogen).

Protein expression and purification

Generally, recombinant proteins were expressed in Escherichia coli BL21 (DE3) cells in LB medium with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C for 12-24 h unless noted. The bacteria were lysed by sonication in buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulfonil fluoride), then proteins were purified using nickel-NTA-agarose beads (GE Healthcare, UK) followed by ion exchange chromatography and finally by size exclusion chromatography in KMEI buffer (150 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10 mM imidazole, pH 7.4, 1 mM Tris(2-carboxyethyl)phosphine, and 10% glycerol). Some proteins were not amenable to ion exchange, and therefore this purification step was omitted. For (SH3-p53)14 and (Smac-SH3)14, the purification was performed by sequentially using nickel-NTA-agarse beads, anion exchange, cleavage by ULP1, a tandem MBP-NTA column to remove the N-terminal tags, and finally size exclusion chromatography with buffer containing 150 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10 mM imidazole, pH 7.4, 1 mM Tris (2-carboxyethyl)phosphine, and 10% glycerol. All proteins were rapidly frozen with liquid nitrogen and stored at −80°C.

PolySUMO-derived proteins (including g(SUMO3)5-YTH/MBD) and SIM6 were individually expressed and affinity-purified with nickel-NTA beads followed by size exclusion chromatography in buffer containing 150 mM KCl, 1 mM MgCl2, 1 mM EGTA, 20 mM HEPES, pH 7.0, 1 mM DTT (the pH is critical for the phase separation of polySUMO and polySIM as revealed by the work of Banani et al.). To achieve active enzyme, SUV39H1 and HP1β were co-expressed in Escherichia coli BL21 (DE3) cells and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C for 16 h. The complex of SUV39H1 and HP1β was affinity-purified with nickel-NTA beads followed by size exclusion chromatography as described above.

SEC-MALS

The analysis was performed as previously described (16). The protein samples were centrifuged at 12,000 × g for 10 min to remove any protein precipitation and then loaded into a gel filtration column (Superdex 200 Increase 10/300GL, GE Healthcare) coupled with an 18 angle light scattering detector (DAWN HELEOS II, Wyatt) and an Optilab DSP interferometric refractometer (Wyatt) in buffer composed of 150 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10 mM imidazole, pH 7.4. Data were analyzed by ASRTA (Wyatt).

Client recruitment and inhibition assays

SmF-derived scaffold proteins, client proteins, and inhibitors were mixed together at the desired concentrations in buffer KMEI (150 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10 mM imidazole, pH 7.4) in a total volume of 20 μl and incubated at 4 °C for at least 2 h to allow the phase-separated droplets to gradually sink down to the bottom of the wells in 384-well microplates. Images for each sample were then automatically acquired at ×63 or ×40 water-immersion objective using identical parameters including laser power, detector gain, etc. with a high-content confocal microscope (Opera Phenix, PerkinElmer Life Sciences, USA). At least five images per well were collected with at least two experimental replicates. Images were analyzed using the manufacturer-provided software Harmony4.8. The region of phase-separated droplets driven by GFP-labeled scaffolds were defined using GFP signal, and subsequently mCherry intensities in the GFP-positive regions were quantified.

Images in Figs. 1 and 2 were taken with a ×100 oil-immersion objective using a confocal laser scanning microscope (Nikon A1RSi, Japan).
Condensates-aided high-throughput drug screening

PolySUMO- and polySIM-based partitioning assay

PolySUMO- and polySIM-based partitioning assays were conducted in buffer containing 20 mM HEPES, pH 7.0, 150 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT. The phase-separated scaffolds and recruited clients were mixed thoroughly followed by incubation at 4 °C for more than 2 h. Images were taken and analyzed by high-content microscopy.

Partitioning assay of SNAP-tagged proteins

A mixture of 1 μM (Smac-SH3)₁₄, 1 μM (gPRM)₁₄, 2 μM XIAP-SNAP, and 1 μM SNAP-Surface 546 fluorophore was incubated with XIAP inhibitor GDC-0152 at the indicated concentration in KMEI buffer supplemented with 2% DMSO overnight at 4 °C for efficient labeling of XIAP-SNAP. Enrichment of XIAP-SNAP in phase-separated green droplets was assessed by imaging and evaluation of the SNAP-Surface 546 signal.

Similarly, 1 μM (SH3-p53)₁₄, 1 μM (gPRM)₁₄, 2 μM MDM2-SNAP, and 1 μM SNAP-Surface 546 fluorophore were incubated with 5 μM MI773 in KMEI buffer supplemented with 2% DMSO overnight at 4 °C. As expected, (SH3-p53)₁₄ and (gPRM)₁₄ formed green droplets via phase separation, into which MDM2-SNAP was recruited. Recruitment of MDM2-SNAP was inhibited by MI773. The phase-separated green droplets were isolated by cautious removal of the surrounding solution after centrifugation. Enrichment of fluorescently labeled MDM2-SNAP in the droplets was assessed by microplate reader via measurement of SNAP-Surface 546 intensity. The Z factor was calculated using the fluorescence intensity of samples treated with DMSO (0% inhibition) as the negative control and samples treated with 5 μM MI773 (100% inhibition) as the positive control.

