Selection of DNA-encoded Chemical Libraries for Compounds that can Induce Protein Ubiquitination

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Abstract: We report a selection method of DNA-encoded libraries (DELs) that can identify the compounds able to induce ubiquitination of the protein of intertest (POI). Since the selection readout of the method is based on POI ubiquitination, rather than POI binding or ternary complex formation, the identified compounds are more functionally relevant and more predicative of active protein degraders. In this study, by selecting a DEL of 950 different combinations of POI ligands, linkers, and E3 ligase ligands against the BD1 domain of bromodomain-containing protein 4 (BRD4-BD1) in the presence and absence of ATP, we have identified a potent BRD4-BD1 degrader (DC_{50}: ~9.7 nM) and also a short-isoform-selective BRD4 degrader (DC_{50}: 0.26 μM). Furthermore, we show that the selection based on POI binding or ternary complex formation identified compounds that may have induced stable complexes but are inactive degraders. This approach may be an efficient and broadly applicable method for discovering functional protein degraders, as well as E3 ligase ligands, by harnessing the vast chemical diversity of DELs.

Introduction: Target protein degradation has emerged to become a novel therapeutic modality in drug discovery.[1-3] PROteolysis TArgeting Chimera (PROTAC) exploits the cellular Ubiquitin-Proteasome System (UPS) through a chimeric molecule consisting of a POI ligand (POI-L) connected with an E3 ligase ligand (E3L) via a linker motif. PROTACs function by simultaneously binding to the POI and the E3 ligase complex to induce ubiquitination of the POI and thereby its subsequent degradation by the proteosome.[2, 4, 5] The distinctive "event-driven" mechanism of PROTACs establishes a potentially revolutionary therapeutic paradigm, demonstrating efficacy against many of the traditionally deemed "undruggable" targets.[2, 6] The catalytic nature of PROTACs also provides pharmacological advantages such as sub-stoichiometric dosing and differentiated pharmacokinetic/pharmacodynamic drug profiles.[7] The development of PROTACs has advanced from academia to industry, and around a dozen PROTAC compounds have been developed into clinical trials in the past decade.[2, 8]

To date, degrader development has primarily relied on the screening of different combinations of E3 ligands, linkers, and POI ligands, whereas productive pairing between an E3 ligase and its neo-substrates enabled by PROTACs are largely unpredictable. Traditional metrics such as target

engagement by a parent ligand or degrader do not correlate well with target degradation.[9, 10]

The human proteome contains more than 600 E3 ligases; however, only ~2% of them have been recruited for target protein degradation.[1, 11, 12] Identifying functional E3 ligase ligands is inherently challenging, arguably due to the limited availability of binding sites on an E3 ligase that are conducive to effective polyubiquitination of the POI. Consequently, the prospect of yielding productive ubiquitination diminishes when a protein substrate binds to an arbitrary site on an E3 ligase.[13] The optimal binding site needs to not only accommodate the interactions with the protein substrates but also to provide adequate spatial dimensions to facilitate processive ubiquitin (Ub) chain elongation on the POI.[13] Previously, rapid synthesis methods,[4, 14-19] screening platforms,[20] biochemical and cell-based assays,[21, 22] and computational tools[23-26] have been developed to accelerate PROTAC discovery and development. Many techniques have also been reported for the discovery and validation of E3 ligases and their ligands. The elegant work by the groups of Cravatt, Nomura, Gray, and others showed that electrophilic screening platform is an efficient way to identify novel covalent E3Ls and E3 ligases.[27-33] The Queisser group reported a novel phenotypic screening approach for identifying E3 ligase binders.[20] Recently, Verdine, McGee, and co-workers reported a phage-display-based screening method for discovering helicons[34] that bind to and modulate E3 ligases across all four major families.[35]

DNA-encoded chemical libraries (DELs or DECLs) have emerged to become as an important tool in drug discovery.[36-38] In DELs, the spatial encoding in traditional chemical libraries is replaced with DNA encoding, thereby allowing the entire library to be synthesized, processed, and selected against the target simultaneously at a minute scale. The selected binders are decoded by PCR amplification and the next-generation sequencing (NGS) to read the DNA barcodes. Because of the high encoding capacity and the amplifiability of DNA molecules, DELs can contain hundreds of millions to many billions of compounds in microliter volumes. In principle, DEL and PROTAC are highly complementary.[37, 38] First, DEL selection is a binding assay; the identified binders may not be functionally active but could be useful in PROTACs. Second, the DNA-tagging site of DEL compounds provides a clearly defined exiting vector for linker attachment. Third, DEL selections identify many micromolar binders, which may not be potent enough to elicit biological responses but are useful for PROTAC develoment.[37, 38] Several DEL platforms designed for discovering potential PROTACs and other types of degraders have been reported.[39-42] DEL selections can identify POI-Ls to be further linked with E3Ls; conversely, E3 ligases may be interrogated with DELs to discover E3Ls.[43, 44] Chen, *et al.* explored DEL selections against the POI and E3 Ligase simultaneously to identify chimeric compounds that can stabilize the POI-ligand-E3 ternary complex.[40] Recently, Schreiber, Liu, Zécri, and co-workers reported an elegant method that can select DELs built on the ligands of the VHL (Von Hippel–Lindau) protein, a widely used E3 ligase in PROTACs,[12, 45] against the POI with and without VHL to discover chemical inducers of proximity (CIPs) of the two preselected proteins.[41] Using the similar concept, Schreiber, Liu, and co-workers also explored the ternary/binary enrichment ratio ("presenter ratio"), as a predictive measure of cooperativity and identified a CIP exhibiting cooperativity comparable to classical molecular glues.[42] All these studies exploited the formation of stable POI-ligand-E3 ligase ternary complexes for the signal readout of compound enrichment; however, studies also showed that productive degraders exhibited diverse complex properties.[46-48] A stable ternary complex may not be sufficient for POI degradation[49-52] For instance, stabilization of a ternary complex that disfavors proper lysine recognition or ubiquitin transfer could inhibit degradation.[52] Thus, the formation of a stable ternary

complex may not be a consistent predictor of protein ubiquitination and degradation.[52, 53]

Herein, we report a "functional" DEL selection method for PROTAC discovery (PROTAC-DEL). Enabled by ubiquitination-based affinity pulldown, the selection depends on not only ternary complex formation, but also POI ubiquitination, which is the key feature of proteosome-mediated protein degradation. This method may also be used to discover new ligands to expand the chemical toolbox targeting E3 ligases.[2] We first conducted methodology development with model systems. Next, a PROTAC-DEL of 950 different "E3L-linker-POI-L" combinations was prepared and selected against the BD1 domain of bromodomain-containing protein 4 (BRD4-BD1), along with E1, E2 and the VHL complex, in the presence and absence of ATP. Several potent PROTACs have been identified and validated, including a potent BRD4 degrader (DC_{50}: 9.7 nM) and a short-isoform-selective BRD4 degrader (DC_{50}: 0.26 μM). In addition, we show that this approach may avoid identifying the compounds that are inactive in POI degradation but can be enriched by affinity pulldown based on stable complex formation.

