Supplementary Information for:

Diversity-oriented stapling yields intrinsically cell-penetrant inducers of autophagy

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*corresponding authors

Supplementary Figures, Tables and Methods

Supplementary Figure 1. Chemical structures of selected compounds.
**SI Table 1. Peptide sequences and observed masses following HPLC purification.**

| Name              | Peptide structure | (cap)-Sequence | Linker | Calculated [M+H⁺] | Observed [M+H⁺] |
|-------------------|-------------------|----------------|--------|-------------------|-----------------|
| Tat-Beclin 1      | YGRKKRRQRRRGG     | TNVFNATFEIWHDGEFGT | -      | 3741.2            | 3742.9          |
| Tat-Bec1 Mini     | YGRKKRRQRRRGG     | VFNATFEIWHD    | -      | 3034.5            | 3034.0          |
| Tat-Bec1 Mini-W2  | YGRKKRRQRRRGG     | VWNATFEIWHD    | -      | 3073.5            | 3075.5          |
| Tat-11mer         | YGRKKRRQRRRGG     | VWNATFIWHD     | -      | 3079.6            | 3079.7          |
| Tat-11mer ΔV1     | YGRKKRRQRRRGG     | VWNATFIWHD     | -      | 2982.4            | 2983.7          |
| Tat-11mer ΔD11    | YGRKKRRQRRRGG     | VWNATFIWHD     | -      | 2966.5            | 2967.8          |
| Tat-11mer ΔV1D11  | YGRKKRRQRRRGG     | VWNATFIWHD     | -      | 2867.3            | 2869.0          |
| Tat-11mer ΔV1H9D11| YGRKKRRQRRRGG     | VWNATFIWH      | -      | 2730.2            | 2732.3          |
| Tat-Bec1 Mini ΔD11| YGRKKRRQRRRGG     | VFNATFEIWH     | -      | 2919.4            | 2920.6          |
| D-(Tat-11mer)     | YRRRQRRKKRGYYGG   | dhwiftanwv     | -      | 3079.7            | 3080.4          |
| Tat-11mer, N3A    | YGRKKRRQRRRGG     | VWAATFIWHD     | -      | 3038.5            | 3039.9          |
| Tat-11mer, T5A    | YGRKKRRQRRRGG     | VWNAAFHIWHD    | -      | 3051.5            | 3053.6          |
| Tat-11mer, F6A    | YGRKKRRQRRRGG     | VWNATAHIWHD    | -      | 3005.4            | 3005.9          |
| Tat-11mer, H7A    | YGRKKRRQRRRGG     | VWNATFAIWHD    | -      | 3015.5            | 3017.5          |
| Tat-11mer, I8A    | YGRKKRRQRRRGG     | VWNATFHAIWHD   | -      | 3039.5            | 3041.1          |
| Tat-11mer, W9A    | YGRKKRRQRRRGG     | VWNATFHIAHD    | -      | 2966.4            | 2968.1          |
| Tat-11mer, H10A   | YGRKKRRQRRRGG     | VWNATFIWAD     | -      | 3015.5            | 3017.4          |
| Tat-11mer, W2A    | YGRKKRRQRRRGG     | VANATFIWHD     | -      | 2966.4            | 2968.1          |
| Tat-11mer, W2S    | YGRKKRRQRRRGG     | VSNATFIWHD     | -      | 2980.6            | 2980.5          |
| Tat-11mer, W2F F6S| YGRKKRRQRRRGG     | VFNATSHIWHD    | -      | 2982.4            | 2983.8          |
| Tat-11Scr         | YGRKKRRQRRRGG     | WNHADEDHTFWI   | -      | 3079.6            | 3079.8          |
| Tat-Bec1Scr       | YGRKKRRQRRRGG     | VQNDFFINHETTGFAEW| -      | 3738.9            | 3738.9          |
| pa-11mer          | (pa)-VWNATFIWHD   | -              |        | 1504.7            | 1504.0          |
| pa-10mer          | (pa)-VWNATFIWH    | -              |        | 1389.6            | 1388.5          |
| D6D10-o           | (pa)-VWNATcHIWc   | ortho-xylene   | 1413.7 | 1411.9            |
| D6D10-m           | (pa)-VWNATcHIWc   | meta-xylene    | 1413.7 | 1413.0            |
| D6L10-o           | (pa)-VWNATcHIWC   | ortho-xylene   | 1413.7 | 1413.2            |
| Compound          |Sequence| Description     | m/z 1   | m/z 2   |
|-------------------|--------|-----------------|---------|---------|
| D6L10-m           | (pa)-VWNATcHIWC | meta-xylene | 1413.7 | 1412.4 |
| D6L10-allyl       | (pa)-VWNATcHIWC | allyl       | 1391.7 | 1391.0 |
