Cell-Based Assay of Pd-Mediated Arylation of Cyclic Peptide Binders of Ubiquitin Chains: Towards Modulating NEMO Liquid-Liquid Phase Separation

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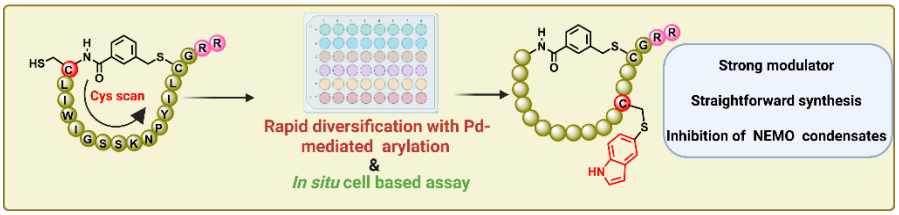
Abstract

Ubiquitination is a critical post-translational modification that regulates key cellular processes such as protein degradation and DNA damage repair. Targeting a specific type of ubiquitin chain (e.g., Lys48 or Lys63-linked ubiquitin chain) via cyclic peptides presents a new strategy to modulate biological processes with therapeutic potential for different diseases. However, such a strategy remains challenging due to the obstacles of cell permeability and bioactivity. Here, we report a new approach to directly examine these parameters by combining palladium-mediated Cys arylation with *in situ* cell-based screening. Using **CP4**, a previously identified cyclic peptide modulator of Lys63-linked ubiquitin chains, we generated a focused library of arylated analogues and optimized the Pd-mediated arylation for cell-based screening. We discovered a new analog, **CP-P12-Ar^{H,}** that demonstrated enhanced binding affinity and robust bioactivity, as evidenced by increased γ-H2AX phosphorylation and apoptosis induction in cancer cells. Furthermore, **CP-P12-Ar^{H}** effectively inhibited the *in vitro* formation of NF-κB essential modulator (NEMO) biomolecular condensates by disrupting the elongation of Lys63-linked ubiquitin chains, offering a novel way to modulate NF-κB signaling. This work establishes a generalizable platform for the rapid optimization of cyclic peptide therapeutics targeting protein-protein interactions.



Introduction

Ubiquitination is a complex and vital post-translational modification in which the small ubiquitin protein is covalently attached mainly to lysine residues on protein substrates through an enzymatic cascade involving E1 activating enzymes, E2 conjugating enzymes, and E3 ligases.[1,2] Notably, ubiquitination is characterized by the formation of various ubiquitin chains that are elongated from any of the eight amino groups (e.g., Lys48, Lys63) through the C-terminus of the subsequent ubiquitin. Ubiquitination is reversed by a family of enzymes known as deubiquitinases (DUBs).[3] This process plays a crucial role in regulating diverse cellular processes such as protein degradation, signaling, and cell cycle control, while aberrations in these processes can lead to various diseases.[4,5] Therefore, modulating ubiquitin-dependent pathways holds significant therapeutic potential. For example, proteasome inhibitors are known to block the degradation of ubiquitinated proteins, resulting in cell death that has led to the development of life-saving drugs (e.g., Bortezomib) for multiple myeloma.[6] Other potential therapeutic approaches include inhibiting the enzymes involved in this process, such as E3 ligases and DUBs.[7] In recent years, proteolysis-targeting chimeras (PROTACs) and molecular glues that trigger the degradation of specific oncogenic proteins have emerged as powerful approaches.[8,9] However, targeting the ubiquitin chains presents substantial challenges as it involves various chains, such as Lys48- and Lys63-linked ubiquitin chains, each with distinct functions, complicating selective modulation of a specific signal. Additionally, the lack of well-defined pockets in these chains and other ubiquitination components and the nature of protein-protein interactions in ubiquitin signaling pose significant hurdles to achieving specific modulation of ubiquitin pathways in disease treatment.[10]

Due to their unique properties, cyclic peptides have gained significant attention as modulators of protein-protein interactions, offering therapeutic potential for various diseases.[11] Recent advancements from our labs have included the use of the Random Non-standard Peptides Integrated Discovery (RaPID) platform, in conjunction with chemical protein synthesis, enabling the discovery of cyclic peptides that selectively bind to Lys48- or Lys63-linked ubiquitin chains.[12–15] Cyclic peptides that bind to Lys48-linked ubiquitin chains inhibit proteasomal protein degradation *in vitro*, in cell lines, and in a mouse model,[16] making them potentially applicable for further development in treating multiple myeloma. Conversely, cyclic peptides that bind to Lys63-linked ubiquitin disrupt DNA damage repair (DDR) pathways via non-homologous end-joining (NHEJ) repair, leading to cell death by apoptosis.[17]

The RaPID technology offers an extraordinary method for screening cyclic peptides containing non-canonical amino acids, enabling the examination of over 10^[12] cyclic peptides against the target of interest.[18] However, the screening selection is based on the binding affinity of the cyclic peptides to a relevant target, excluding their cellular effectiveness, particularly for intracellular targets. Despite efforts to incorporate various motifs to enhance cell permeability,[19] it still cannot fully address cell permeability and direct biological activity to intracellular targets. Improving the effectiveness of cyclic peptides post-RaPID screening requires complementary methods to overcome these limitations.

