Supporting Information of

Click Chemistry Facilitates Formation of Reporter Ions and Simplified Synthesis of Amine-Reactive Multiplexed Isobaric Tags for Protein Quantification

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2. Materials

Allyl bromide-d_5 was purchased from C/D/N isotopes Inc. (Quebec, Canada). MagneHis Ni-Particles and sequencing grade trypsin was purchased from Promega (Madison, WI). The model peptide, VIP (residue 1-12), HSDAVFTDNYTR, was acquired from Anaspec (Fremont, CA). High Capacity Neutravidin Agarose Resin, n-dodecyl-β-D-maltoside, and SuperSignal West Dura Extended Duration chemiluminescent substrate were from Thermo Scientific (Rockford, IL). Lysyl endopeptidase (LysC) was from Wako Chemicals USA (Richmond, VA). Cell culture reagents, Flip-In T-REx 293 cells, plasmids, and monoclonal antibodies for Cul1 and Cand1 were from Invitrogen (Carlsbad, CA). Plasmid DNA containing the human Cul1 sequence was purchased from Open Biosystems (Huntsville, AL). MLN4924 was a generous gift from Millennium: The Takeda Oncology Company (Cambridge, MA). All other general chemicals were purchased from Fisher Scientific (Hampton, NH), VWR International (West Chester, PA), and Sigma-Aldrich (St. Louis, MO) and used as received without further purification.

3. Synthesis of Caltech Isobaric Tags

Figure S1. Synthesis of CIT

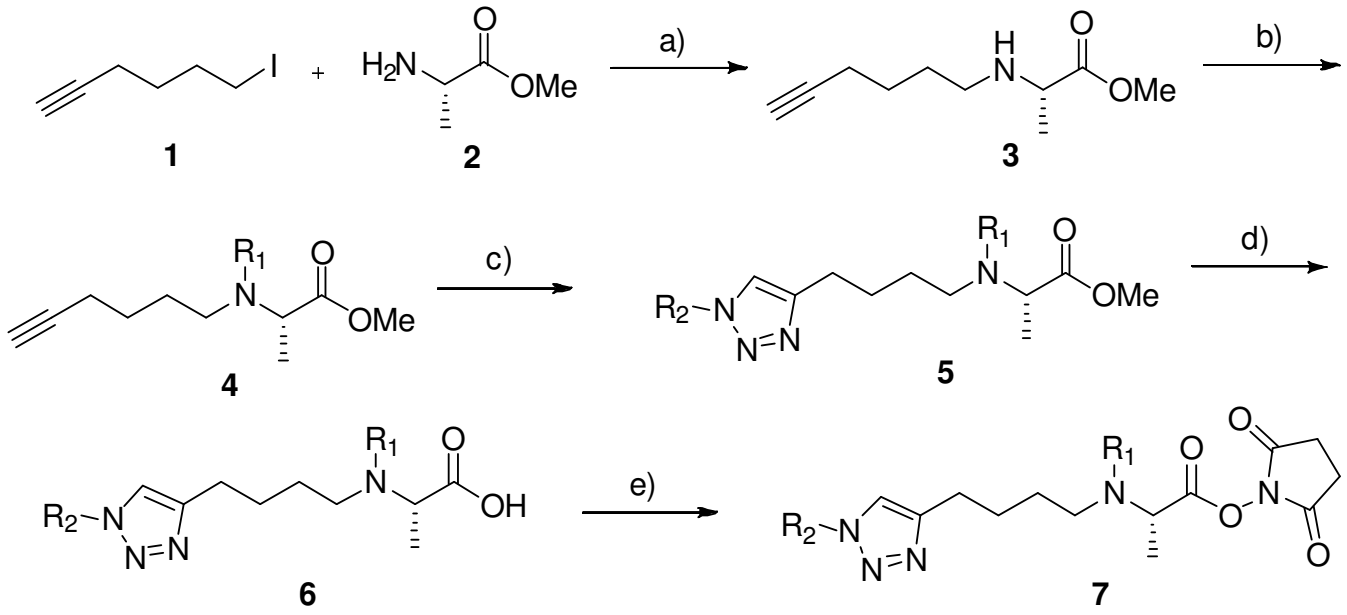

Figure S1. Synthesis of CIT reagents. a) THF, K_2CO_3, TEAI, reflux 18 h, 56%. b) THF, K_2CO_3, TEABr, reflux 18 h, R_1 = Allyl-d_0-bromide, 56% (**4a**), R_1 = Ally-d_5-bromide, 67% (**4b**). c) 0.4 eq Na ascorbate, 0.1 eq CuSO4, 0.01 eq TBTA, DMSO/H_2O, RT 4 h, R_2 = Allyl-d_5-azide, 72% (heavy tag, **5a**), R_2 = Allyl-d_0-azide, 69% (light tag, **5b**). d) 2M KOH, THF, RT overnight, *quantitative* (heavy tag, **6a**), 97% (light tag, **6b**). e) TFA-NHS, DMF, RT overnight, 24% (heavy tag, **7a**), 23% (light tag, **7b**). THF = tetrahydrofuran, TEAI = tetraethylammonium iodide, TEABr = tetraethylammonium bromide, TBTA = tris[(1-*t*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, DMSO = dimethyl sulfoxide, TFA-NHS = trifluoroacetic *N*-hydroxysuccinimide ester, DMF = *N,N'*-dimethyl formamide.