| D6D11-o           | (pa)-VWNATFcIWHc | ortho-xylene | 1560.9 | 1561.6 |
| D6D11-m           | (pa)-VWNATFcIWHc | meta-xylene | 1560.9 | 1562.0 |
| D6D11-allyl       | (pa)-VWNATFcIWHc | allyl       | 1538.9 | 1539.1 |
| DD6-o             | (pa)-VcNATcHIWH | ortho-xylene | 1364.6 | 1363.9 |
| DD6-m             | (pa)-VcNATcHIWH | meta-xylene | 1364.4 | 1363.9 |
| DD6-allyl         | (pa)-VcNATcHIWH | allyl       | 1342.6 | 1342.7 |
| DD6-nap           | (pa)-VcNATcHIWH | 2,6-naphthlene | 1414.7 | 1415.0 |
| DD6-phe           | (pa)-VcNATcHIWH | 4,4-biphenyl | 1440.7 | 1440.1 |
| DL6-allyl         | (pa)-VcNATcHIWH | allyl       | 1342.6 | 1341.9 |
| LD6-allyl         | (pa)-VcNATcHIWH | allyl       | 1343.6 | 1342.1 |
| LL6-p             | (pa)-VCNATCHIWH | para-xylene | 1364.6 | 1363.6 |
| LL6-nap           | (pa)-VCNATCHIWH | 2,6-naphthlene | 1414.7 | 1412.5 |
| LL6-phe           | (pa)-VCNATCHIWH | ortho-xylene | 1440.7 | 1441.2 |
| DD5-o             | (pa)-VcNACFHIWH | ortho-xylene | 1410.7 | 1410.8 |
| DD5-m             | (pa)-VcNACFHIWH | meta-xylene | 1410.7 | 1411.2 |
| DD5-p             | (pa)-VcNACFHIWH | para-xylene | 1410.7 | 1410.8 |
| DD5-allyl         | (pa)-VcNACFHIWH | allyl       | 1388.7 | 1388.8 |
| DL5-o             | (pa)-VcNACFHIWH | ortho-xylene | 1410.7 | 1411.3 |
| LD5-o             | (pa)-VCNACFHIWH | ortho-xylene | 1410.7 | 1411.1 |
| LL5-o             | (pa)-VCNACFHIWH | ortho-xylene | 1410.7 | 1410.9 |
| Ac-DD5-o          | (acetyl)-VcNACFHIWH | ortho-xylene | 1326.6 | 1327.6 |
| H2N-DD5-o         | VcNACFHIWH      | ortho-xylene | 1284.5 | 1284.1 |
| nicot-DD5-o       | (nicot)-VcNACFHIWH | ortho-xylene | 1435.7 | 1436.2 |
| phenyl-DD5-o      | (phenyl)-VcNACFHIWH | ortho-xylene | 1448.7 | 1449.6 |
| benzo-DD5-o       | (benzo)-VcNACFHIWH | ortho-xylene | 1434.7 | 1433.8 |
| hexano-DD5-o      | (hexano)-VcNACFHIWH | ortho-xylene | 1428.7 | 1428.6 |
| hexyno-DD5-o      | (hexyno)-VcNACFHIWH | ortho-xylene | 1424.7 | 1424.3 |
| cyano-DD5-o       | (cyano)-VcNACFHIWH | ortho-xylene | 1411.7 | 1411.6 |
| pentene-DD5-o     | (pentene)-VcNACFHIWH | ortho-xylene | 1412.7 | 1412.6 |
| DD5-o His10Ala    | (pa)-VcNACFHIWA | ortho-xylene | 1345.6 | 1345.2 |
| DD5-o Trp9Ala     | (pa)-VcNACFHIAH | ortho-xylene | 1296.5 | 1296.3 |
| DD5-o Ile8Ala     | (pa)-VcNACFHAWH | ortho-xylene | 1369.6 | 1369.5 |
| DD5-o His7Ala     | (pa)-VcNACFALH | ortho-xylene | 1345.6 | 1344.7 |
| DD5-o Phe6Ala     | (pa)-VcNACFIWH | ortho-xylene | 1335.6 | 1335.0 |
| DD5-o Asn3Ala     | (pa)-VcNAcFIWH | ortho-xylene | 1368.6 | 1368.2 |
| DD5-o Val1Ala     | (pa)-AcNACFHIWH | ortho-xylene | 1383.6 | 1383.0 |
| HT-DD5-o          | (HT)-VcNACFHIWH | ortho-xylene | 1636.4 | 1636.5 |
| HT-DD5-neg        | (HT)-VcNACFHIWH | acetic acid   | 1650.3 | 1649.4 |
| HT-Tat-Beclin 1   | (HT)-YGRKKRRQRRRGG TNVFNATFEIWHGDGEFGT | - | 4046.0 | 4048.0 |
| HT-Tat-11mer      | (HT)-YGRKKRRQRRRGG VWNATFHIWHD | - | 3386.3 | 3386.5 |
SI Table 1. Lowercase letters denote D-amino acids. **Linker** specifies the chemical group attached to the two cysteines via thioether bonds. Peptides with the allyl linker are linear peptides in which both cysteines were alkylated using allyl bromide. Peptides had free N-termini, or were N-terminally capped with following carboxylic acids: (pa) = pentynoic acid, (ac) = acetic acid, (nicot) = nicotinic acid, (phenyl) = phenylacetic acid, (benzo) = benzoic acid, (hexane) = hexanoic acid, (hexyno) = hexynoic acid, (cyano) = 3-cyanopropanoic acid, (pentene) = 4-pentenoic acid, (HT) = HaloTag carboxylic acid, as shown for HT-DD5-o in SI Fig 16. Masses of purified peptides were measured by MALDI-TOF mass spectrometry.