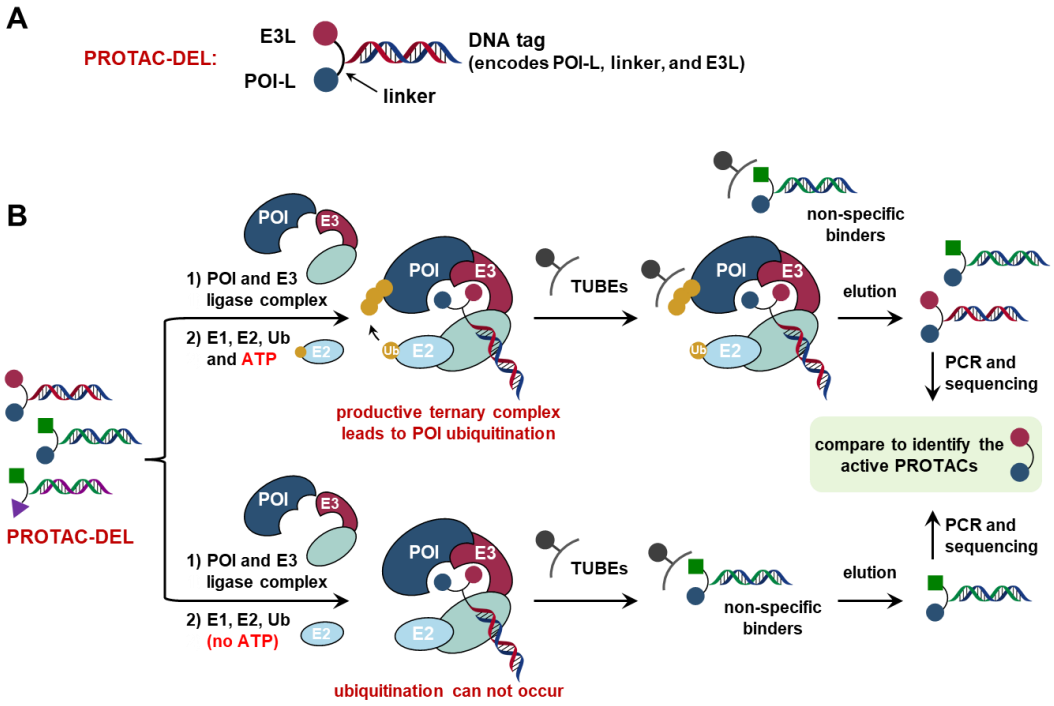

Figure 1. Selection of PROTAC-DELs. (A) The architecture of PROTAC-DEL. (B) The proposed scheme for PROTAC-DEL selection. The library is incubated with the POI and the E3 ligase complex, followed by adding E1, E2, Ub, and ATP. Next, TUBEs are used to capture and isolate the ubiquitinated complex containing the enriched compounds for hit decoding. The selection results are compared with the control selection without ATP to identify the active PROTACs. TUBEs: Tandem Ubiquitin Binding Entities.[54]

Results:

As shown in Figure 1A, PROTAC-DEL comprises the POI-L, the linker motif, and the E3L, all encoded by the DNA tag. The three components resemble the building blocks in a typical 3-cycle DEL.[38, 55] In a selection, the library is incubated with the POI and the E3 ligase complex, followed by adding E1, E2, Ub, and ATP (Figure 1B). The potential PROTACs in the library will bring the POI to the proximity of the protein complex and may also facilitate protein-protein interaction between the POI and the E3 ligase, thereby promoting POI poly-ubiquitination. Next, tandem ubiquitin binding entities (TUBEs)[54] will be used to capture and isolate the ubiquitinated ternary complex containing the enriched compouds,[54] which will be further decoded by PCR amplification