6-Iodo-hex-1-yne (**1**)

In a flame-baked, one neck 250 mL round bottom flask, 40 mmol of 6-chloro-1-hexyne (~5 mL) was dissolved in 25 mL of acetone with 80 mmol of sodium iodide (~12 g) and refluxed for 2 days with thin layer chromatography (TLC) check. After filtration, the mixture was diluted by diethylether. The organic layer was washed with water, $Na_2S_2O_4$ and brine and dried over $MgSO_4$. The resulting solution was concentrated *in vacuo* with caution (the product is slightly volatile). The desired product, 6-iodo-1-hexyne (5.58 g, 26.4 mmol, **1**) was acquired as a brownish oil. Yield: 66%

N-(5-hexynyl) L-alanine methyl ester (**3**)

In a flame-baked, two neck 100 mL round bottom flask, 20 mmol of L-alanine methyl ester hydrochloride, **2** (2.8 g), 40 mmol of K_2CO_3 (5.53 g), and 20 mmol of tetrabutylammonium iodide (TBAI, 7.39 g) were charged under a stream of dry N_2 gas. 35 mL of tetrahydrofuran (THF) was slowly added and stirred for 15 min at RT. Then 2.6 mL of 6-iodo-hex-1-yne was added dropwise while the mixture was stirred. The reaction mixture was refluxed at ~70°C for 15-18 h with TLC check. After consumption of the starting material, the mixture was cooled to RT, diluted with diethylether, and filtered. The filtrate was further diluted with diethylether and filtered again. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography on silica gel (1:2 = Hexane/EtOAc, 1% triethylamine) to give *N*-(5-hexynyl) L-alanine methyl ester (2.044 g, 11.2 mmol, **3**) as a yellow oil. Yield: 56%. R_f = 0.27 (1:1 = Hexane/EtOAc); ESI-MS $[M+H]^+$ = *m/z* 184.1; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.70 (s, 3H), 3.32 (q, *J* = 7.0 Hz, 1H), 2.53 (m, 2H), 2.18 (m, 2H), 1.92 (t, *J* = 2.6 Hz, 1H), 1.56 (m, 5H), 1.27 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.29, 84.21, 68.41, 56.61, 51.74, 47.41, 29.20, 26.01, 19.11, 18.23.

N,N-(5-hexynyl)(allyl-d_0) L-alanine methyl ester (**4a**) *and N,N-(5-hexynyl)(allyl-d_5) L-alanine methyl ester* (**4b**)

In a flame-baked, two neck 50 mL round bottom flask, 10 mL THF was charged under a stream of dry N_2 gas. 6 mmol of K_2CO_3 (0.83 g), and 6 mmol of tetrabutylammonium bromide (TBAB, 1.93 g) were slowly added and stirred for 15 min at RT. 3 mmol (0.55 g) of **3** , and 4.5 mmol of allyl bromide-d_0 (0.544g, 0.38 mL) were slowly added dropwise using a syringe while the mixture was stirred. The reaction mixture was heated at ~55°C and reacted for 8 h with TLC check. After consumption of the starting material, the mixture was cooled to RT, diluted with diethylether and filtered. Filtration was repeated twice to remove the remaining TBAB completely. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography on silica gel (20:1 = Hexane/EtOAc) to give **4a** (0.378 g, 1.69 mmol) as a transparent oil. Yield: 56%. R_f = 0.4 (5:1 = Hexane/EtOAc); ESI-MS

$[M+H]^+$ = *m/z* 224.2; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.79 (m, 1H), 5.12 (m, 2H), 3.67 (s, 3H), 3.54 (q, *J* = 7.3 Hz, 1H), 3.18 (m, 2H), 2.53 (m, 2H), 2.18 (m, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.52 (m, 4H), 1.24 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.45, 136.86, 116.78, 84.47, 68.24, 57.79, 54.20, 51.21, 49.85, 27.40, 26.07, 18.26, 14.88.

0.457g of *N,N*-(5-hexynyl)(allyl-d_5) L-alanine methyl ester (**4b**) (2.0 mmol) was obtained by the same procedure described above using 3.55 mmol of allyl bromide-d_5 (0.448 g). Yield: 67%. R_f = 0.4 (5:1 = Hexane/EtOAc); ESI-MS $[M+H]^+$ = *m/z* 229.3; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.68 (s, 3H), 3.54 (q, *J* = 7.08 Hz, 1H), 2.53 (m, 2H), 2.18 (m, 2H), 1.93 (t, *J* = 2.68 Hz, 1H), 1.52 (m, 4H), 1.25 (d, *J* = 7.08 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.47, 136.16 (t, *J* = 23.48 Hz), 116.25 (quintet, *J* = 23.47 Hz), 84.47, 68.27, 57.76, 53.28 (quintet, *J* = 18.41 Hz), 51.22, 49.79, 27.39, 26.07, 18.26, 14.90.

Allyl-d_0 azide and allyl-d_5 azide

0.5 M NaN_3 in DMSO was prepared as described in the literature by stirring the mixture at room temperature overnight.[1] 1.1 eq of 0.5 M NaN_3 solution was mixed with the appropriate amount of allyl bromide (d_0/d_5) and stirred overnight. TLC monitored for the complete consumption of the starting material; unwanted dimerization of allyl azides was not observed in this condition. The resulting mixtures were used for the next steps without further purification or analysis.