SI Figure 2. **Truncation and substitution of Tat-Beclin 1 to produce Tat-11mer.** Autophagy induction in HeLa cells, analyzed using p62 and LC3 immunoblots, for truncations of Tat-Beclin 1 and analogs of the truncated sequences. Sequences are shown in SI Table 1. Tat-11mer is the optimized peptide derived from Tat-Beclin 1.

SI Figure 3. **Identification of key residues for Tat-11mer.** a. Autophagy induction in HeLa cells, analyzed using p62 and LC3 immunoblots, for key analogs of Tat-11mer. Two exposures are shown for p62 blots, short and long. Actin is shown as a loading control. Alanine scan peptides for Tat-11mer were tested, as well as peptide Tat-Bec1 Mini Δ11, and the retro inverso version of Tat-11mer, D-(Tat-11mer). All peptides were tested at 10 μM, except for D-(Tat-11mer) tested at 10 and 20 μM. b. Sequences of Tat-11mer alanine scan peptides tested in a. c. Additional controls for Tat-11mer including Phe to Ser substitutions and a scrambled version of Tat-11mer, Tat-11scr. All peptides were tested at 10 μM.
SI Figure 4. \textit{pa-10mer has minimal effect on autophagic flux.} A GFP-LC3 puncta assay in HeLa cells, identical to the assay shown in Fig. 3f, was used to quantify the effect of \textit{pa-10mer} on autophagic flux. \textit{pa-10mer} showed a very mild effect on autophagic flux, and this effect was not dose-dependent. GFP-LC3 HeLa were treated with peptide at the indicated concentration, with or without bafilomycin A1 (BafA1). Similar results were obtained in at least 3 independent experiments. Bars represent mean +/- s.e.m. for triplicate samples (at least 100 cells analyzed per sample). * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$ by t-test for indicated group vs. DMSO control.

SI Figure 5. Location of staple within peptide sequence affects activity. Autophagy induction in HeLa cells, analyzed using p62 and LC3 immunoblots. Actin is shown as a loading control. These blots show that no autophagy induction was observed when the staple was moved to the C-terminal end of the Beclin 1-derived sequence. The stereochemistry of the cysteines was varied and three different linkers were used (ortho-xylene, meta-xylene and allyl), but for all peptides no autophagy induction was observed. Concentrations are noted in micromolar. Peptide sequences are given in SI Table 1.
SI Figure 6. Linker structure affects autophagy-inducing activity for DD6-series peptides. Autophagy induction in HeLa cells for additional peptides, analyzed using p62 and LC3 immunoblots. Actin is shown as a loading control. Peptide Tat-Bec1 Mini ΔD11 (labeled #25) is shown as a positive control. A variety of DD6-m analogs were tested, varying the size and type of linker as well as the stereochemistry of the cysteines. DD6-m and DL6-allyl induced autophagy at 100 μM. Concentrations are noted in micromolar. Peptide sequences are given in SI Table 1.