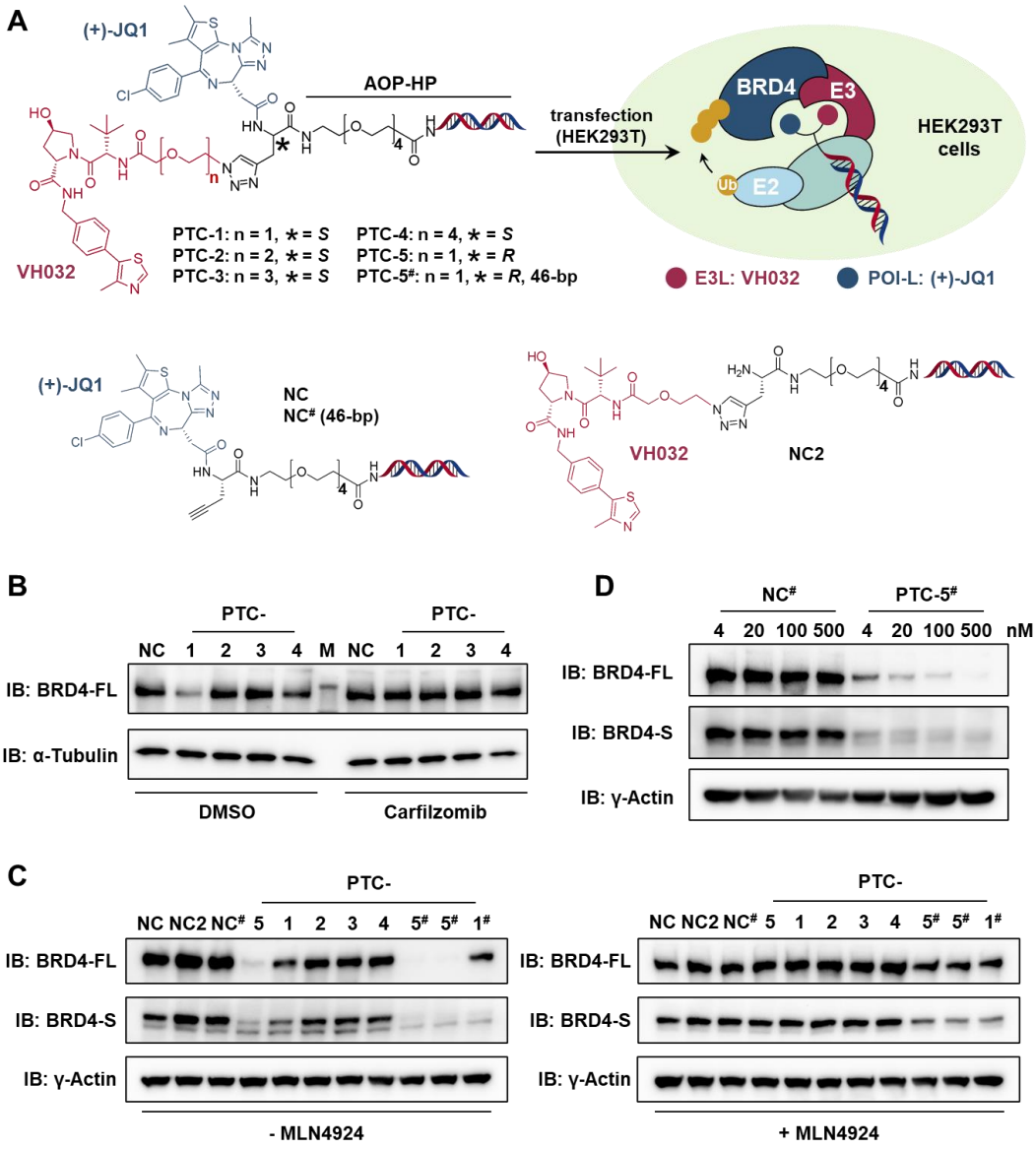

Figure 2. Degradation assay of DNA-conjugated chimeras in HEK293T cells. (A) A series of DNA-conjugated (+)-JQ1-VH032 chimeras (2 μM) were transfected into HEK293T cells, respectively, by using RNAiMAX according to the manufacturer's protocol. Structures of PTC-1 to PTC-5/5# and the negative controls (NC/NC#, NC2) are shown. The asterisk indicates the stereogenic center. (B) Degradation of the endogenous BRD4 was assessed with Western blot (24 hr. at 37 ºC). NC: negative control without VH032; M: protein ladders; carfilzomib: 0.5 μM; BRD4-FL: full-length BRD4. (C) Western blot analysis of BRD4-FL and BRD4-S degradation after pretreating HEK293T cells (1 hr.) with DMSO or MLN4924 (1 μM) (1 hr at 37 ºC), followed by transfection of the DNA-conjugated chimeras (0.5 μM). The hash symbol indicates the chimeras with a 46-bp DNA tag. NC2: negative control without (+)-JQ1. BRD4-FL: BRD4 full length; BRD4-S: BRD4 short isoform. (D) Dose-dependent degradation of BRD4-FL and BRD4-S in HEK293T cells with PTC-5# (24 hrs. at 37 ºC).

and DNA sequencing. Since ATP is required for POI ubiquitination,[56] the selection result will be compared with a control selection without ATP to identify the active PROTACs.

As an initial validation, we first verified whether DNA-conjugated PROTACs can induce POI ubiquitination and degradation. We used cellular BRD4 as the POI and the VHL E3 ligase complex as the model system.[57] The BRD4 inhibitor (+)-JQ-1 was used as the POI-L, which is connected with the VHL ligand VH032 through a trifunctional linker (Figure 2A). Both (+)-JQ-1 and VH032 have been used in many targeted protein degradation studies.[58] A branching vector on the linker was

used to conjugate with the "AOP headpiece" DNA (**AOP-HP**, 6-bp with a two-base 3' overhang; Figure 2A and S1),[55] which has a long PEG linker that can minimize the potential interference of the DNA tag.[41] A series of DNA-conjugated chimeras with different numbers of PEG units were synthesized (**PTC-1** to **PTC-5**; Figure 2A and S2). Next, we transfected HEK293T cells with the DNA conjugates for 24 h, respectively, and used Western blot to assess their effects on BRD4 degradation. As shown in Figure 2B, the conjugate with one PEG unit connecting the VHL ligand (**PTC-1**) resulted in modest BRD4 degradation at 2 μM, which could be rescued by the proteosome inhibitor carfilzomib, whereas the conjugates with more PEG units (**PTC-2**, **3**, and **4**) showed little activity. Interestingly, **PTC-5,** whose chimera is the diastereomer of the one in **PTC-1**, exhibited significantly better degradation activity than **PTC-1**, suggesting the importance of the chirality at the branching point of the linker (Figure 2C; left panel). Furthermore, we ligated **PTC-5** with a longer DNA (46-bp; **PTC-5$^{\#}$**), so that the conjugate has the similar length of DNA tag to typical DELs. **PTC-5$^{\#}$** retained BRD4 degradation activity and pretreatment with the NAE1 inhibitor MLN4924 rescued BRD4 stability, suggesting that the longer DNA tag did not affect the complex formation and target ubiquitination (Figure 2C; right panel). Dose-dependent degradation of BRD4 was also evaluated; **PTC-5$^{\#}$** exhibited potent BRD4 degradation with a half-maximal degradation concentration (DC_{50}) value of ~4 nM and a maximum level of target degradation (D_{max}) value of 99% after 24 hrs. of treatment (Figure 2D). In addition, co-treatment of cells with **PTC-5$^{\#}$** and MLN4924 completely reversed the degradation (Figure S3). All negative controls without either VH032 (**NC**/**NC$^{\#}$**) or (+)-JQ-1 (**NC2**) did not show any degradation.