To enhance the cell permeability, solubility, and bioactivity of the cyclic peptide **CP1** targeting Lys63-linked ubiquitin chains, we modified the cyclization linker, exo-cyclic region, and internal residues beyond RaPID constraints (**Figure 1**). A Cys scan led to **CP2** with better activity. [12] Further modifications resulted in **CP4** (benzoyl linker) and **CP7** (thioacetal linker), both of which exhibited improved cellular activity. [17] However, **CP7's** synthesis is limited by a low-yield

thioacetal formation step (1–2%), and despite its better cell permeability, it binds Lys63-diUb less effectively than **CP4**. Considering these limitations, starting with a straightforwardly prepared selected cyclic peptide and combining rapid diversification with cell-based screening could provide a practical approach to generate an effective compound with enhanced properties.

Herein, we report on Pd-mediated Cys arylation of **CP4**-based cyclic peptides to develop a focused library for *in situ* screening using a cell-based assay. Applying this strategy, we discovered a potent cyclic peptide (**CP-P12-Ar^{H}**), which markedly increases γ-H2AX levels and promotes apoptotic cell death in cancer cells. Furthermore, this compound effectively inhibits the *in vitro* formation of NF-κB essential modulator (NEMO) protein condensates driven by Lys63-linked ubiquitin chains.

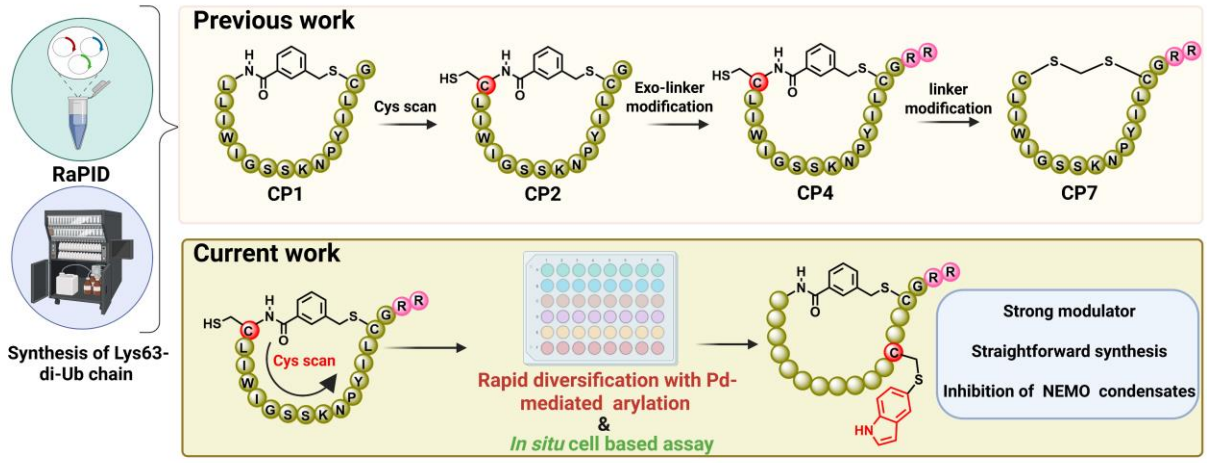


Figure 1. Previous and present work for the discovery and development of cyclic peptides that modulate Lys63-linked ubiquitin chains.

Results and Discussion

Palladium-mediated Cys arylation is a highly efficient, chemoselective process that forms carbon-sulfur (C(sp3)–S) bonds by activating aryl halides.[20,21] This chemistry offers several advantages: high selectivity for Cys residues, rapid reaction rates, compatibility with various functional groups, and the ability to function at room temperature and neutral pH, making it suitable for biological applications.[22–24] Its significance lies in its capacity to modify biomolecules accurately without damaging sensitive structures,[25] thereby facilitating the development of bioconjugates,[26] macrocycles,[27] engineered proteins,[28–30] and protein post-translational modification (PTM) mimics. [31] Recent studies from our group and several others have demonstrated the compatibility of using Pd chemistry in a cell-based environment[32] and in living systems to decage various protecting groups in proteins and small molecules.[33] For example, we have shown the use of thiazolidine to mask an aldehyde in the C-terminus of ubiquitin,[34] which was decaged in cells to serve as an activity-based probe for DUBs.[35] Other studies have shown that Pd complexes can promote depropargylation and deallylation reactions in living mammalian cells,[36] enabling intracellular chemical transformations, cleaving propargyloxy groups, and removing alkoxy protecting groups from genetically expanded protected amino acids under biological conditions, albeit with varying efficiencies.[37] In addition, the decaging of a prodrug containing allyl ether and carbamate masking groups[38], and thioether linkers[39] for spatiotemporal control of drug activation was also demonstrated.

Although there has been significant advancement in Pd-mediated S-arylation, its application within living cells or *in situ* cell-based screening remains unexplored. The latter could hold great potential for drug discovery, allowing for the rapid selection of lead molecules. Therefore, we

aimed to utilize water-soluble palladium (II) oxidative addition complexes (OACs) supported by the sSPhos ligand [21] for *in situ* arylation of ubiquitin chain modulators and a cell-based assay.

We aimed to verify this concept by examining the bioactivity of straightforwardly synthesized **CP4,** where each internal residue was substituted with Cys (**Figure 2A**), resulting in 14 different cyclic peptides that facilitate site-specific arylation for *in situ* cell-based screening **(Figure S1, Table S2)**. Additionally, we designed 10 different aryl Pd(II) OACs (**Pd-A-J)** with various substituents (**Figure 2B, S2**) to enable late-stage arylation of the Cys analogues, producing 140 distinct modified cyclic peptides (**Figure 2C**).