SI Figure 7. N-terminal cap of DD5-o affects autophagy induction. Autophagy induction in HeLa cells for additional peptides, analyzed using p62 and LC3 immunoblots. Actin is shown as a loading control. Activity was observed for analogs of DD5-o with a benzoic acid cap, a hexanoic acid cap and a pentenoic acid cap at 50 μM. In contrast, no activity was observed for the nicotinic acid cap. Concentrations are noted in micromolar. Peptide sequences are given in SI Table 1.

SI Figure 8. Alanine scan for DD5-o. Autophagy induction in HeLa cells for additional peptides, analyzed using p62 and LC3 immunoblots. Actin is shown as a loading control. Analogs of DD5-o with alanine substitutions in each position (except D-Cys2, Ala4, and D-Cys5) were synthesized and tested. Substitution of Ile8, Phe6, or Val1 leads to complete loss of activity. Trp9Ala has only mild activity at 100 μM. His10Ala is 5-fold worse in activity than DD5-o, with induction at 100 μM. Concentrations are noted in micromolar. Peptide sequences are given in SI Table 1.
**SI Methods: solution structure NMR of DD5-o in methanol**

All the NMR experiments were carried out using a Bruker 500 MHz spectrometer. Peptide DD5-o was dissolved in CD$_3$OH at a concentration of roughly 2 mM. Complete resonance assignments were achieved using a combination of homonuclear $^1$H-$^1$H COSY, TOCSY and ROESY experiments at 289 K. Standard pulse programs available in the Bruker library were used for all experiments. The residual methyl signal in CD$_3$OH was used as an internal standard for chemical shift referencing.

NMR spectra were processed in Bruker Topspin software and imported into CcpNMR Analysis v2.4.2 for assignments and to generate distance constraints. A total of 114 NOEs were compiled, including 12 medium- and long-range NOEs. Three phi dihedral angle constraints, derived from JN$_{H-C_\alpha}$H coupling constants, were also compiled. These were used as constraints in simulated annealing experiments using CNS Solve version 1.3. Simulated annealing involved a high-temperature annealing stage of 1000 steps, followed by two slow-cooling stages, each 1000 steps, which was then followed by a 10 cycles of 200 steps of energy minimization. Structure calculations were iterated until the distance and dihedral violations were completely resolved. A total of 25-lowest energy structures with no NOE and dihedral angle violation greater than 0.1 Å and 5°, respectively, were then selected for further analysis.

**SI Figure 9.** $^1$H 1D NMR spectrum of DD5-o in CD$_3$OH at 289 K. The well-resolved, sharp peaks are indicative of a high degree of overall structure.
SI Figure 10. $^1$H-$^1$H COSY NMR spectrum of DD5-β in CD$_3$OH at 289 K.
SI Figure 11. $^1$H-$^1$H TOCSY NMR spectrum of DD5-o in CD$_3$OH at 289 K.
SI Figure 12. $^1$H-$^1$H ROESY NMR spectrum of DD5-o in CD$_3$OH at 289 K.
## SI Table 2. \(^1\)H chemical shifts for DD5-o at 289 K in CD\(_3\)OH.