N,N-(4-(1-allyl-d_5-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_0) L-alanine methyl ester (**5a**) *and N,N-(4-(1-allyl-d_0-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_5) L-alanine methyl ester* (**5b**)

The literature procedure was followed with some modifications.[2] To *in situ* prepared allyl-d_5 azide solution (~1.2 eq), 1.7 mmol (0.378 g) of **4a**, 0.17 mmol of $CuSO_4 \cdot 5H_2O$ (42.5 mg, 0.1 eq), 0.68 mmol of sodium ascorbate (134.7 mg, 0.4 eq), 0.017 mmol of tris[(1-*t*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (7.3 mg, 0.01 eq), and an additional 2 mL of DMSO were added and stirred for 2 h at RT. Then, 2.4 mL of water was added and stirred for additional 2 h with TLC monitoring. After the complete consumption of the starting material, 4 mL of 1M NH_4OH was added to remove residual CuN_3 and $(Cu)_2N_3$. The mixture was diluted using additional water and ethyl acetate. The

aqueous layer turned blue due to the coordination of ammonia to copper ions. The organic layer was separated, and further extracted by ethyl acetate three times. The combined organic layer was then washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (1:1 = Hexane/EtOAc, 1% triethylamine) to give **5a** (0.379 g, 1.22 mmol) as a transparent oil. Yield: 72%. R_f = 0.3 (1:1 = Hexane/EtOAc); ESI-MS $[M+H]^+$ = *m/z* 312.3, CID of $[M+H]^+$ produced *m/z* 169.1 fragment; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.25 (s, 1H), 5.76 (m, 1H), 5.14 (dd, *J* = 17.1, 1.22 Hz, 1H), 5.04 (d, *J* = 10 Hz, 1H), 3.64 (s, 3H), 3.51 (q, *J* = 7.08 Hz, 1H), 3.21 (dd, *J* = 14.6, 5.6 Hz, 1H), 3.09 (dd, *J* = 14.4, 6.9 Hz, 1H), 2.68 (t, *J* = 7.6 Hz, 2H), 2.52 (m, 2H), 1.54 (m, 4H), 1.21 (d, *J* = 7.08 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.46, 148.43, 136.81, 130.94 (t, *J* = 24.9 Hz), 120.40, 119.21 (quintet, *J* = 24.9 Hz), 116.82, 57.85, 54.25, 51.85 (quintet, *J* = 23.9 Hz), 51.24, 50.20, 27.97, 27.06, 25.54, 14.83.

1.59 mmol of **4b** (0.363 g) was used for the same reaction described above to give **5b** (0.341 g, 1.10 mmol) as a transparent oil. Yield: 69%. R_f = 0.3 (1:1 = Hexane/EtOAc); ESI-MS $[M+H]^+$ = *m/z* 312.3, CID of $[M+H]^+$ produced *m/z* 164.1 fragment; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.25 (s, 1H), 5.98 (m, 1H), 5.30 (dd, *J* = 10, 0.9 Hz, 1H), 5.25 (d, *J* = 17.1 Hz, 1H), 4.91 (d, *J* = 6.1 Hz, 2H), 3.65 (s, 3H), 3.51 (q, *J* = 7.1 Hz, 1H), 2.69 (t, *J* = 7.6 Hz, 2H), 2.52 (m, 2H), 1.55 (m, 4H), 1.22 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.49, 148.45, 136.12 (t, *J* = 23 Hz), 116.29 (quintet, *J* = 22.6 Hz), 120.43, 119.78, 57.84, 53.33 (quintet, *J* = 19.3 Hz), 52.55, 51.24, 50.15, 27.98, 27.07, 25.55, 14.85.

N,N-(4-(1-allyl-d_5-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_0) L-alanine (**6a**) *and N,N-(4-(1-allyl-d_0-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_5) L-alanine* (**6b**)

1.22 mmol of **5b** (0.375 g) was charged to a 10mL one neck flask with 2 mL of THF and 2 mL of 2M KOH and stirred at RT for 10 h. The reaction was monitored by TLC and ESI-MS until the starting material was completely consumed. Upon completion of the reaction, THF was removed *in vacuo* and the aqueous layer was neutralized by ~2 mL of 2M HCl. Water was then completely removed *in vacuo* and the residue was reconstituted in acetonitrile (ACN). Insoluble KCl salt was filtered and ACN was

removed *in vacuo*. The free acid of the alanine derivative, *N,N*-(4-(1-allyl-d_5-1*H*-1,2,3-triazol-4-yl)butyl)(allyl-d_0) L-alanine (**6a**) was recovered as a pale yellow oil. Yield: *quantitative*. ESI-MS $[M+H]^+$ = *m/z* 298.1; ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.62 (br, 1H), 7.90 (d, *J* = 2.2 Hz, 1H), 6.05 (m, 1H), 5.53 (d, *J* = 17.1 Hz, 1H), 5.45 (d, *J* = 10.5 Hz, 1H), 4.18 (q, *J* = 7.1 Hz, 1H), 3.83 (m, 2H), 3.15 (br, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 1.69 (m, 4H), 1.52 (d, *J* = 6.9 Hz, 3H).

Hydrolysis of **5b** (0.247 g, 0.79 mmol) using above-described procedure gave 0.230 g of **6b** (0.773 mmol) as a pale yellow oil. Yield: 97%. ESI-MS $[M+H]^+$ = *m/z* 298.1; ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.54 (br, 1H), 7.89 (d, *J* = 4.2 Hz, 1H), 6.03 (m, 1H), 5.25 (m, 1H), 5.16 (m, 1H), 4.98 (m, 2H), 4.18 (q, *J* = 7.1 Hz, 1H), 3.15 (br, 2H), 2.64 (t, *J* = 7.3 Hz, 2H), 1.69 (m, 4H), 1.52 (d, *J* = 7.1 Hz, 3H).

N,N-(4-(1-allyl-d_5-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_0) L-alanine N-hydroxylsuccinimide ester (**7a**) *and N,N-(4-(1-allyl-d_0-1H-1,2,3-triazol-4-yl)butyl)(allyl-d_5) L-alanine N-hydroxylsuccinimide ester* (**7b**)

In a flame-baked 50 mL one neck flask, 2.75g of *N*-hydroxysuccinimide was added to 14 mL of trifluoroacetic anhydride at RT under a stream of dry N_2 gas and stirred for 4 h. The solvent was removed *in vacuo* overnight. A white crystalline product, trifluoroacetic *N*-hydroxysuccinimide ester (TFA-NHS) was obtained, stored in a dry desiccator, and used just before activation of free acids.