Encouraged by the high potency of **PTC-5** in cells, we performed *in vitro* ubiquitination assay with the BRD4-BD1 by using an *in vitro* ubiquitination kit containing the protein complex VHL/Cul2/ELOB/ELOC/Rbx1 and E1/E2/Ub (LifeSensors, catalog #: PA770). The result is consistent with the cellular degradation assay. **PTC-5** induced significant BD1 ubiquitination and **PTC-1** produced modest BD1 ubiquitination, while the other DNA-conjugated chimeras did not show obvious effect (Figure 3A). Dose-dependent experiments showed the hook effect when the concentration of the chimeras became significantly higher than BD1 (Figure 3B). Next, to validate that the DNA tag encoding the active PROTACs can be enriched from the selection, we mixed **PTC-5$^{\#}$** (46-bp) and a negative control (**NC$^{\#}$**, without the E3 ligand VH032; Figure 3C) with orthogonal primer binding sites at 1:10 ratio. The mixture was subjected to the selection with BRD4-BD1 in the presence of VHL/Cul2/ELOB/ELOC/Rbx1 and E1/E2/Ub. After affinity pulldown using TUBEs,[54] the recovery of each DNA conjugate was determined with quantitative PCR (qPCR). The recovery of **PTC-5$^{\#}$** is ~3.3%, approximately 45.6-fold higher than **NC$^{\#}$** (Figure 3D). Notably, the selection without ATP gave a ~189-fold decrease of **PTC-5$^{\#}$** recovery, proving that the selection readout depends on ATP, which is required for POI ubiquitination.[59] In addition, pulldown experiments using the magnetic beads without the Ub-binding entities did not show any enrichment, confirming that the enrichment is specific to ubiquitination (Figure 3D). Furthermore, higher concentration of BD1 gave higher enrichment of **PTC-5$^{\#}$** (Figure 3E); we reason that sufficient BD1 is needed in the selection to avoid the competition between the productive BD1-**PTC-5$^{\#}$**-VHL ternary complex and the possibly stable but non-productive binary complexes. Using herring sperm DNA and bovine serum albumin (BSA) as the blocking agents in the selection slightly increased the **PTC-5$^{\#}$** enrichment folds (Figure S4).

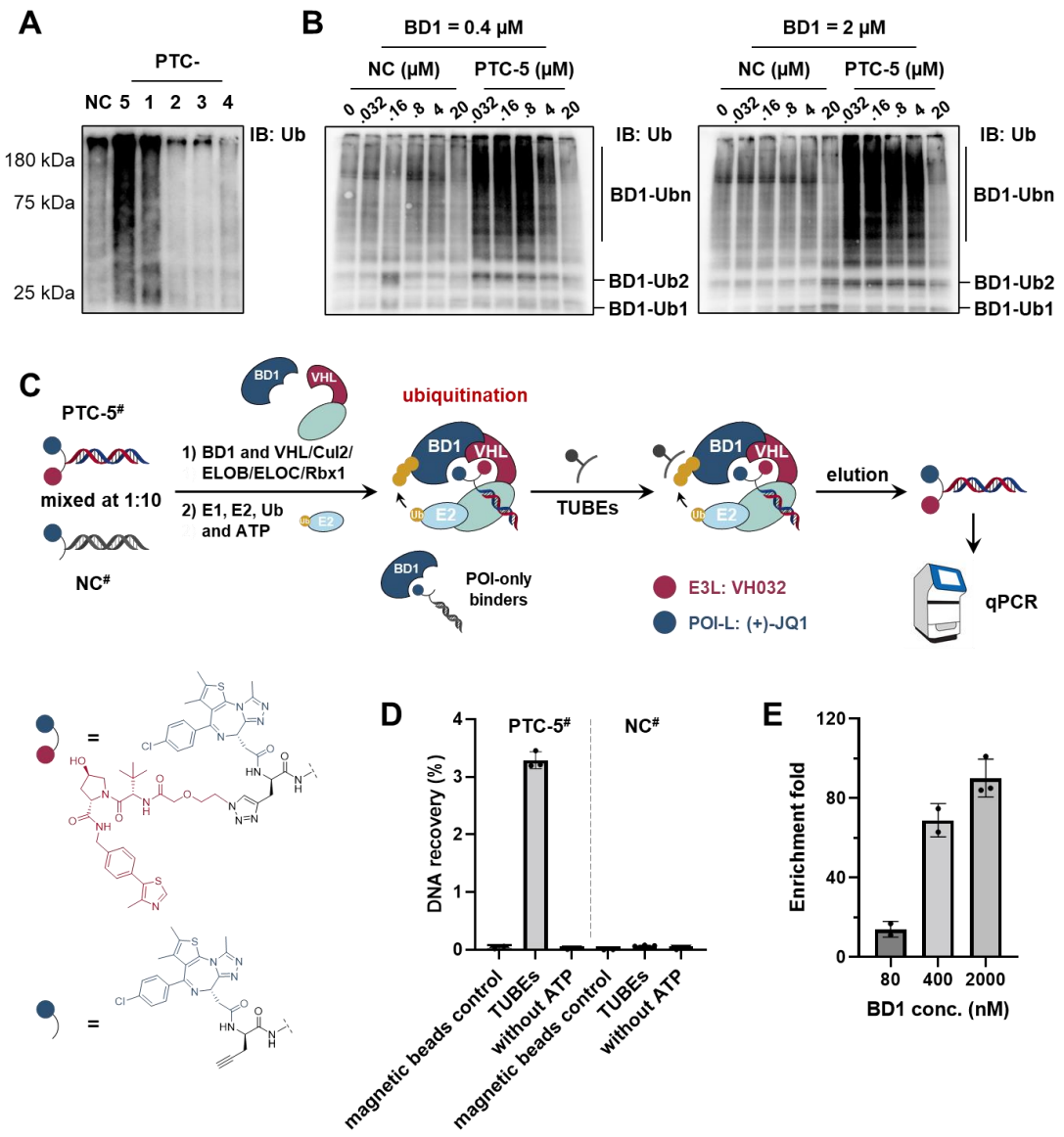

Figure 3. Affinity enrichment of DNA-conjugated PROTAC based on POI ubiquitination. (A) *In vitro* ubiquitination assay of BRD4-BD1 (2 μM) with different DNA-conjugated chimeras (PTC-1 to 5; 160 nM) and the negative control (NC, without VH032, 160 nM). (B) Dose-dependent *in vitro* ubiquitination assay of BRD4-BD1 (0.4 or 2 μM) with PTC-5 or NC. Ubn: poly-ubiquitination; Ub2: di-ubiquitination; Ub1: mono-ubiquitination. (C) Scheme of the model selection; the structures of the PTC-5# and NC are shown on the lower left. The mixtures of PTC-5# (8 nM) and NC# (80 nM) were selected against BRD4-BD1 (2 μM) in the presence of VHL/Cul2/ELOB/ELOC/Rbx1 and E1/E2/Ub, followed by TUBEs pulldown and qPCR analysis. (D) Recovery of the DNA conjugates, quantified by qPCR. (E) Model selections performed with different concentrations of BD1. in D and E, $n = 3$ independent experiments; data are presented as mean values ± s.d. Enrichment fold is given as the fold change in recovery of PTC-5# relative to NC#. See experimental details in the Supporting Information.