| Residue | \(H_N\) | \(H_{\alpha}\) | \(H_{\beta}\) | other protons |
|---------|---------|---------|---------|--------------|
| Val2    | 8.54    | 3.86    | 2.10    | \(\gamma = 1.11, 1.10\) |
| D-Cys3  | 8.82    | 4.63    | 3.06, 3.38 | - |
| Asn4    | 8.43    | 4.46    | 2.79, 2.92 | \(\delta_2 = 7.12, 7.74\) |
| Ala5    | 8.32    | 4.07    | 1.45    | - |
| D-Cys6  | 7.87    | 3.65    | 2.92, 3.02 | - |
| Phe7    | 7.93    | 4.31    | 3.02, 3.18 | \(\delta = 7.17; \varepsilon = 7.17; \zeta = 7.16\) |
| His8    | 8.15    | 4.42    | 3.29, 3.39 | \(\delta_2 = 7.48; \epsilon_1 = 8.81\) |
| Ile9    | 7.84    | 3.96    | 1.84, 2.12 | \(\gamma_1 = 1.16, 1.63; \gamma_2 = 0.84; \delta_1 = 0.75\) |
| Trp10   | 8.06    | 4.47    | 3.06, 3.16 | \(\delta_1 = 7.23; \epsilon_1 = 10.51; \epsilon_3 = 7.55; \zeta_2 = 7.34; \zeta_3 = 7.13; \eta_2 = 7.01\) |
| His11   | 7.94    | 4.59    | 2.82, 3.19 | \(\delta_2 = 7.16; \epsilon_1 = 8.68\) |

Note: for software compatibility purposes, the pentylic acid cap was numbered residue 1, the first amino acid (Val) was numbered 2, and so on.

## SI Table 3. List of NOE-derived distance constraints and \(\phi\) dihedral angle restraints used to calculate solution NMR structures of DD5-o.

**Distance constraints:**

assign ( resid 2 and name HN ) ( resid 2 and name HA ) 3.1 0.6 0.6
assign ( resid 2 and name HN ) ( resid 2 and name HB ) 3.0 0.6 0.6
assign ( resid 2 and name HN ) ( resid 2 and name HG2# ) 3.1 0.6 0.6
assign ( resid 2 and name HA ) ( resid 2 and name HB ) 3.0 0.6 0.6
assign ( resid 2 and name HA ) ( resid 2 and name HG2# ) 2.7 0.5 0.5
assign ( resid 2 and name HA ) ( resid 2 and name HG1# ) 2.7 0.5 0.5
assign ( resid 2 and name HA ) ( resid 3 and name HB1 ) 4.2 0.7 0.7
assign ( resid 2 and name HA ) ( resid 5 and name HB1 ) 3.8 0.8 0.8
assign ( resid 3 and name HN ) ( resid 2 and name HA ) 2.6 0.5 0.5
assign ( resid 3 and name HN ) ( resid 2 and name HB ) 4.3 0.9 0.9
assign ( resid 3 and name HN ) ( resid 2 and name HG2# ) 4.0 0.8 0.8
assign ( resid 3 and name HN ) ( resid 3 and name HA ) 3.0 0.7 0.7
assign ( resid 3 and name HN ) ( resid 3 and name HB2 ) 3.9 0.8 0.8
assign ( resid 3 and name HN ) ( resid 3 and name HB1 ) 3.9 0.8 0.8
assign ( resid 3 and name HA ) ( resid 3 and name HB2 ) 3.1 0.6 0.6
assign ( resid 3 and name HA ) ( resid 3 and name HB1 ) 3.2 0.7 0.7
assign ( resid 3 and name HB1 ) ( resid 3 and name HB2 ) 2.3 0.5 0.5
assign ( resid 3 and name HN ) ( resid 4 and name HN ) 3.5 0.8 0.8
assign ( resid 4 and name HN ) ( resid 2 and name HA ) 4.1 0.8 0.8
assign ( resid 4 and name HN ) ( resid 3 and name HA ) 3.5 0.8 0.8
assign ( resid 4 and name HN ) ( resid 4 and name HA ) 3.0 0.6 0.6
assign ( resid 4 and name HN ) ( resid 4 and name HB1 ) 3.6 0.7 0.7
assign ( resid 4 and name HN ) ( resid 4 and name HB2 ) 2.9 0.6 0.6
assign ( resid 4 and name HD21 ) ( resid 4 and name HB1 ) 2.9 0.7 0.7
assign ( resid 4 and name HD21 ) ( resid 4 and name HB2 ) 3.3 0.7 0.7
assign ( resid 4 and name HA ) ( resid 4 and name HB2 ) 3.0 0.6 0.6
assign ( resid 4 and name HA ) ( resid 4 and name HB1 ) 2.8 0.6 0.6
assign ( resid 4 and name HD22 ) ( resid 4 and name HB1 ) 3.4 0.7 0.7
assign ( resid 4 and name HD21 ) ( resid 4 and name HA ) 4.2 0.8 0.8
assign ( resid 5 and name HN ) ( resid 2 and name HA ) 4.1 0.9 0.9
assign ( resid 5 and name HN ) ( resid 4 and name HA ) 3.8 0.8 0.8
assign ( resid 5 and name HN ) ( resid 4 and name HB1 ) 3.6 0.8 0.8