In a flame-baked 50 mL one neck flask, 87 mg of **6a** (0.29 mmol) and 75 mg of TFA-NHS were added to 1 mL of dry DMF, and stirred overnight at RT. After the complete consumption of the starting material (monitored by TLC), the reaction mixture was separated by flash chromatography on silica gel (1:1 = Hexane/EtOAc) and yielded 28 mg of **7a** (~0.7 mmol) as a yellow oil. (Note: the poor recovery yields for the final products are observed due to the retained products on silica gel through the coordination of the highly *N*-substituted residues such as the 1,2,3-triazole and the tertiary amine. Other purification methods such as crystallization would improve the overall yield.) Yield: 24%. ESI-MS (100% ACN) $[M+H]^+$ = *m/z* 395.1. The stock solution of the heavy tag (*m/z* 169 reporter ion) was prepared without further analysis by adding 20 μL dry DMSO to 1 mg of the NHS-ester product into each vial. Stock solutions were stored at −80°C. Each vial contained 1 mg of the reagent and was used

for each labeling experiment.

The same procedure was used for NHS ester activation of 52 mg of **6b** (0.175 mmol) and yielded 16 mg of the NHS-ester product **7b**. Yield: 23%. ESI-MS (100% ACN) $[M+H]^+$ = *m/z* 395.1. The stock solution vials of the light tag (*m/z* 164 reporter ion) were prepared as described above and stored at −80 °C.

4. Synthesis of iTRAQ-113 Reagent

Methyl 2-(4-methylpiperazin-1-yl)acetate (**8**)

To a mixture of 5 mmol of 1-methyl piperazine (0.5 g) and 6 mmol of K_2CO_3 (0.83 g) in a flame-baked, two neck 100 mL round bottom flask under a stream of dry N_2 gas was slowly added 15mL of tetrahydrofuran (THF) and stirred for 15 min at RT. While the mixture was stirred, 0.56 mL of methyl boromoacetate was added dropwise. The mixture was reacted at RT for 15-18 h with TLC check. After consumption of the starting material, the mixture was diluted with diethylether, and filtered. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography on silica gel (5:1 = $CHCl_3$/MeOH) to methyl 2-(4-methylpiperazin-1-yl)aceate (0.111 g, 0.65 mmol) as a pale yellow oil. Yield: 13%. R_f = 0.36 (5:1 = $CHCl_3$/MeOH); ESI-MS $[M+H]^+$ = *m/z* 173.1, CID of $[M+H]^+$ produced *m/z* 113.0 fragment; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.68 (s, 3H), 3.18 (s, 2H), 2.52 (m, broad, 8H), 2.26 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.70, 59.36, 54.71, 52.94, 51.70, 45.91.

2-(4-methylpiperazin-1-yl)acetic acid (**9**)

To a 10mL one neck flask with 2 mL of THF and 2 mL of 2M KOH, 51.3 mg of methyl 2-(4-methylpiperazin-1-yl)acetate was added and stirred at RT for 10 h. The reaction was monitored by TLC and ESI-MS until the starting material was completely consumed. Upon completion of the reaction, THF was removed *in vacuo* and the aqueous layer was neutralized using ~2 mL of 2M HCl. Water was then completely removed *in vacuo* and the residue was reconstituted by acetonitrile (ACN). Insoluble KCl salt was filtered and ACN was removed *in vacuo*. The free acid of the product was recovered as a

transparent gleasy oil. Yield: *quantitative*. ESI-MS $[M+H]^+$ = *m/z* 159.1.

N-hydroxylsuccinimide 2-(4-methylpiperazin-1-yl)acetate ester (iTRAQ-113) (**10**)

In a flame-baked 15 mL glass vial, ~0.65 mmol of 2-(4-methylpiperazin-1-yl)acetic acid and 33.0 mg of TFA-NHS were added to 0.5 mL of dry DMF and 101 mg of *N*,*N*-diisopropylethylamine, and stirred overnight at RT. After the complete consumption of the starting material (TLC monitoring), the reaction mixture was used for peptide labeling without further purification. ESI-MS (100% ACN) $[M+H]^+$ = *m/z* 256.1. The stock solution vials of the iTRAQ-113 reagent (*m/z* 113 reporter ion) were prepared as described above and stored at −80 °C.

5. Protein Sample Preparation

Protein mixture digestion

Equal amounts (by weight) of protein mixtures, bovine serum albumin, ovalbumin, α- and β-caseins, lysozyme were dissolved in water, reduced by 10 mM dithiothreitol (DTT) for 30 min at room temperature, alkylated by 5 mM iodoacetamide for 1 h in the dark and digested by trypsin (50:1, w/w) in 50 mM ammonium bicarbonate buffer for 15 h at 37 °C. The reaction was quenched by 5% formic acid, and the resulting peptide products were desalted with C_{18} spin columns (Satorious Stedim Biotech, Aubagne Cedex, France).