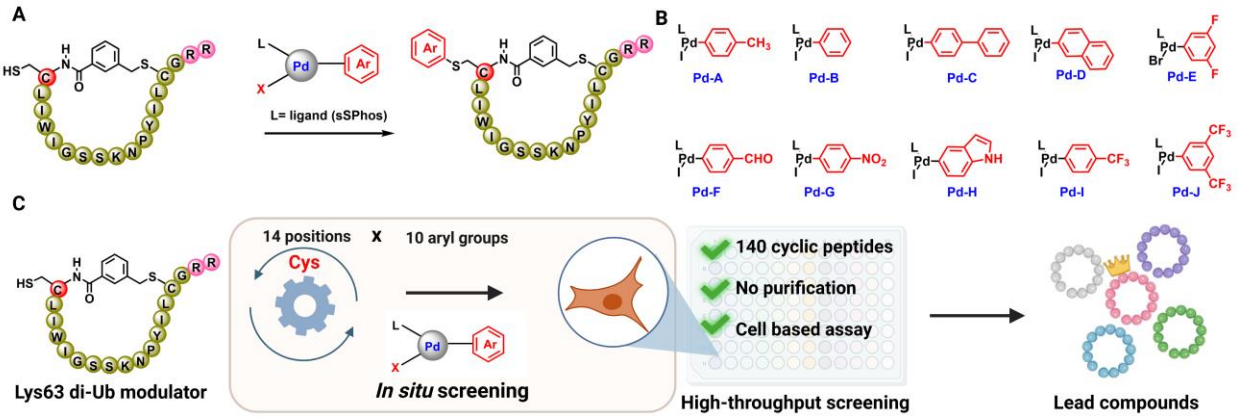


Figure 2. Schematic presentation of our strategy for *in situ* Cys arylation combined with a cell-based assay. (A) cyclic peptide modification with Pd(II)-mediated arylation, (B) Aryl groups used in this study, (C) in situ arylation of 14 different Cys residues with 10 different aryls.

In our design, we wished to take advantage of our cyclic peptide's ability to induce DNA damage repair and increase the phosphorylation of histone H2AX, generating γ-H2AX.[40] Using high-throughput microscopy, we should be able to identify and quantify the phosphorylation level with the specific antibody.

Initially, we optimized the Pd(II)-mediated arylation reaction for **CP4** with **Pd-A** aryl (**Figure 3A** and **B**) in various aqueous buffers, adjusting reaction times, concentrations, and temperatures. The

Pd-A aryl was stable under air and aqueous conditions with minimal ligand oxidation and the aryl group release (**Figure 3C, S3-S5**). The best conditions were determined to be Tris buffer, pH 7.5, at 37°C, affording the arylated product in over 95% yield within 45 minutes (**Figure 3C, S6-S15**). Additionally, we observed a minor product of the Pd-complex that coordinated with the arylated peptide (**Figure 3D, S3-5**).