Next, we prepared a model library containing the active degrader **PTC-5**, three non-degrader chimeras (**PTC-2**, **PTC-3**, and **PTC-4**), and a pool of background DNAs of 729 different sequences at equal ratio (without the VHL ligand; Figure 4A and S5). The model library was selected against BRD4-BD1 with and without ATP following the same conditions and procedure as in Figure 3, except that the selected compounds were decoded with NGS. The sequencing data were processed with a custom script to quantitatively tally the codons for each compound and calculate the enrichment fold (post-selection fraction)/(pre-selection fraction) as we previously reported.[60, 61] The

selection results are shown in 2D scatter plots (Figure 4B). Compounds with relatively high enrichment fold and post-selection sequence count are considered to be potential hits.[62] Notably, in the presence of ATP, **PTC-5** was significantly more enriched than **PTC-2**, **PTC-3**, **PTC-4**, and also the background DNAs, whereas no **PTC-5** enrichment was observed without ATP. The enrichments with and without ATP were also compared in the form of normalized *z*-scores,[63] which again confirmed that only the active degrader **PTC-5** was strongly enriched from the selection (Figure 4C).

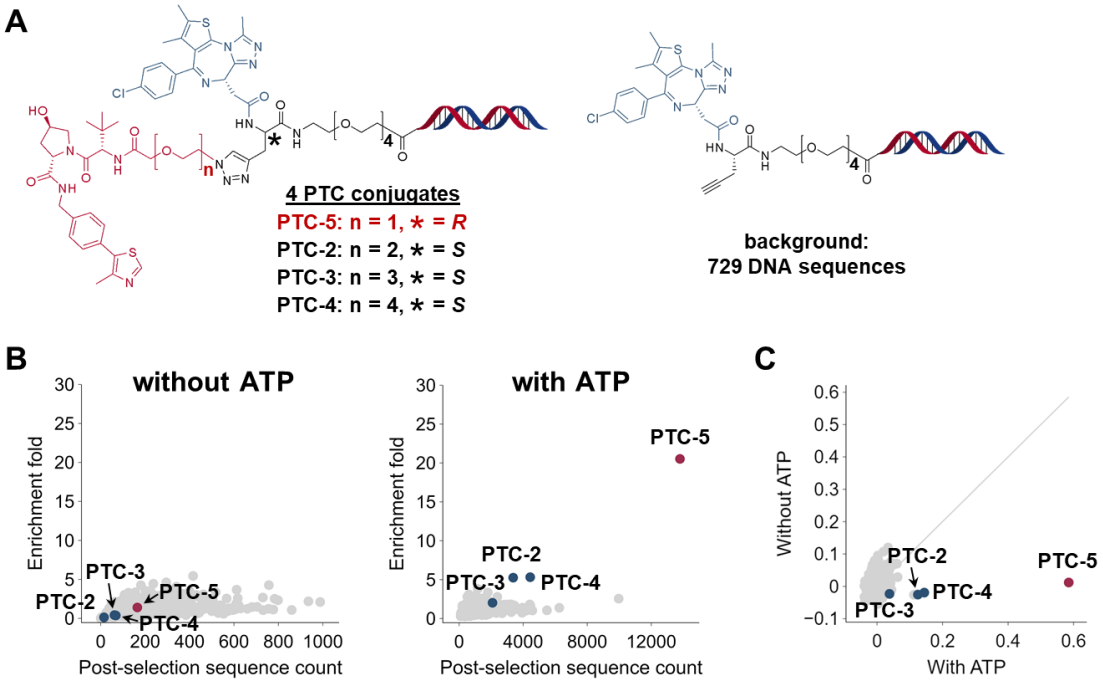

Figure 4. Model PROTAC-DEL selection. (A) Composition of the model library; see Figure S5 for details. (B) The selections were performed with and without of ATP; the results are shown in 2D scatter plots; *x*-axis: post-selection sequence count; *y*-axis: enrichment fold; enrichment fold = (post-selection fraction)/(pre-selection fraction).[60, 61] Data points of the PTC conjugates are highlighted; the active degrader (PTC-5) is highlighted in red and the non-degrader chimeras (PTC-2 to PTC-4) are highlighted in blue. (C) The enrichments with and without ATP are compared in the form of the normalized *z*-score;[63] the diagonal *y* = *x* line is shown as the reference.