Affinity purification and digestion of Cul1 and its associated proteins

The applicability of CIT to the quantification of protein complexes in the cell was evaluated with Cul1. Cul1 is a ubiquitin ligase that attaches a ubiquitin chain on target substrates for proteasome-catalyzed degradation.[3] Cul1 is a prototype of the cullin ligase family, and constitutes modular ligase complexes with many confirmed binding partners. The purification of Cul1 and its binding partners from the cells was carried out as described previously with minor modifications.[4] Briefly, to facilitate the purification of Cul1, a HEK 293-derived stable cell line capable of expressing tagged Cul1 upon tetracycline treatment was constructed using the T-REx™ (Tetracycline-regulated Expression) system

(Invitrogen, Carlsbad, CA). The tandem tag we adopted consists of a hexa-histidine sequence and a biotinylation signal sequence.[5] Biotinylation is catalyzed by endogenous biotin ligases, which are present in all eukaryotic cells.[6] A specific lysine residue in the biotinylation signal sequence functions as an acceptor site for biotin *in vivo*.[7]

Tagged Cul1 was induced with 1.0 μg/mL tetracycline for 4 h in the experiments aimed to quantify purified Cul1 complexes with an initial 1:2 mixing ratio for the light and heavy CIT labeling. For the quantification of differentially expressed Cul1 in the cell, 0.5 or 2.0 μg/mL tetracycline were added to the growth medium for 1 h or 4 h, respectively. Twenty-four hours after induction, cells were harvested and lysed for 30 min at 4 °C with the lysis buffer (0.050 M HEPES, pH 7.5, 0.0050 M Mg(OAc)2, 0.070 M KOAc, 10% glycerol, and 0.4% IGEPAL CA630). The lysate was centrifuged at 16,600 g at 4 °C for 20 min and the supernatant was used for purification. Tandem purification of tagged Cul1 and associated proteins was carried out using MagneHis Ni-particles from Promega and Streptavidin-coupled Dynabeads from Invitrogen. Purified proteins were subjected to reduction by 10 mM DTT and alkylation by 5 mM chloroacetamide, sequential on-bead protease digestions, first with Lys-C (35 ng/mg lysate) for 4 h at 37 °C in 8 M urea (freshly prepared), and then with trypsin (30 ng/mg lysate) for 12 h at 37 °C in 2 M urea (freshly prepared). The resulting tryptic peptides were desalted with C_{18} spin columns.

CIT Labeling

The model peptide, VIP (residue 1-12, HSDAVFTDNYTR; 50 μg), was dissolved in 50 μL of 100 mM tetraethylammonium bicarbonate (pH 8.5) and 100 μL of ACN (66.7% organic phase) and labeled with 5 μL of 5 μg/μL DMSO stock solution of either light or heavy tag, by incubating for 2 h at RT. The reaction was quenched by adding 50 μL of 100 mM hydroxylamine and incubated for 7 h at RT. The mixture was acidified by adding 4 μL of formic acid and completely dried *in vacuo*. The residue was reconstituted using 100 μL of 0.1% formic acid, desalted using the C_{18} desalting tip, and eluted to 100 μL of 0.1% formic acid, 50% ACN and 50% water. The CIT labeled VIP peptide eluent was diluted

(~X20), and analyzed by various mass spectrometers. The iTRAQ-113 reagent labeling was performed using the same conditions for the CIT reagents.

An aliquot of 1 μg of the protein digest was labeled using light or heavy CIT reagents under the same solvent system used for the model peptide, and mixed with a 1:1 ratio. The combined samples were desalted, and injected to a nanoLC-LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) at Caltech. Approximately 3 μg of Cul1 digests were labeled by light or heavy CIT reagents with the 1:2 ratio under the same solvent system used for the model peptide. After conjugation, light and heavy CIT-labeled Cul1 digests were combined, desalted and injected to a nanoLC-LTQ-Orbitrap mass spectrometer at Caltech. For HCD/CID experiments, differentially expressed Cul1 digest samples were labeled by light or heavy CIT reagents. The resulting peptides were subject to a nanoLC-LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) at UCLA for HCD/CID analyses.

6. Instrumentation Setup

Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) spectra were acquired using Voyager DE-Pro mass spectrometer (Applied Biosystems, Foster City, CA) in the reflectron mode with a 20 kV acceleration voltage, a 150 ns delay extraction time and a 75% grid voltage. A 0.5 μL sample of the derivatized peptide solution was mixed with 0.5 μL of 10 mg/mL CHCA matrix solution in 0.1% TFA, 50% ACN, and 50% H_2O and the mixed spots were dried and introduced to the mass spectrometer for analyses. For all spectra, 100 shots were averaged.

PQD experiments via direct infusion for model peptide studies were performed by ion trap scans in an LTQ-FTICR mass spectrometer (Thermo, San Jose, CA) equipped with the Nanomate (Advion BioSciences Inc., Ithaca, NY) nanospray unit. The spraying voltage was 1.4 kV and the gas pressure was 0.3 psi. Critical parameters of the mass spectrometer include capillary voltage 49 V, capillary temperature 200 °C, and tube lens voltage 180 V. Other ion optic parameters were optimized by the auto-tune function in the LTQ tune program for maximizing the signal intensity. The parameters for

PQD experiments are the precursor isolation window 2.0 *m/z*, collision energy 29%, isolation Q 0.70, and activation time 0.1 ms. For PQD spectra, 100 scans were recorded.

The samples were also analyzed by a nanoflow HPLC, Proxeon easy-nLC-System (Proxeon Biosystems) coupled on-line via a nanoelectrospray ion source (Proxeon Biosystems) to an LTQ-Orbitrap mass spectrometer. Samples were loaded onto a C_{18}-reversed phase column (15 cm long, 75 µm inner diameter, packed in-house with ReproSil-Pur C_{18}-AQ 3 µm resin (Dr. Maisch)) in buffer A (5% ACN, 0.2% formic acid) with a flow rate of 500 nl/min for 24 min and eluted with a linear gradient from 0% to 36% buffer B (80% ACN 0.2% formic acid) over 110 minutes, followed by 10 minutes at 100% buffer B, at a flow rate of 350 nl/min. The column was re-equilibrated with buffer A. Mass spectra were acquired in the positive ion mode applying data-dependent acquisition with automatic switching between survey scan and tandem mass spectrum acquisition. Samples were analyzed with a top 5 method; acquiring one Orbitrap survey scan in the mass range of *m/z* 400-1600 followed by MS/MS of the five most intense ions in the LTQ in the mass range of *m/z* 100-1600. The target value in the LTQ-Orbitrap was 500,000 for survey scan at a resolution of 60,000 at *m/z* 400. Fragmentation in the LTQ was performed by PQD with a target value of 5,000 ions. Selected sequenced ions were dynamically excluded for 30 s. General mass spectrometric conditions were: spray voltage, 2.4 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 200 °C; normalized collision energy (29%) using wide band activation mode for MS/MS. An activation of q = 0.55 and delay time of 0.4 ms were applied in MS/MS acquisitions.[8]