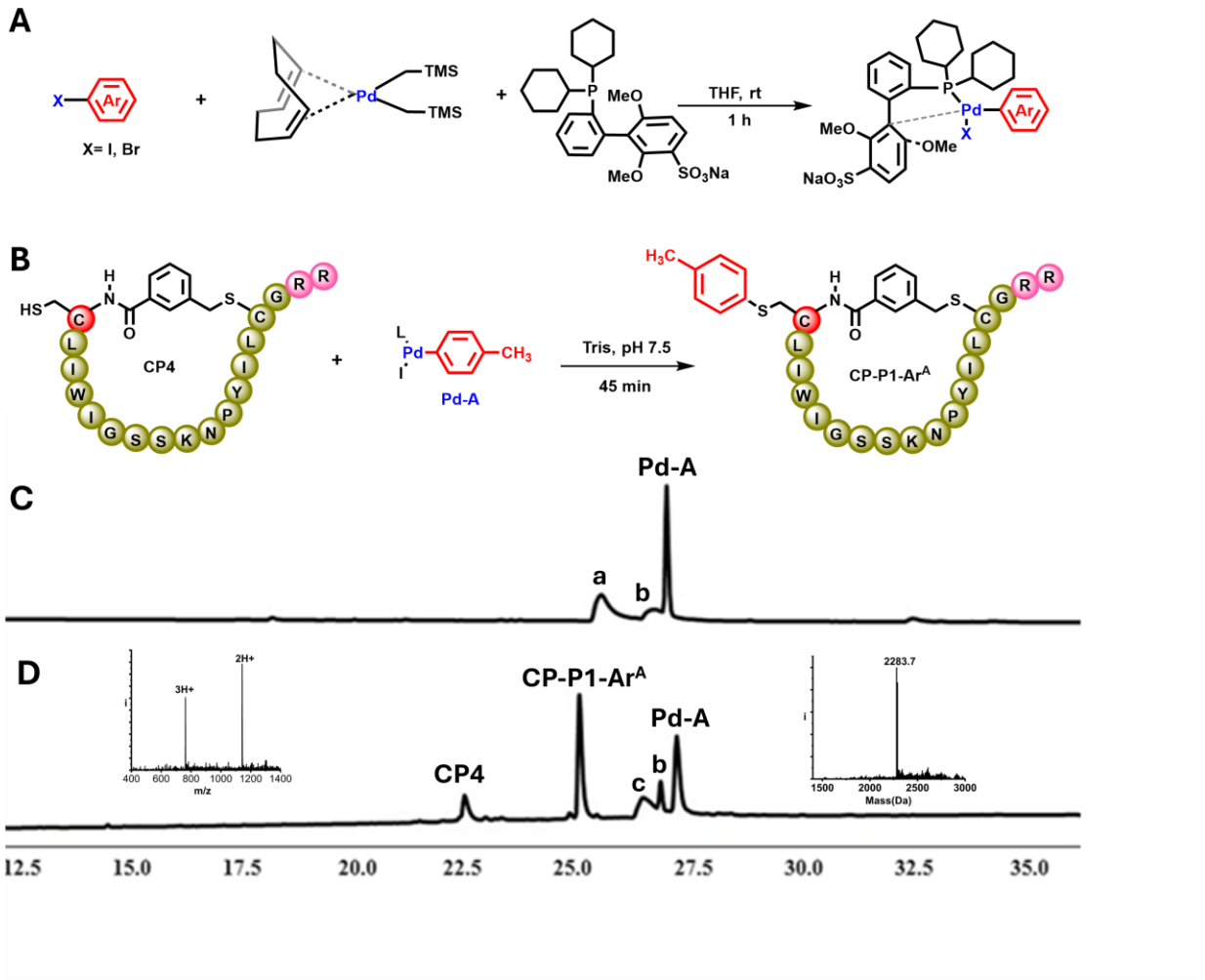


Figure 3. Schematic presentation of the general synthesis of Pd(II)-aryl complex (A) and the Cys arylation of the cyclic peptide with **Pd-A** (B). (C, D) HPLC and MS analyses of the arylation reaction of **CP4** peptide with **Pd-A** to produce **CP-P1-Ar^A**, observed mass: 2283.7± 0.2 Da (calculated mass: 2284.1 Da). The reaction was monitored at t=0, showing **Pd-A** without peptide (C) and after 45 minutes with **CP4** (D). **a** corresponds to the oxidized ligand, **b** corresponds to the Pd complex without the aryl group, and **c** corresponds to the arylated peptide coordinated with the Pd complex (See **Fig. S5** for more information).

With this high-yielding reaction and minimal side products, we were encouraged to perform the arylation reaction for direct screening with live cells. Therefore, we applied these conditions to **CP4** using the ten Pd-aryls catalysts shown in **Figure 2B**, and each catalyst's reaction mixture was incubated with HeLa cells (**Figure 4A**) to monitor the level of γ-H2AX.