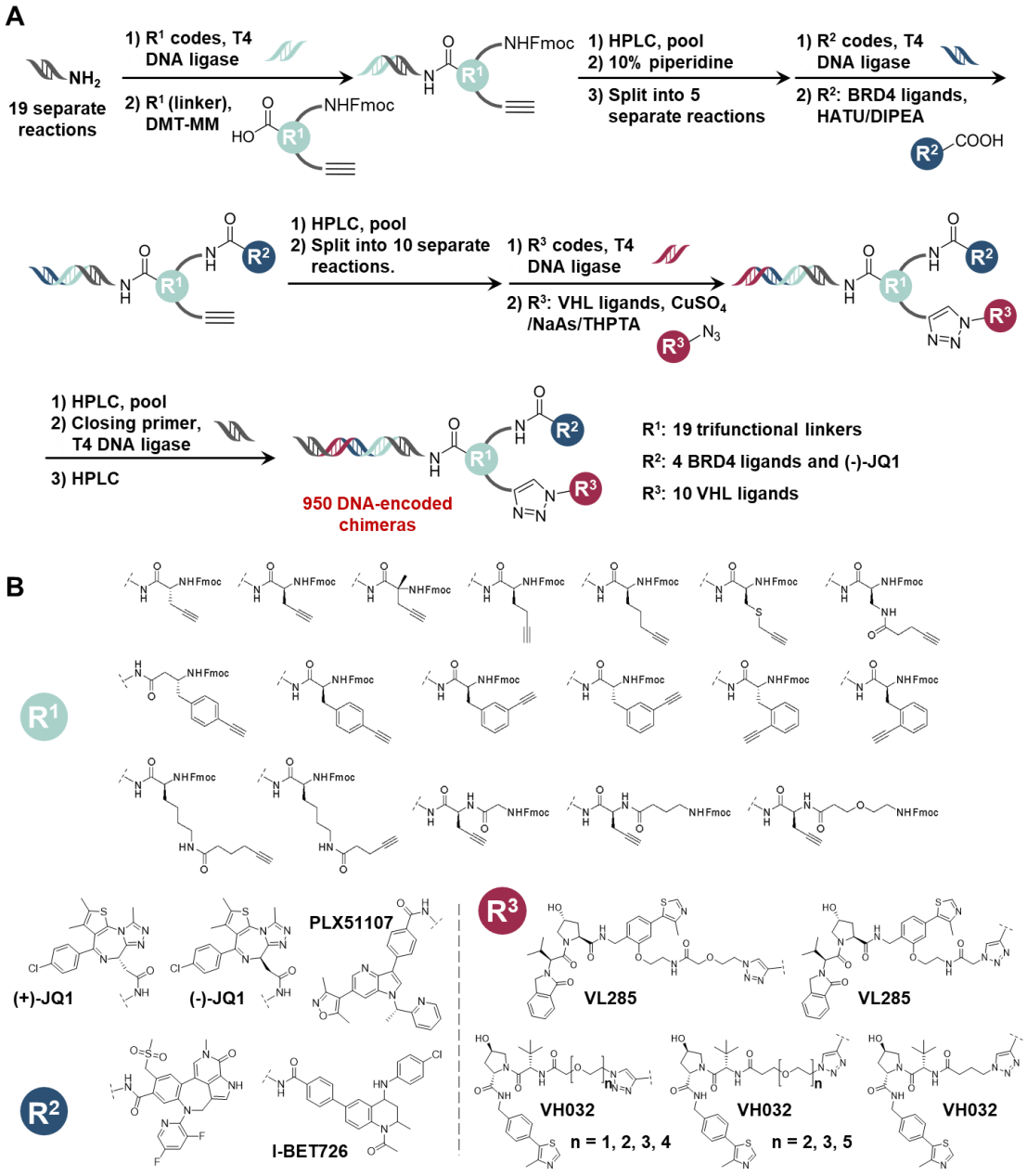

Figure 5. Synthesis of the PROTAC-DEL of 950 "E3 ligand-linker-POI ligand" combinations. (A) Library synthesis scheme. (B) Structures of the three sets of the building blocks.

Next, aiming to discover new degraders, we prepared a PROTAC-DEL using the split-and-pool technique to encode the three components of the chimeras (Figure 5). The library includes 19 trifunctional linkers (R^1), 4 BRD4-BD1 ligands and (-)-JQ1 as the negative control (R^2), and 10 E3 ligase ligands (R^3).[58, 64] The final library was HPLC-purified and characterized with mass spectrometry (MS), which showed the expected mass range, and the DNA sequence of the library was confirmed with Sanger sequencing (Figure S6). The library (60 nM) was selected against BRD4-BD1 (2 μM) with and without ATP, and the selections were performed in triplicates. After the selection, the *z*-scores of the enriched compounds were plotted (Figure 6A; top panel). The compounds with high *z*-scores with ATP and low *z*-scores without ATP are considered as potential hits.[62] Two chimeras **1-1-1** and **15-1-3** were significantly more enriched with ATP than without ATP.

Notably, **1-1-1** has the same chimera structure as **PTC-5** and **15-1-3** has a much longer spacer between the VHL ligand and (+)-JQ1 (Figure 6B). In contrast, when (+)-JQ1 was replaced with (-)-JQ1 in the two chimeras (**1-5-1** and **15-5-3**), no preferential enrichment was observed in the presence of ATP. In addition, we intentionally encoded **1-1-1** with two different DNA tags, and both were significant enriched, further supporting the selection reliability (circled in Figure 6A, top panel).

For hit validation, **1-1-1** and **15-1-3** were synthesized off-DNA and their activities were tested in HEK293T cells. Dose-dependent degradation was observed for both **1-1-1** and **15-1-3** with DC_{50} values of approximately 9.7 nM and 60 nM and D_{max} values of 99% and 66%, respectively (Figure 6C). Co-treatment with the NEDDylation inhibitor MLN4924 or the proteasome inhibitor carfilzomib completely reversed the degradation (Figure 6D). For comparison, we synthesized **1-1-2** (one PEG unit longer than **1-1-1**), a moderately enriched chimera with ATP, and **15-1-2** (one PEG unit shorter than **15-1-3**), which was not enriched in the selection. As expected, **1-1-2** showed decreased potency in BRD4 degradation and hook effect at 10 μM**,** and **15-1-2** did not induce BRD4 degradation up to 10 μM (Figure 6E). Furthermore, we performed the selection of the same library but the affinity enrichment was based on the fused His-tag on BD1 with Ni-NTA beads, which is expected to enrich the chimeras that induces the formation of stable ternary complexes or BD1-POI-L binary complexes.[41, 42] The His-tag pulldown selection result is shown in a 2D scatter plot (Figure 6A, bottom panel); similar to Figure 2B, the enriched compounds are expected to be in the upper right corner of the plot. Both the active degraders (**1-1-1, 1-1-2** and **15-1-3**) and the inactive degrader (**15-1-2**) have been enriched, suggesting that **15-1-2** may have formed stable ternary/binary complex but the complex formation did not result in target ubiquitination, which is required for proteasomal degradation. The enrichment levels of **1-1-1, 1-1-2** and **15-1-3** are consistent with the TUBEs pulldown, suggesting they form productive complexes and the stabilities are correlated with target ubiquitination. In addition, all the "negative" compounds with (-)-JQ1 are concentrated at the lower left corner of the plots (light green dots; Figure 6A) exhibiting little enrichment in both selections. Collectively, these results have indicated that, since the formation of stable complexes may not always lead to protein ubiquitination and degradation,[49-52] the selection based on ubiquitination is more predicative of active POI degraders.