For HCD/CID analyses, CIT-labeled peptides were loaded and washed on a 100 μm×35 mm CVC Microtech (Fontana, CA). Peptide trapping was performed using a New Objective Halo C_{18} 75μm × 100 mm, 90 Å, 2.7 μm (Woburn, MA) column by flushing a mobile phase of 0.1% formic acid in water (A). Peptides were subsequently eluted from the column at 300 nL/min using an Eksigent nanoLC 2D pump (Dublin, CA) with a 110 min gradient (0.1% formic acid in water (buffer A) and ACN containing 0.1% formic acid (buffer B); 0-30% phase B over 90min, 30-80% phase B over 20 min). The HPLC system

was coupled to an LTQ-Orbitrap XL mass spectrometer and the source conditions were as follows: capillary temperature, 180 °C; capillary voltage, 49 V; ESI spray voltage, 1.8 kV. The automatic gain control target was fixed at 500,000 ions for MS and 50,000 for MS/MS scans. The instrument was operated in data-dependent acquisition mode, with MS survey scan (*m/z* 400-1400*)* performed in the Orbitrap using a resolution set at 60,000. CID and HCD activations were performed on the 3 most abundant ions over 5,000 counts (charge state +1 rejected) using normalized collision energies of 30 and 40, respectively, and detected using the linear ion trap. Ions with masses within 10 ppm of previously fragmented ions were excluded for 120s.

For HCD analysis, a LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA) was used with an Eksigent nanoLC 2D pump (Dublin, CA) as for HCD/CID analyses. Peptides were eluted from the column at 350 nL/min with a 80 min gradient (0.1% formic acid in water (buffer A) and ACN containing 0.1% formic acid (buffer B); 0-40% phase B over 70 min, 40-80% phase B over 10 min) and sprayed to the mass spectrometer. Samples were analyzed with a top 5 HCD method; acquiring one Orbitrap survey scan in the mass range of *m/z* 400-1800 followed by MS/MS of the five most intense ions in the HCD cell in the mass range of *m/z* 100-1600 at a resolution of 7,500. The target value in the LTQ-Orbitrap was 500,000 for survey scan at a resolution of 60,000 at *m/z* 400. Fragmentation in the HCD cell was performed with a target value of 50,000 ions, 40% normalized collision energy and 2.0 m/z isolation window. Selected sequenced ions were dynamically excluded for 30 s. The critical parameters for the instruments were as follows: source voltage, 2.50 kV; capillary temperature, 180 °C; capillary voltage 49 V.

7. Data Processing

The raw files from LTQ-Orbitrap mass spectrometers were converted to Mascot generic format (MGF) files using ReAdW4Mascot (version 20090305a, available from the National Institute of Standards and Technology at http://peptide.nist.gov/software/ReAdW4Mascot2_20090305a.zip). PQD

of the CIT labeled model peptide acquired for testing of the linear dependency in reporter ion formation was then analyzed using in-house software and best-fit lines were calculated using linear regression. Error bars are displayed for the middle 95% reported ion ratios. For the five protein mixture, a database was constructed containing the five protein sequences as well as a small contaminant protein database. For the Cul1 pull-down search, a target sequence database was constructed from the human IPI database (version 3.54) and a small contaminant protein database. A decoy database was constructed from the target following the protocol as described elsewhere.[9] The decoy database was then appended to the target and used to estimate the false discovery rate of the database search. The database search was performed using Mascot (version 2.2.06, Matrix Science, http://www.matrixscience.com). The database search parameters were as follows: 0.5 Da fragment ion mass tolerance, 10 ppm precursor ion mass tolerance, trypsin enzyme specificity, up to two missed cleavages, fixed carbamidomethyl (57.02146 Da) modification of cysteine, variable modifications of oxidation (15.99491 Da) of methionine, carbamylation (43.005814 Da) of the N-terminal, and quantitation enabled. The Mascot quantitation parameters were as follows: fixed the N-terminal modification of 279.210745 Da with reporter ions at *m/z* 164.1188 and 169.1502, respectively. Reported proteins have at least one unique peptide sequence and two peptide ratios. Reported peptide ratios are included for those peptides whose score is above the homology level and outlier peptide ratios are discarded using the Mascot auto outlier detection. Reported Mascot protein quantitation ratios are the median of the top scoring peptide reporter ion ratios. The confidence intervals at 95% confidence level were determined by the home-built pearl script using the Bootstrap algorithm as implemented in pearl(x,y) 2.7.2.0.

8. Density Functional Calculation

The formation of the reporter ion is simulated by the *N*-protonated *N,N*-dimethyl-4-(1-methyl-1*H*-1,2,3-triazol-4-yl)butan-1-amine. Initial coarse geometries were constructed by the MC/MM conformer search using Macromol 8.0 (Schrödinger Inc., Portland, OR, USA) as implemented in Maestro 8.0

(Schrödinger Inc., Portland, OR, USA). The OPLS 2005 was used for the force field model. Within 5 kcal/mol energy, all low energy conformers were initially recorded. Low energy conformers were selected for further structure optimization by density functional theory (DFT). Each conformer was subject to a geometry optimization using Jaguar 7.5 (Schrödinger Inc., Portland, OR, USA) at the B3LYP/6-311++G(d,p) level. Thermochemical parameters of optimized conformers were estimated by vibrational frequency calculation at 1 atm and 298.15 K at the same level of theory. The transition state structures were searched using the QST method by interpolating initial guesses for reactants, products and transition states. All calculations were performed using computational resources kindly provided by the Material and Process simulation center at the Beckman Institute, Caltech.