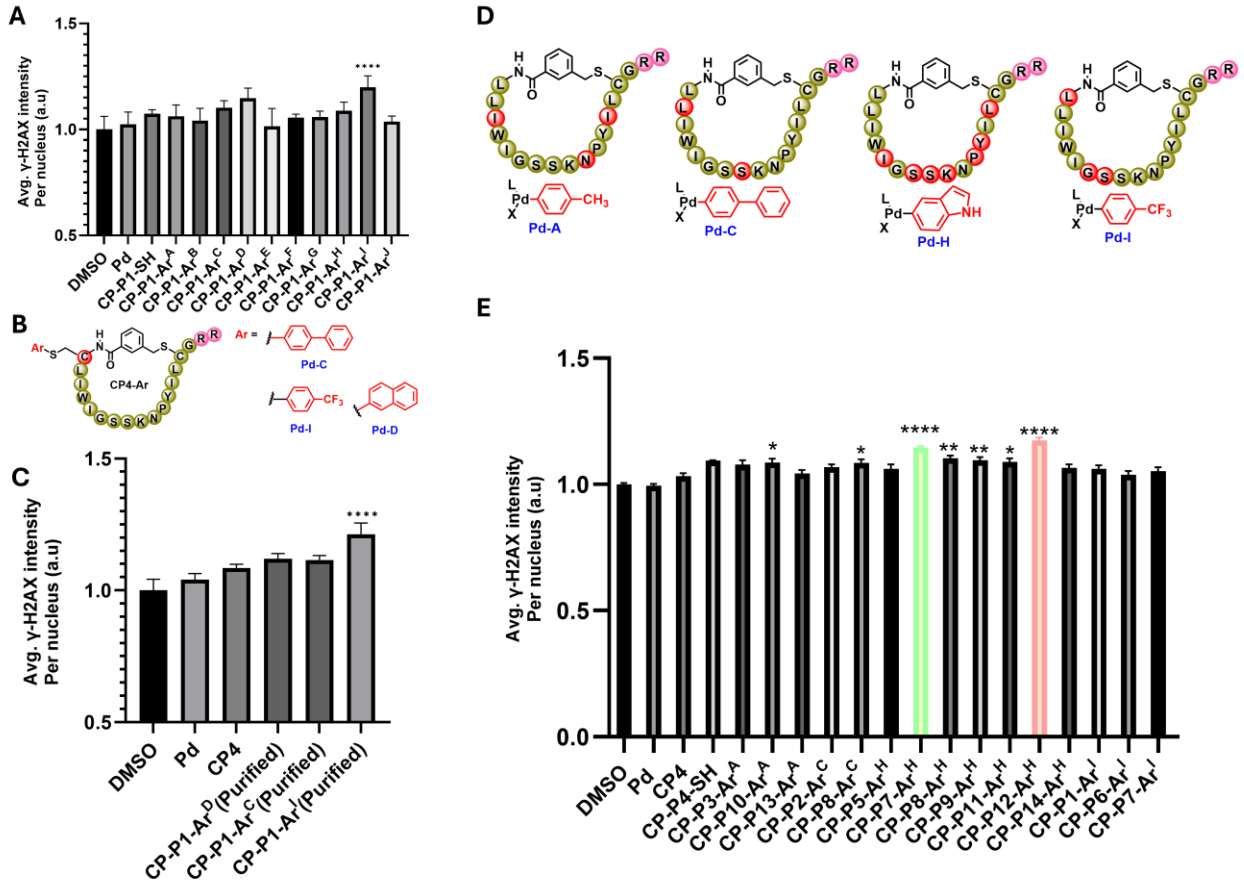


Figure 4. (A, B) represents an example of *in situ* screening of the arylation of **CP4** with ten Pd-aryls Pd-(A-J). (A) Quantification of relative histone H2AX phosphorylation in the cell nucleus from high-content live-cell fluorescent microscopy with **CP4** and its derivatives having different arylated groups (n=8). (B) cyclic peptides' structure of the leading **CP4** derivatives. (C) Quantification of relative γ-H2AX with purified **CP4** derivatives generated from Pd-C, D, and I (n=8). (D) Cyclic peptides highlighting the leading position, among the 14 residues, with the specific aryl group. (E) Final screening of the leading derivatives compared to **CP4**. High-throughput microscopy-based analysis was done with 16 replicates per sample. Data are presented as mean values ± SE. (Two-tailed, paired t-test ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.1$).

We examined the impact of arylated peptides compared to **CP4** after 24 hours, during which **CP4** is known to have little effect on γ-H2AX, with significant activity of **CP4** observed only after 48 hours.[17] This helped us identify compounds that exhibit better activity within the initial signal range of **CP4** and facilitate the discovery process (24 h vs 48 h). These results were compared to cells treated only with DMSO or Pd-aryl, which, to our satisfaction, demonstrated no effect on the γ-H2AX level. To exclude any influence on the γ-H2AX level due to the Pd-Ary or decomposed intermediates, the three most active arylated cyclic peptides were purified and compared to the crude reaction (**Figure 4B, S16-18**), which showed consistent results with the non-purified cyclic peptides (**Figure 4C**).

The compatibility of the arylation chemistry for direct live cell screening allowed us to conduct rapid testing of the arylation of each of the fourteen **CP4** analogues with the ten Pd-aryls. Each crude reaction was subsequently incubated with cells for 24 hours. After fixation and labeling with γ-H2AX antibody, we identified the leading arylated cyclic peptide for each position using high-throughput microscopy (**Figure 4D, S25-S38**). Finally, from each screen, we selected the most active cyclic peptide that exhibited statistically significant changes in the γ-H2AX level and performed secondary screening to extract the best modulators (**Figure 4E, S39-40**).

Interestingly, we identified two leading cyclic peptides at positions 7 and 12 featuring the same indole aryl group (**CP-P7-Ar^{H}** and **CP-P12-Ar^{H}, Figure 4E)**. To validate these results, we purified the two **CP4** derivatives (**Figure S19-20**) and conducted further screening of the purified peptides and their reaction mixture (**Figure S41**). **CP-P12-Ar^{H}** demonstrated a higher induction of the γ-H2AX level (**Figure S42)** compared to **CP-P7-Ar^{H}** and **CP4**. Next, we compared their relative binding affinity[12,41] to that of **CP4** and its free Cys analogs, specifically **CP-P7-SH** and **CP-P12-SH.** Additionally, we included two negative controls of **CP4**: one with a 1,3-difluoroaryl

at position 7, **CP-P7-Ar^{E} (Figure S21)**, which showed a lower effect on the γ-H2AX level, and the one with the scrambled sequence of **CP4 (sc-CP4),** as illustrated in **Figure 5A**. Our results indicated an increase in the relative binding of **CP-P12-Ar^{H}** and **CP-P7-Ar^{H}** compared to the other two analogs, with **CP-P12-Ar^{H}** being the strongest variant **(Figure 5B)**.