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assign (resid 5 and name HN) (resid 4 and name HB2) 4.1 0.8 0.8
assign (resid 5 and name HN) (resid 4 and name HN) 3.4 0.7 0.7
assign (resid 5 and name HN) (resid 5 and name HA) 2.9 0.6 0.6
assign (resid 5 and name HN) (resid 5 and name HB1) 3.0 0.6 0.6
assign (resid 5 and name HA) (resid 5 and name HB#) 2.7 0.5 0.5
assign (resid 5 and name HN) (resid 6 and name HN) 3.1 0.7 0.7
assign (resid 6 and name HN) (resid 2 and name HA) 4.2 0.8 0.8
assign (resid 6 and name HN) (resid 5 and name HA) 3.9 0.8 0.8
assign (resid 6 and name HN) (resid 5 and name HB#) 2.8 0.5 0.5
assign (resid 6 and name HN) (resid 6 and name HA) 2.7 0.5 0.5
assign (resid 6 and name HN) (resid 6 and name HB1) 3.0 0.6 0.6
assign (resid 6 and name HN) (resid 7 and name HN) 3.4 0.7 0.7
assign (resid 7 and name HN) (resid 4 and name HA) 3.3 0.6 0.6
assign (resid 7 and name HN) (resid 6 and name HA) 3.1 0.6 0.6
assign (resid 7 and name HA) (resid 7 and name HB2) 2.9 0.6 0.6
assign (resid 7 and name HA) (resid 7 and name HB1) 2.9 0.6 0.6
assign (resid 7 and name HN) (resid 7 and name HB1) 3.0 0.6 0.6
assign (resid 7 and name HN) (resid 7 and name HB2) 3.1 0.7 0.7
assign (resid 7 and name HN) (resid 4 and name HA) 3.3 0.6 0.6
assign (resid 7 and name HN) (resid 6 and name HA) 3.1 0.6 0.6
assign (resid 7 and name HN) (resid 7 and name HA) 3.1 0.6 0.6
assign (resid 7 and name HA) (resid 7 and name HB2) 2.9 0.6 0.6
assign (resid 7 and name HA) (resid 7 and name HB1) 2.9 0.6 0.6
assign (resid 7 and name HN) (resid 7 and name HB1) 3.0 0.6 0.6
assign (resid 7 and name HN) (resid 7 and name HB2) 3.1 0.7 0.7
assign (resid 8 and name HN) (resid 5 and name HA) 3.8 0.8 0.8
assign (resid 8 and name HD2) (resid 5 and name HA) 3.6 0.7 0.7
assign (resid 8 and name HN) (resid 7 and name HA) 3.3 0.7 0.7
assign (resid 8 and name HN) (resid 7 and name HB2) 3.6 0.7 0.7
assign (resid 8 and name HN) (resid 7 and name HB1) 3.1 0.6 0.6
assign (resid 8 and name HN) (resid 8 and name HA) 3.0 0.6 0.6
assign (resid 8 and name HN) (resid 8 and name HB2) 2.8 0.7 0.7
assign (resid 8 and name HD2) (resid 8 and name HB2) 3.6 0.7 0.7
assign (resid 8 and name HD2) (resid 8 and name HB1) 3.6 0.7 0.7
assign (resid 8 and name HD2) (resid 8 and name HA) 3.8 0.7 0.7
assign (resid 8 and name HD2) (resid 8 and name HG1) 4.4 0.9 0.9
assign (resid 8 and name HD2) (resid 9 and name HA) 4.4 0.9 0.9
assign (resid 8 and name HN) (resid 9 and name HA) 3.8 0.7 0.7
assign (resid 9 and name HB) (resid 6 and name HB1) 3.5 0.7 0.7
assign (resid 9 and name HN) (resid 8 and name HA) 3.2 0.6 0.6
assign (resid 9 and name HN) (resid 8 and name HB2) 4.1 0.8 0.8
assign (resid 9 and name HN) (resid 9 and name HB) 3.6 0.8 0.8
assign (resid 9 and name HN) (resid 9 and name HB) 3.2 0.6 0.6
assign (resid 9 and name HN) (resid 9 and name HB) 2.8 0.6 0.6
assign (resid 9 and name HN) (resid 9 and name HG1) 3.8 0.7 0.7
assign (resid 9 and name HN) (resid 9 and name HG2) 3.2 0.7 0.7
assign (resid 9 and name HN) (resid 9 and name HG2) 3.2 0.7 0.7
assign (resid 9 and name HG1) (resid 9 and name HB) 3.6 0.7 0.7
assign (resid 9 and name HG1) (resid 9 and name HB) 3.4 0.7 0.7
assign (resid 10 and name HN) (resid 7 and name HA) 4.2 0.8 0.8
assign (resid 10 and name HE3) (resid 7 and name HA) 4.2 0.8 0.8
assign (resid 10 and name HN) (resid 9 and name HA) 4.2 0.8 0.8
assign (resid 10 and name HN) (resid 9 and name HN) 3.6 0.8 0.8
assign (resid 10 and name HN) (resid 9 and name HB) 3.6 0.8 0.8
assign (resid 10 and name HN) (resid 9 and name HB) 3.8 0.7 0.7
assign (resid 10 and name HD1) (resid 9 and name HD1) 4.0 0.8 0.8
assign (resid 10 and name HD1) (resid 9 and name HB) 4.0 0.8 0.8
assign (resid 10 and name HN) (resid 10 and name HA) 3.1 0.6 0.6
assign (resid 10 and name HN) (resid 10 and name HB1) 3.0 0.6 0.6
assign (resid 10 and name HN) (resid 10 and name HB2) 3.3 0.7 0.7
assign (resid 10 and name HA) (resid 10 and name HB1) 3.0 0.7 0.7
assign (resid 10 and name HA) (resid 10 and name HB2) 2.8 0.6 0.6
assign (resid 10 and name HE3) (resid 10 and name HB1) 3.2 0.8 0.8
assign (resid 10 and name HE3) (resid 10 and name HB2) 3.2 0.7 0.7
assign (resid 10 and name HE3) (resid 10 and name HA) 3.8 0.6 0.6
assign (resid 10 and name HD1) (resid 10 and name HB2) 3.5 0.7 0.7
assign (resid 10 and name HD1) (resid 10 and name HB1) 3.3 0.7 0.7
Si Table 4. NMR structural data and refinement statistics for DD5-o.