9. Cartesian Coordinates of DFT Optimized Model Systems

All structures were optimized by the B3LYP/6-311++G(d,p) level of theory.

1) Reactant

atom	x	y	z
	angstroms		
N1	1.3378230058	1.1723051240	-1.8024698392
C2	-0.0629676997	1.5132733209	-2.1984521898
C3	1.4656684295	-0.2796917251	-1.4780601238
C4	1.8408983511	2.0507353878	-0.6744919783
C5	2.2377104457	3.4659278441	-1.1400895017
C6	3.7521721248	3.7368693445	-1.2316306171
C7	4.5909867621	2.7848483225	-2.1135964343
H8	-0.7388229186	1.2628353367	-1.3803908379
H9	-0.3257265354	0.9424578502	-3.0878938414
H10	5.6250327369	3.1363732819	-2.1051606316
H11	3.8818645436	4.7618501939	-1.5911611586
H12	4.6191390850	1.7854593289	-1.6645966977
C13	4.1323001212	2.6475962527	-3.5331461445
C14	4.6629211763	3.1084022064	-4.7187392055
N15	3.8202480429	2.6536925068	-5.6813765904
N16	2.8201422634	1.9526548570	-5.1525067194
N17	3.0085582355	1.9460337336	-3.8563937191
H18	5.5413189394	3.6896777795	-4.9424101893
C19	3.9059421071	2.8311772357	-7.1313110211
H20	3.9975408588	1.8578717122	-7.6116644340
H21	3.0097519476	3.3362482810	-7.4891386668
H22	4.7827886419	3.4353453166	-7.3542535708
H23	-0.1289385801	2.5760735228	-2.4211008542
H24	2.5153601615	-0.5156446294	-1.3096069123
H25	0.8861759251	-0.5058095374	-0.5826119503
H26	1.0923225524	-0.8651563082	-2.3170852985
H27	1.0463999570	2.0768828191	0.0732203034
H28	2.6905548437	1.5315446748	-0.2288663027
H29	1.7649350548	3.6867614214	-2.1008627154
H30	1.8158404483	4.1865797885	-0.4357196883
H31	4.1791760855	3.7121232108	-0.2236838232
H32	1.9570279710	1.3760677814	-2.6511743781

The zero point energy (ZPE): 181.178 kcal/mol
is not included in U, H, or G in the table below

T = 298.15 K

	U	Cv	S	H	G	ln(Q)
trans.	0.889	2.981	41.522	1.481	-10.899	18.39467
rot.	0.889	2.981	31.563	0.889	-8.522	14.38288
vib.	6.568	44.348	40.636	6.568	-5.548	9.36330
elec.	0.000	0.000	0.000	0.000	0.000	0.00000
total	8.346	50.309	113.721	8.938	-24.968	42.14084

Total internal energy, Utot (SCFE + ZPE + U): -573.022984 hartrees
Total enthalpy, Htot (Utot + pV): -573.022040 hartrees
Total Gibbs free energy, Gtot (Htot - T*S): -573.076073 hartrees

2) Transition state

		angstroms	
atom	x	y	z
C1	-2.6041842460	1.0544796483	-1.1194187389
N2	-1.5579899142	1.1108219162	-0.2595038202
N3	-1.1874925718	-0.0797439873	0.1274401846
N4	-2.0021185818	-0.9348210093	-0.4855180876
C5	-2.9006267189	-0.2845250729	-1.2696915238
C6	-1.8602118951	-2.3744454219	-0.2631524588
H7	-3.6482458388	-0.8016398124	-1.8474004714
H8	-1.6416106769	-2.8732714384	-1.2072862316
H9	-2.7790153610	-2.7730587377	0.1668462654
H10	-1.0352481236	-2.5176992799	0.4302920384
C11	-0.6308338039	2.8133139270	0.4072010224
C12	-3.1669175845	2.3160926647	-1.6932156524
C13	-2.7593690749	3.5439864973	-0.8631877832
C14	-1.2365024023	3.7083118676	-0.6581010269
H15	0.2892146950	2.2786355962	0.2471789613
H16	-1.1402488051	2.6667342068	1.3455253218
H17	-4.2563193161	2.2497315384	-1.7472710539
H18	-2.8133754008	2.4356246984	-2.7239595237
H19	-3.1301205978	4.4354356608	-1.3735370266
H20	-3.2582460283	3.5143892722	0.1115370915
H21	-1.0565026365	4.7480373111	-0.3778430109
H22	-0.7106663248	3.5515574565	-1.6042725096
N23	0.5922876320	4.2049968666	1.4269163409
C24	1.0286171012	3.5706943350	2.6834534042
C25	1.7189277228	4.6446919132	0.5848468104
H26	0.1594706697	3.3314362111	3.2979028429
H27	1.6974768779	4.2206227658	3.2562720187
H28	1.5601863403	2.6446606922	2.4536317916
H29	2.3023665369	3.7719250716	0.2833477133
H30	2.3806880243	5.3336363262	1.1193368335
H31	1.3436861936	5.1420963168	-0.3101004968
H32	0.0020818173	5.0044351458	1.6451627981

Imaginary vibrational frequency: -451.28 cm**-1

The zero point energy (ZPE): 179.336 kcal/mol
is not included in U, H, or G in the table below

T = 298.15 K

	U	Cv	S	H	G	ln(Q)
trans.	0.889	2.981	41.522	1.481	-10.899	18.39467
rot.	0.889	2.981	31.944	0.889	-8.635	14.57465
vib.	7.296	46.706	48.442	7.296	-7.147	12.06272
elec.	0.000	0.000	0.000	0.000	0.000	0.00000
total	9.073	52.668	121.908	9.666	-26.681	45.03204