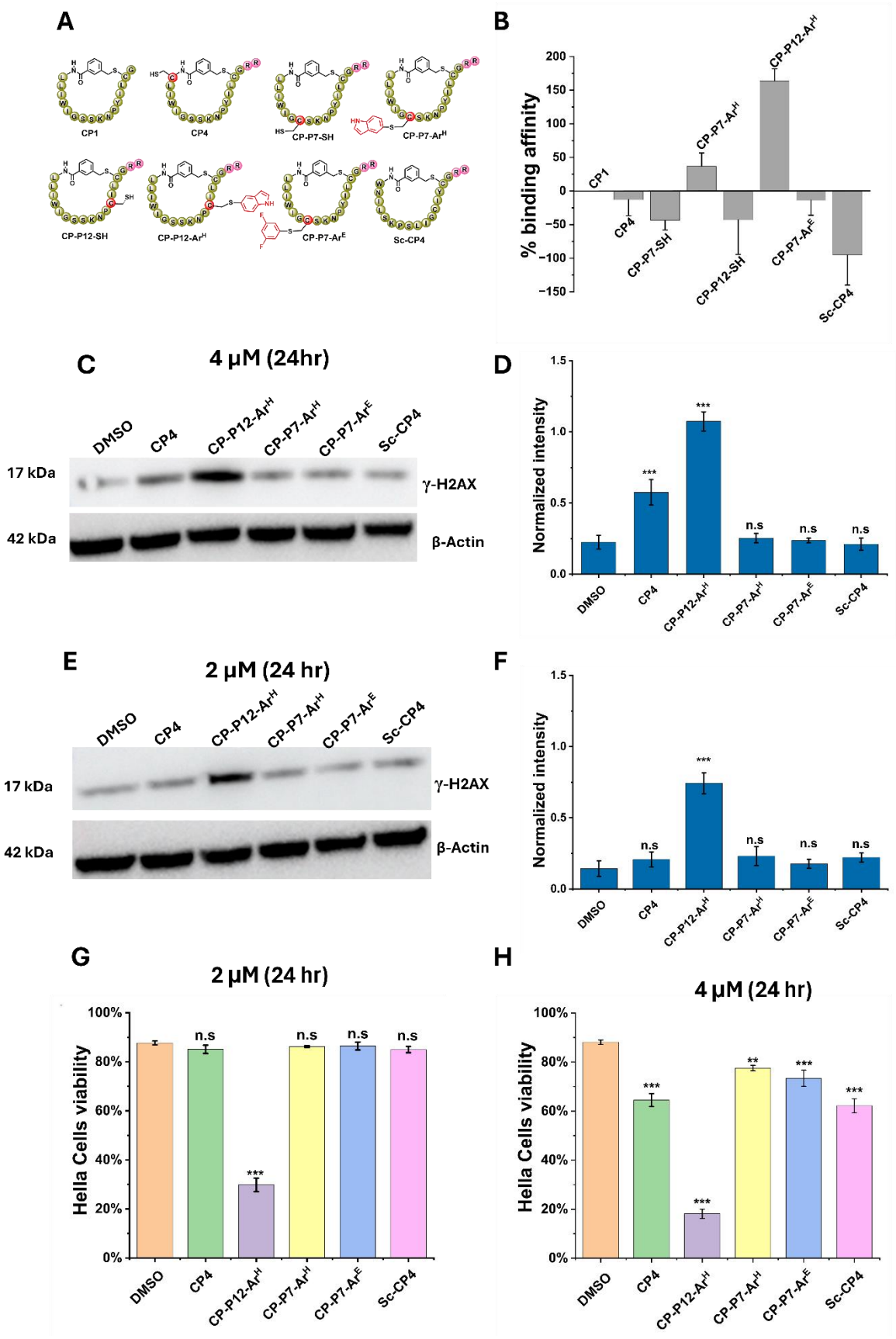


Figure 5. (A) The structure of **CP1**, **CP4**, leading derivatives **CP-P7-Ar^{H}** and **CP-P12-Ar^{H}**, non-modified peptides **CP-P7-SH** and **CP-P12-SH,** and negative controls **CP-P7-Ar^{E}** and **sc-CP4**. (B) Relative binding affinity of the indicated cyclic peptides to Lys-63-di-Ub, normalized to **CP1**. (C-D) and (E-F) WB analysis and band intensity quantification of γ-H2AX with the indicated cyclic peptides after incubation for 24 hours with HeLa cells at 4 μM and 2 μM, respectively. Data are presented as band intensity ± SE for three independent repeats. (G-H) HeLa cell viability of the cyclic peptide derivatives after incubation for 24 hours with HeLa cells at 2 μM or 4 μM. Data are presented as percentages of cell viability using apoptosis/necrosis assay ± SEM of three independent repeats with >7000 cells. n.s. indicates non-significant results; the level of significance between the indicated treatments is determined as follows: *p<=0.05, **p<0.01, ***p<0.001.

To validate the activity of our selected cyclic peptide (**CP-P12-Ar^{H}**), we tested its ability to induce the phosphorylation of H2AX using western blot analysis and subsequent apoptosis compared to **CP4**, **CP-P7-Ar^{E}**, **CP-P7-Ar^{H}**, and **sc-CP4**. Therefore, we synthesized and purified the cyclic peptides and incubated them with HeLa cells at concentrations of 2 μM and 4 μM for 24 hours. Following cell lysis, the western blot revealed that **CP-P12-Ar^{H}** treatment resulted in the highest increase in γ-H2AX compared to the other variants, including **CP-P7-Ar^{H}** (**Figure 5C-F**). Furthermore, we evaluated the ability of these derivatives to induce apoptosis and necrosis in HeLa cells. Consistent with the binding affinity and western blot experiments, our results indicated that **CP-P12-Ar^{H}** is the most effective modulator for inducing apoptosis (**Figure 5 G-H).**

Although the **CP7** peptide featuring a thioacetal linker[17] demonstrated an excellent ability to induce the phosphorylation of H2AX and trigger apoptosis, its binding affinity was lower than that of **CP4**. The cell permeability of **CP7** was dramatically increased, compensating for its lower binding affinity. However, while **CP7** exhibits high activity in disrupting DNA damage repair and inducing apoptosis, its lower affinity for Lys63-linked ubiquitin chains may limit its ability to modulate pathways that depend on high binding affinity for Lys63-specific interactions. We reasoned that the strong affinity of the selected cyclic peptide could be leveraged to modulate

Lys63-linked ubiquitin chain-dependent pathways that rely on a dynamic network of multivalent interactions between the chains and other proteins, such as in phase separation.