| NMR distance and dihedral constraints | DD5-o |
|--------------------------------------|-------|
| **Distance constraints**             |       |
| Total NOE                            | 114   |
| Intra-residue                        | 72    |
| Inter-residue                        | 42    |
| Sequential (|i – j| = 1) | 30    |
| Medium-range to long range (|i – j| ≥ 2) | 12    |
| φ dihedral angle restraints          | 3     |

| Structure statistics                 |       |
|--------------------------------------|-------|
| Violations                           | 0     |
| Distance violations >0.1 (Å)         | 0     |
| Dihedral angle violations > 5 (°)    | 0     |
| Deviations from idealized geometry   |       |
| Bond lengths (Å)                     | 0.0043 ± 0.00017 |
| Bond angles (°)                      | 0.4159 ± 0.0181 |
| Impropers (°)                        | 0.2210 ± 0.0167 |
| Coordinate precision                 |       |
| Heavy (Å)                            | 1.101 |
| Backbone (Å)                         | 0.449 |
Figure 1. Secondary structure analysis of DD5-o using NMR chemical shift values and CD spectroscopy. a. Residue-by-residue deviation of Hα chemical shifts with respect to random coil values. Large, negative deviations across the peptide are consistent with a well-folded helical structure for DD5-o. For the alkylated D-Cys residues, the Hα chemical shift for an oxidized L-Cys was used. Random coil Hα chemical shifts were obtained from BMRB-Biological Magnetic Resonance Bank database, which are reported in water. However, a good correlation has been found between Hα chemical shifts in CD3OD and those reported in water.1 b. CD spectrum of 0.1 mg/mL DD5-o in methanol. While it is not straightforward to calculate percent helicity using methanol-derived CD data, the helical signature supports the NMR results.