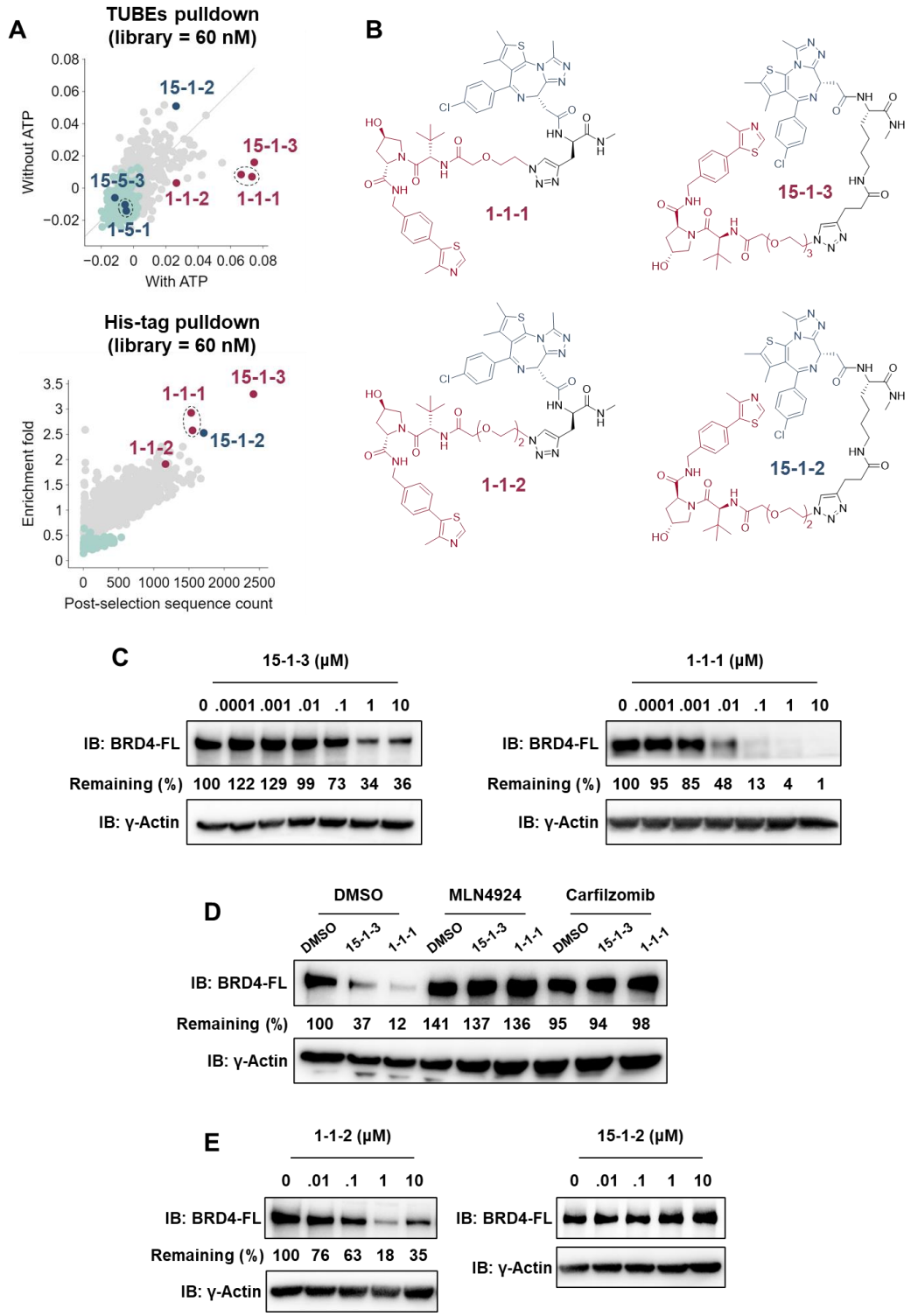

Figure 6. PROTAC-DEL selection result analysis and hit characterization. (A) Top panel: 2D scatter plot showing normalized *z*-scores of the enriched compounds from the TUBEs pulldown selection; *x*-axis: with ATP, *y*-axis: without ATP. Bottom panel: 2D scatter plot showing the enriched compounds from the His-tag pulldown; *x*-axis: post-selection sequence count; *y*-axis: enrichment fold; enrichment fold = (post-selection fraction)/(pre-selection fraction).[60, 61] Red: selected hits; blue: selected controls; light green: compounds containing (-)-JQ-1. Selection conditions are the same as in Figure 4; library: 60 nM. The circled dots are the same compound encoded by two different codes. (B) Structures of the selected hits and control compounds. (C) Dose-dependent degradation of BRD4 in HEK293T cells treated with 1-1-1 or 15-1-3 (24 hrs, 37 °C). (D) 1-1-1 (0.1 μM) and 15-1-3 (1 μM) induced BRD4 degradation is rescued by the proteasome inhibitor carfilzomib (0.5 μM) or the neddylation inhibitor MLN4924 (1 μM). (E) Western blot analysis of BRD4 degradation with 1-1-2 and 15-1-2. See experimental details in the Supporting Information.