Lys63-linked ubiquitin chains are involved in diverse cellular signaling,[42] one of which is the NF-κB pathway.[43] Recently, it has been shown that the NEMO protein forms membrane-less organelles (MLO) driven by Lys63-linked and/or linear Met1-linked ubiquitin chains.[44,45] These condensates result from liquid-liquid phase separation (LLPS) driven by multivalent interactions between NEMO and ubiquitin chains. They are dynamic structures that can concentrate signaling molecules, thereby facilitating the activation of downstream signaling pathways, specifically the activation of the IKK complex in NF-κB signaling. These findings highlight the significance of such MLO in NF-κB signaling and the possible role of Lys63-linked ubiquitin chains in regulating downstream signaling and gene expression levels. However, the exact contributions and cross-linking of these chains to this process remain unclear.

Inspired by these findings, we sought to explore the ability of our Lys63-linked ubiquitin binders to modulate NEMO LLPS (**Figure 6A**) by disrupting the interaction between NEMO proteins and Lys63-linked ubiquitin chains. Therefore, we tested the capacity of the leading peptide, **CP-P12-Ar^{H}**, to inhibit this process *in vitro*.

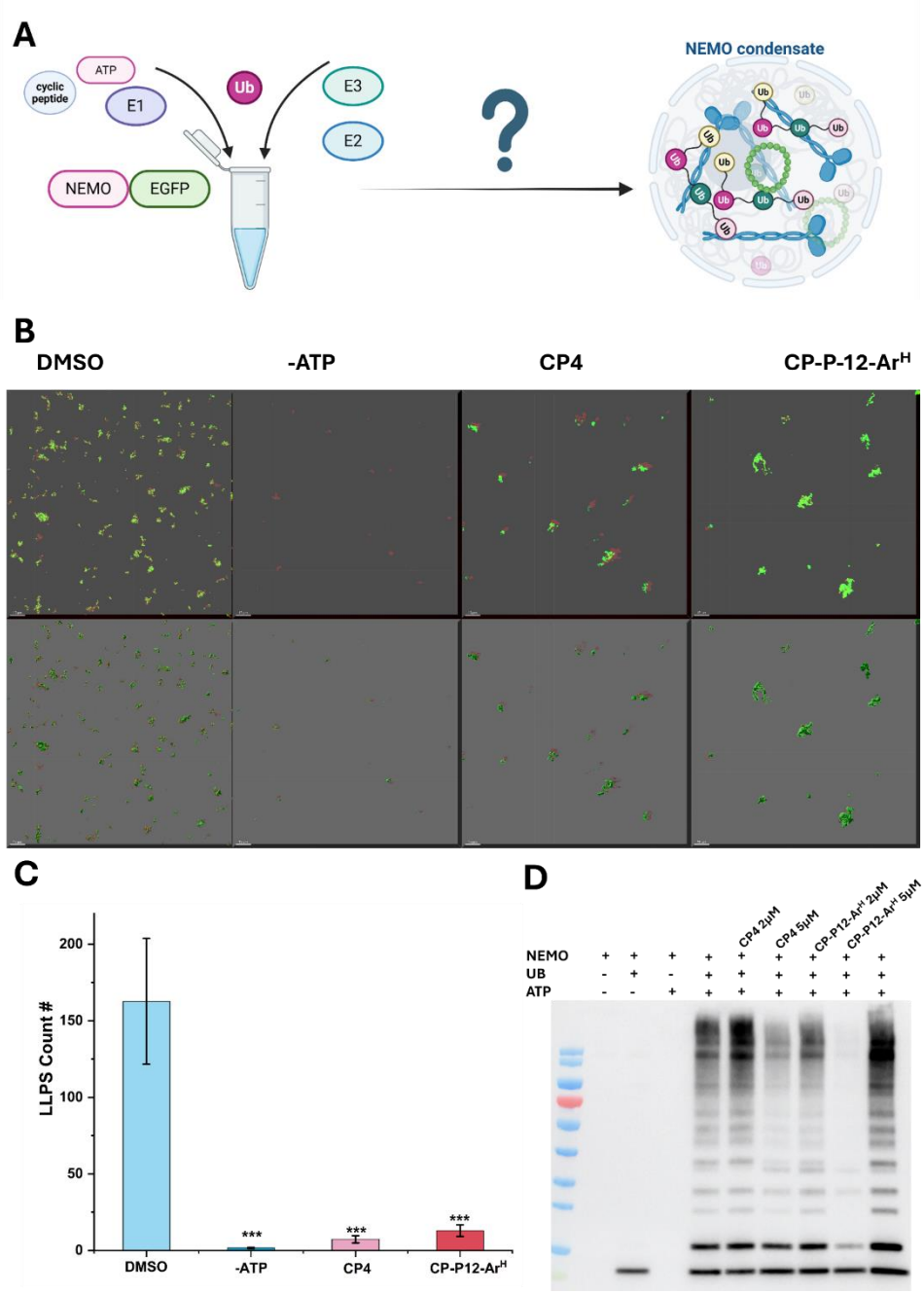


Figure 6. (A) A schematic representation of the inhibition of NEMO condensate using a cyclic peptide modulator of Lys-63 di-Ub. (B) Confocal microscopy images depict the inhibition of NEMO condensate under the indicated conditions and cyclic peptide treatment (5μM). The upper panel shows the overlay of the NEMO-GFP signal and Ub-TAMRA. The lower panel illustrates a surface reconstruction of the NEMO biomolecular condensates. (C) The count of NEMO condensates for the indicated treatment ± SEM from three different images. "n.s." indicates non-significant results, and the level of significance between the indicated treatments is determined as:

*p≤0.05, **p<0.01, ***p<0.001. (D) An in vitro ubiquitination assay using the enzymatic machinery for assembling Lys63-linked ubiquitin chains, which were treated with our cyclic peptides.