Competitive FP assay

The FP assay was performed as described by Czarna et al. (33). The probe was a p53-derived synthetic peptide P4 modified with FITC. The interacting protein was recombinant human MDM2 (residues 10-110) with an N-terminal His₆ tag. The FP assay was performed with 5 nM FTIC-P4 and 80 nM His-MDM2 in the presence of different concentrations of MI773, Nutlin-3b, and VER. All assay components were mixed thoroughly in buffer (40 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 5% DMSO) with a total volume of 10 μl. The FP value was measured by using a versatile microplate reader (EVI-SION, PerkinElmer, USA) after 30 min incubation at room temperature.

In vitro methylation and detection of H3K9 peptide

The synthetic peptide substrate H3K9(me0)-PV (8 μM) was in vitro-methylated by SUV39H1-HP1β complex in the presence of 20 μM SAM. The reaction was conducted in buffer containing 50 mM Tris, pH 8.4, 5 mM MgCl₂, 4 mM DTT by incubation at room temperature for 2 h and ended by heating at 95 °C for 10 min to inactivate the enzyme. Subsequently, 5 μl of the reaction product was analyzed in the CEBIT system consisting of 1 μM (gSH3-CD)₁₄, 1 μM (gPRM)₁₄, 1 μM (mPD2)₆, in a final volume of 20 μl in KMEI. After incubation for 2 h at 4 °C, images were taken by high-content microscopy. mCherry signal in the phase-separated droplets was analyzed. To determine the time course of enzyme activity, the reaction was stopped by quick-freezing with liquid nitrogen followed by heating at 95 °C for 10 min to inactivate the enzyme. Then the methylation of H3K9(me0)-PV peptide was assessed as indicated.

High-throughput screening

The assay system for screening compounds that disrupt the p53/MDM2 interaction was comprised of 0.5 μM (gSH3-p53)₁₄, 0.5 μM (gPRM)₁₄, and 1 μM mMDM2 in KMEI buffer supplemented with 2% DMSO in a total volume of 20 μl in a 384-well microplate (Greiner Bio-One, catalog number 781090). High-throughput screening was performed using compounds in a commercial library (Selleck-2148). This library, containing 2148 small molecules, was supposed to include 6 reported MDM2 inhibitors (Nutlin-3, Nutlin-3a, Nutlin-3b, YH239-EE, MI773, and RG7388). However, the solution corresponding to RG7388 in the stock library failed to inhibit the p53/MDM2 interaction after repeated trials, whereas separately purchased RG7388 potently inhibited p53/MDM2 using CEBIT. We concluded that RG7388 in the library was mislabeled, and 5 known MDM2 inhibitors (Nutlin-3, Nutlin-3a, Nutlin-3b, YH239-EE, and MI773) actually existed in the library. Each compound was used at a concentration of 20 μM. After thoroughly mixing all the components (scaffolds, client proteins, and compounds), the microplates were incubated at 4 °C overnight before imaging and data collection.

In the screen for inhibitors of the methyltransferase SUV39H1, compounds in the Selleck-2148 library were used at a final concentration of 30 μM. Each compound was added to an in vitro methylation reaction comprising 8 μM H3K9(me0)-PV, 0.4 μM SUV39H1, and 20 μM SAM. The mixture was incubated at room temperature for 2 h before the reaction was stopped by heating at 60 °C for 30 min. Subsequently, 5 μl of product from the reaction was assessed using the indicated methylation assay system. After incubation at 4 °C for 2 h, images were taken by high content microscopy and data were collected.

Statistical analysis

Statistical analysis was carried out on GraphPad version 7.0 (La Jolla, CA, USA) software. The multiple t test (two-tailed) and one-way ANOVA were employed to assess the difference between groups. The statistical significance was defined as follows: *, p < 0.05; **, 0.01 < p < 0.005; ***, 0.001 < p < 0.001; and ****, p < 0.0001.

Curve fitting

Curve fitting was done using GraphPad Prism version 7.0. The curve fitting in Figs. 2f and 6c was done using a linear regression model. For the curve fitting in Figs. 3, and 6, d, e, and h, the “inhibitor versus response” variable slope (four parameters) logistic equation: y = B + (T-B)/(1 + ((X - H)/(IC₅₀ ^ H))), in which H stands for Hill coefficient, T and B are the top and bottom plateaus in the units of y axis, respectively, was used to
obtain IC50 values with respective 95% confidence intervals and dose-response curves. For curve fitting in Figs. 4, 5, and 6b, the “agonist versus-response variable slope (four parameters) logistic equation”: 

\[ y = B + (X \times H) \times \frac{(T - B)}{(X \times H + EC_{50} \times H)} \]

in which H stands for Hill coefficient, T and B are the top and bottom plateaus in the units of y axis, respectively, was used.

Data availability

All data and materials in this study are available. Pilong Li, pilongli@mail.tsinghua.edu.cn.

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Conflict of interest—The authors declare that they have no competing interests.

Abbreviations—The abbreviations used are: CEBIT, condensate-aided high-throughput drug screening; Condensates-aided high-throughput drug screening.

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Condensates-aided high-throughput drug screening

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