We also varied the amount of the library in the selection. At lower library concentration (0.4 or 4 nM), a new chimera **4-4-10**, was significantly enriched in the presence of ATP, while its close analogs **2-4-10** (only one methylene shorter) and **5-4-10** (one methylene longer) were not enriched (Figure 7A). With His-tag pulldown, **4-4-10** was only moderately enriched to the level similar to **2-4-10** and **5-4-10** (Figure 7B). Moreover, the active degraders **1-1-1** and **15-1-3** identified above were enriched by both pulldown methods. Instead of (+)-JQ-1, **4-4-10** contains I-BET726, a high-affinity BRD4 tandem bromodomain ligand (Figure 7C; K_d: 4 nM),[65] We hypothesize that **4-4-10** might form productive but relatively less stable ternary complex of negative cooperativity. Indeed, the Ciulli group also reported that tetrahydroquinoline-based BRD4 degraders showed negative cooperativities of ternary complex formation, despite being derivatized from a highly potent BET inhibitor.[66] In Figure 5A, the library concentration (60 nM) is higher than VHL (~30 nM); thus, most VHL molecules might be occupied by other library compounds that can form more stable ternary complexes and **4-4-10** had little chance to form the productive complex to ubiquitinate BD1. With lower library concentration (0.4 or 4 nM), there is less competition and sufficient VHL for complex formation. Next, **4-4-10** was synthesized and tested in HEK293T cells. Interestingly, it appeared that **4-4-10** is a moderate but selective degrader of the short isoform of BRD4 (BRD4-S; ~61% at 5 μM) without degrading the full-length BRD4 (BRD4-FL; Figure 7D). Such a selectivity of **4-4-10** is intriguing and warrants further investigation, especially in light of the previous reports indicating the tumor-suppressive functions of the full-length BRD4 and the oncogenic roles associated with the short BRD4 isoform.[67, 68] Finally, the amount of TUBEs did not influence the selection outcome significantly (Figure S7).

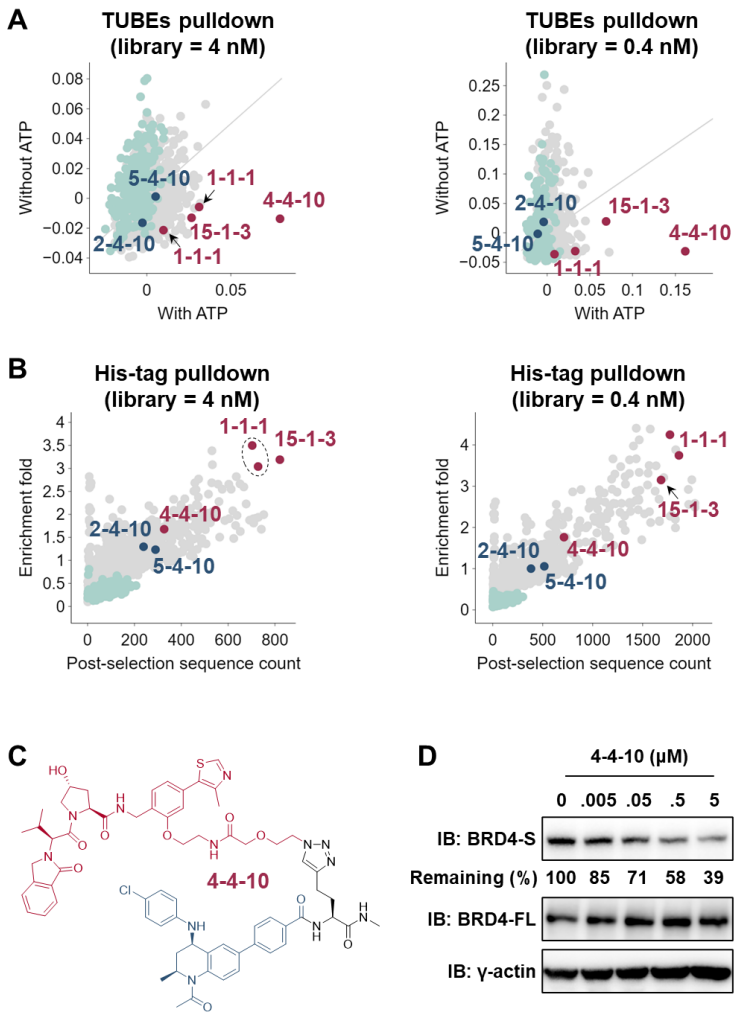

Figure 7. PROTAC-DEL selection results at lower library concentration (4 nM and 0.4 nM). (A) 2D scatter plots showing the enriched compounds with TUBEs pulldown; (B) 2D scatter plots showing the enriched compounds with His-tag pulldown. The axes are the same as in Figure 6. Red: the selected hits; blue: the selected control compounds; light green: compounds containing (-)-JQ-1. (C) Structure of 4-4-10. (D) Western blot analysis of BRD4-FL and BRD4-S degradation with 4-4-10. See experimental details in the Supporting Information.

Discussion.

Despite the intense research efforts and the ever-increasing understanding of the PROTAC-mediated protein degradation, the design and development of PROTACs is still highly empirical. The process can involve iterated rounds of synthesis and testing of large number of compounds, which is laborious and time-consuming.[4, 18, 19] Often a slight change of the chimeric structure leads to large difference in degradation efficiency,[69, 70] such as observed with **1-1-1** vs. **1-1-2**, **15-1-2** vs. **15-1-3** in this study. Sometimes, even the formation of stable ternary complexes is not predictive of functional degraders, adding another layer of complexity to PROTAC development.[49-52] It is still challenging to find a general guideline for PROTAC design.[53] Recent studies have nicely shown that DELs can be a powerful tool in PROTAC discovery.[39-43, 71, 72] Here, we developed a "function-first" DEL selection approach, in which the compound enrichment depends on target ubiquitination; therefore, the method is more likely to identify active degraders, whereas the selection based on POI-containing complex formation may also identify inactive chimeras, such as **15-1-2** in the His-tag pulldown (Figure 6A). Currently, we are synthesizing more compounds identified from the

selections for further testing. More importantly, we are characterizing the stabilities of the complexes in order to obtain more mechanistic insights of these enriched compounds with different degradation activities.

A major issue in PROTAC research is that the E3 ligase space remains heavily underexplored, arguably due to the lack of small molecule E3-recruiting ligands.[13, 73] We anticipate this method can be used to discover novel E3 ligase ligands that can form productive degradation complexes. For instance, existing DELs with an appending functional group, such as amino or clickable azido/alkynyl groups, may be conjugated with a POI ligand to rapidly prepare large PROTAC-DELs without library redesign and resynthesis. Finally, on a technical note, this method does not require protein immobilization and thus may have a wide target scope. In this study, the *in vitro* selection results correlate well with the cellular degradation results and the DNA-conjugated PROTACs showed good activities after delivering into cells. We will further develop the selection method, aiming to realize intracellular PROTAC-DEL selections and apply to other POIs and E3 ligases.

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Author Contributions

The manuscript was written through the contributions of all authors.

Notes

The authors declare no competing financial interests

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