Methods: molecular dynamics simulations of DD5-o in explicit water

Molecular dynamics (MD) simulations were performed with the Gromacs 4.6.7 engine2 in conjunction with the CHARMM22 force field3 with CMAP correction.4 The parameters for the D-Cys residue and the o-xyl linker were determined based on chemical similarity to already-defined atom types (see SI Fig. 14). The TIP3P water model5 was used for solvent molecules. The average NMR structure, as solved in methanol, was used as the input configuration for the simulation. In the MD simulation, the N-terminal 4-pentynoic acid cap was replaced by an acetyl group, and the double protonation state for the two His residues was used. After an energy minimization of 1000 steps in vacuum, the peptide was solvated in a cubic water box. The dimension of the water box was chosen such that the minimum distance between the peptide and the box edges was 10 Å. Two Cl− ions were added to neutralize the net charge of the system. The solvated system was optimized for 5000 steps using the steepest descent algorithm to remove any bad contacts. With all peptide heavy atoms restrained to their initial positions, the minimized system was heated from 5K to 300K within 20 ps and relaxed for additional 30 ps. Before production, the system was further equilibrated for 100 ps with the peptide backbone atoms remain fixed.

The production simulation was performed in the NPT (isothermal-isobaric) ensemble at 300K / 1 bar. The temperature was controlled using the Nosé-Hoover thermostat6,7 with a coupling constant of 1.0 ps. To alleviate the “hot-solvent/cold-solute” artifact,8,9 two separate thermostats were applied to both the peptide and the solvent molecules. The pressure of the system was maintained using an isotropic Berendsen barostat,10 with a coupling time of 2.0 ps and a compressibility of 4.5×10−5 bar−1. All bonds were constrained with the LINCS algorithm11 to enable the use of a 2 fs time step with the leap-frog algorithm.12 The non-bonded interactions (Lennard-Jones and Columbic) were truncated at 8 Å. Long-range Columbic interactions beyond the cut-off distance were treated using the particle mesh Ewald (PME) summation method.13 A long-range analytic dispersion correction was applied to both the energy and pressure to account for the truncation of Lennard-Jones interactions.14 The production simulation was performed for 100 ns. In the production simulation, the C-terminal residues underwent side-chain reorganization and formed a related (slightly more α-helical) structure in water. Once formed, the structure was relatively stable during the rest of the simulation (see Supplemental Movie S1 for a representative 100 ns trajectory). This behavior was observed in three independent runs, which each started from different initial velocities (SI Fig. 15).
SI Figure 14. Atom type definitions applied to linker atoms for molecular dynamics simulations. CHARMM atom types and charges are noted for each atom in each D-Cys residue and the ortho-xylene linker.

SI Figure 15. Three independent 100-ns trajectories for DD5-o. These graphs illustrate overall secondary structure, as calculated using STRIDE, for residue during each 100 ns production run.
SI Figure 16. HT-DD5-o induces autophagy similarly to DD5-o. Autophagy induction in HeLa cells for HaloTag-labeled DD5-o, analyzed using p62 and LC3 immunoblots. Actin is shown as a loading control. These results show that HT-DD5-o induces autophagy at 20 μM, to a similar extent as the pentylnyl-capped DD5-o. Concentrations are noted in micromolar. Peptide sequences are given in SI Table S1.

SI Figure 17. Flow cytometry data from CAPA. These plots show a representative replicate of raw data obtained from CAPA. a. Each measurement involved 10,000 cells, gated as shown to count only live cells expressing Halo-GFP-Mito. b. Histogram of a representative set of CAPA experiments, showing the dose-dependence of CAPA signal for peptide HT-DD5-o between 0.08 and 20 μM. For each experiment, mean fluorescence intensity values were calculated from these histograms. These mean intensity values were normalized to the no-TAMRA signal (No TMR, shown in gray) as the 0% value, and the no-peptide signal (TMR, shown in red) as the 100% value. Three independent replicates of each experiment were performed.
SI Figure 18. Autophagy induction in a cellular model of Huntington’s disease. Number of small htt103Q aggregates < 1µm per cell (left) and percentage of cells with aggregates (right) in HeLa cells expressing doxycycline (Dox)-repressible CFP-htt103Q. Bars represent mean ± s.e.m. for triplicate samples (100-150 cells analyzed per sample). Similar results were obtained in three independent experiments. * denotes P<0.05 and ** denotes P<0.01 by t-test for indicated group versus vehicle control.

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