We expressed and purified the NEMO protein tagged with EGFP **(Figure S44)** and mixed it with the enzymatic machinery of Lys63-linked ubiquitin chains to form biomolecular condensates *in vitro*. Notably, it was shown that the NEMO condensates increased with the length of the Ub chain.[44,45] Considering that our modulator binds to Lys63-linked ubiquitin chains, we hypothesized that the cyclic peptide should inhibit the NEMO condensate through two possible mechanisms: first, by hindering the binding of the ubiquitin-binding domain of NEMO to the Lys63-linked ubiquitin chains, and second, by inhibiting ubiquitin elongation to form longer chains.

We tested our lead cyclic peptide, **CP-P12-Ar^{H}**, and compared its ability to inhibit NEMO condensate formation with that of **CP4**. We performed the ubiquitination reaction and observed NEMO condensate formation using a confocal microscope. Significant inhibition of NEMO puncta was observed when the samples were treated with either **CP4** or **CP-P12-Ar^{H}**, showing no considerable difference (**Figure 6B-C**). We then assessed the cyclic peptides' effectiveness in inhibiting the formation of Lys63-linked ubiquitin chains. Notably, **CP-P12-Ar^{H}** exhibited a significant reduction in ubiquitination compared to **CP4** (**Figure 6D**). Overall, although both cyclic peptides displayed similar inhibition in reducing the number of NEMO condensates, the inhibition of ubiquitination was significantly greater with **CP-P12-Ar^{H}** treatment.

Our results suggest that **CP4** may still interfere with the ubiquitin chain and NEMO protein, as its binding plays a crucial role in the formation of NEMO biomolecular condensates. However, the ability of **CP-P12-Ar^{H}** to inhibit NEMO condensates arises from its different binding affinity to the Lys63-linked ubiquitin chains and its interference with chain elongation. When we tested the

ability of **CP7** to inhibit NEMO condensates and chain elongation (**Figure S47**), we found minimal inhibition of these processes compared to **CP4**, **CP1**, and **CP-P12-Ar^{H}**, possibly due to its lower binding affinity.

Conclusion

This work demonstrates the successful integration of palladium-mediated Cys arylation with *in situ* cell-based screening to optimize cyclic peptide modulators targeting Lys63-linked ubiquitin chains rapidly. We identified **CP-P12-Ar^{H}** cyclic peptide, which is straightforwardly synthesized, with enhanced binding affinity and robust biological activity, including the promotion of DNA damage responses and apoptosis in cancer cells. Furthermore, it effectively inhibited Lys63-linked ubiquitin chain elongation and reduced NEMO protein biomolecular condensate formation. These findings lay the groundwork for a new avenue to specifically modulate Lys63-linked ubiquitin chain processes, either by inhibiting their binding to other proteins or by preventing chain elongation. These modulators should enable us to gain insights into the downstream signaling of the NF-κB pathway, which is mainly regulated by linear and Lys63 chains. Although extensive reports have been published on this area, the crosstalk and interplay between linear and Lys63 chains remain elusive.[42,45,46] By leveraging the advantages of our modulator and combining it with recently reported HOIPIN inhibitors,[47,48] we believe that NF-κB pathway modulation can be further advanced, providing more comprehensive details on NF-κB signaling.

Regarding Pd-mediated S-arylation and its compatibility for enabling rapid diversification of lead compounds, along with in situ cell screening, this approach can be extended to various biological systems by leveraging the vast array of commercially available aryl halides, which facilitates high-throughput structure–activity relationship studies. Importantly, the power of organometallic chemistry in this context is not limited to palladium and Cys arylation. Several groups have also

demonstrated that other metals, such as gold,[49] and nickel[50] enable high-fidelity Cys arylation, thereby broadening the toolkit for such an approach.[51] Furthermore, similar strategies can be developed for lysine arylation,[52] and possibly other side chains, which could expand the chemical space accessible for late-stage diversification and functional interrogation of bioactive molecules.

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Author contribution

M.H. performed the purification of NEMO, carried out the *in vitro* LLPS experiments, executed the W.B. analyses, conducted the apoptosis assays, measured binding affinities, analyzed data for the screening experiments, and assisted with peptide synthesis. N.V. synthesized the cyclic peptide and the Pd-aryls and performed the screening experiments. U.G. and Y.S. contributed to peptide synthesis. J.G. synthesized the TAMRA–ubiquitin conjugate. A.B. conceptualized and designed the study, supervising the entire project. H.S. was responsible for selecting the early cyclic peptides used in this project. A.B. and M.H. wrote the manuscript with contributions from all authors.

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