Total internal energy, Utot (SCFE + ZPE + U): -572.969213 hartrees
Total enthalpy, Htot (Utot + pV): -572.968269 hartrees
Total Gibbs free energy, Gtot (Htot - T*S): -573.026191 hartrees

3) Product I: 2-methyl-4,5,6,7-tetrahydro-2H-[1,2,3]triazolo[1,5-a]pyridin-8-ium (reporter ion)

		angstroms	
atom	x	y	z
C1	-2.5004769446	1.2031337574	-1.2609782572
N2	-1.8896131518	1.6261477460	-0.1158108415
N3	-1.2521044682	0.6623024704	0.5146893836
N4	-1.4441149367	-0.4048904562	-0.2267839541
C5	-2.1991135267	-0.1403216784	-1.3256155941
C6	-0.8497742704	-1.6873934675	0.1781659719
H7	-2.4590602782	-0.8940797615	-2.0498189669
H8	-0.1189425949	-1.9959482718	-0.5685929154
H9	-1.6390201794	-2.4325398530	0.2687750186
H10	-0.3643878976	-1.5349802048	1.1384700574
C11	-1.9450974004	2.9956376917	0.4512238036
C12	-3.2649716362	2.1392095081	-2.1447990530
C13	-3.6038139186	3.4459709350	-1.4050222894
C14	-2.3850020916	3.9750457339	-0.6367233053
H15	-0.9553380281	3.2182941144	0.8480096489
H16	-2.6568954783	2.9668235114	1.2791620408
H17	-4.1670305321	1.6409720658	-2.5067027692
H18	-2.6509531315	2.3546502561	-3.0264957638
H19	-3.9433108849	4.1873575553	-2.1293458509
H20	-4.4350134593	3.2761145219	-0.7127186426
H21	-2.6161431288	4.9266561247	-0.1546590078
H22	-1.5550486853	4.1611591605	-1.3260235595

The zero point energy (ZPE): 121.442 kcal/mol
is not included in U, H, or G in the table below

T = 298.15 K

	U	Cv	S	H	G	ln(Q)
trans.	0.889	2.981	40.680	1.481	-10.648	17.97112
rot.	0.889	2.981	29.241	0.889	-7.830	13.21471
vib.	3.780	28.001	23.261	3.780	-3.155	5.32508
elec.	0.000	0.000	0.000	0.000	0.000	0.00000
total	5.558	33.962	93.182	6.150	-21.632	36.51092

Total internal energy, Utot (SCFE + ZPE + U): -437.889571 hartrees
Total enthalpy, Htot (Utot + pV): -437.888627 hartrees
Total Gibbs free energy, Gtot (Htot - T*S): -437.932901 hartrees

4) Product II: dimethylamine

atom	x (angstroms)	y (angstroms)	z (angstroms)
N23	1.1266745940	3.6313289106	1.5354574288
C24	1.4245055540	2.7223683368	2.6354993163
C25	2.1306322775	3.6108186510	0.4787356059
H26	0.6622602060	2.8211127866	3.4117687676
H27	2.4147072872	2.8854224495	3.0967179610
H28	1.3950939160	1.6907830016	2.2715297716
H29	2.1375066420	2.6247497587	0.0042594286
H30	3.1566440547	3.8190464459	0.8305161802
H31	1.8735199140	4.3451286367	-0.2881029631
H32	1.0138958118	4.5752239952	1.8873702930

The zero point energy (ZPE): 57.706 kcal/mol
is not included in U, H, or G in the table below

T = 298.15 K

	U	Cv	S	H	G	ln(Q)
trans.	0.889	2.981	37.342	1.481	-9.652	16.29104
rot.	0.889	2.981	22.331	0.889	-5.769	9.73727
vib.	1.000	8.005	5.277	1.000	-0.573	0.96699
elec.	0.000	0.000	0.000	0.000	0.000	0.00000
total	2.778	13.966	64.949	3.370	-15.994	26.99530

Total internal energy, Utot (SCFE + ZPE + U): -135.113157 hartrees
Total enthalpy, Htot (Utot + pV): -135.112213 hartrees
Total Gibbs free energy, Gtot (Htot - T*S): -135.143072 hartrees

10. References

(1) Alvarez, S. G.; Alvarez, M. T. *Synthesis* **1997**, 413.

(2) Kacprzak, K. *Synlett* **2005**, 943.

(3) Deshaies, R. J. *Annu. Rev. Cell Dev. Biol.* **1999**, *15*, 435.

(4) Lee, J. E.; Sweredoski, M. J.; Graham, R. L. J.; Kolawa, N. J.; Smith, G. T.; Hess, S.; Deshaies, R. J. *Mol. Cell. Proteomics* **2010**, *Electronic preprint. doi:10.1074/mcp.M110.006460*.

(5) Tagwerker, C.; Flick, K.; Cui, M.; Guerrero, C.; Dou, Y. M.; Auer, B.; Baldi, P.; Huang, L.; Kaiser, P. *Mol. Cell. Proteomics* **2006**, *5*, 737.

(6) Cronan, J. E. *J. Biol. Chem.* **1990**, *265*, 10327.

(7) Kulman, J. D.; Satake, M.; Harris, J. E. *Protein Expr. Purif.* **2007**, *52*, 320.

(8) Bantscheff, M.; Boesche, M.; Eberhard, D.; Matthieson, T.; Sweetman, G.; Kuster, B. *Mol. Cell. Proteomics* **2008**, *7*, 1702.

(9) Cox, J.; Mann, M. *Nat. Biotechnol.* **2008**, *26*, 1367.