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An Alkyne–Aspirin Chemical Reporter for the Detection of Aspirin-Dependent Protein Modification in Living Cells

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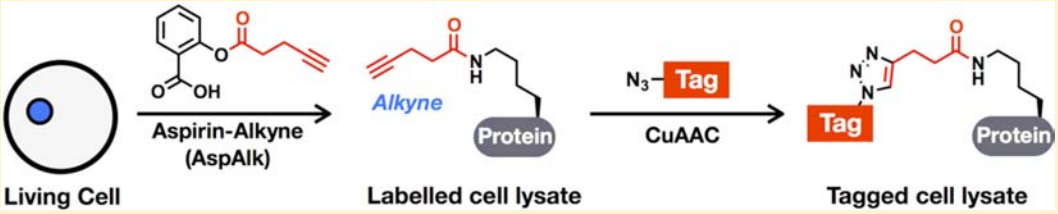
Supporting Information



ABSTRACT: Aspirin (acetylsalicylic acid) is widely used for the acute treatment of inflammation and the management of cardiovascular disease. More recently, it has also been shown to reduce the risk of a variety of cancers. The anti-inflammatory properties of aspirin in pain-relief, cardio-protection, and chemoprevention are well-known to result from the covalent inhibition of cyclooxygenase enzymes through nonenzymatic acetylation of key serine residues. However, any additional molecular mechanisms that may contribute to the beneficial effects of aspirin remain poorly defined. Interestingly, studies over the past 50 years using radiolabeled aspirin demonstrated that other proteins are acetylated by aspirin and enrichment with antiacetyl-lysine antibodies identified 33 potential targets of aspirin-dependent acetylation. Herein we describe the development of an alkyne-modified aspirin analogue (AspAlk) as a chemical reporters of aspirin-dependent acetylation in living cells. When combined with the Cu(I)-catalyzed [3 + 2] azide–alkyne cycloaddition, this chemical reporter allowed for the robust in-gel fluorescent detection of acetylation and the subsequent enrichment and identification of 120 proteins, 112 of which have not been previously reported to be acetylated by aspirin in cellular or in vivo contexts. Finally, AspAlk was shown to modify the core histone proteins, implicating aspirin as a potential chemical-regulator of transcription.

INTRODUCTION

Aspirin, a nonsteroidal anti-inflammatory drug (NSAID), is one of the most common small-molecule treatments in the world, and has been utilized in different forms for over a thousand years for the reduction of inflammation, pain, and fever. Aspirin has at least two biochemical mechanisms by which it produces these anti-inflammatory effects. First, the salicylate moiety of aspirin has been shown to down-regulate the NF-κB signaling pathway through inhibition of IKK-β.[1,2] Second, the acetate group of aspirin is directly transferred to and thereby inhibits the cyclooxygenase (Cox) enzymes, preventing the production of pro-inflammatory prostaglandins.[3,4] Specifically, the acetylation occurs in a 1:1 stoichiometry on serine 530 in Cox1 and serine 516 in Cox2 preventing the binding of the lipid substrate arachidonic acid. This acetylation is irreversible, requiring the translation of a new Cox enzyme for prostaglandin production. Interestingly, low-dose aspirin has also been shown to lower the rates of heart attack and stroke in patients with cardiovascular disease,[5,6] and more recently, a variety of observational studies and trials have demonstrated that chronic aspirin use greatly reduces the incidence of cancer and cancer mortality, with the largest decrease in gastrointestinal cancers.[7−13]

While the majority of effort toward understanding aspirin cardioprotection and chemoprevention has focused on the NFκB and Cox pathways, and much of the observed effects are clearly due to inhibition of these pathways,[8,14−17] aspirin is known to modify other proteins by chemical transfer of its acetate group to amino acid side-chains.[18] This modification, which we have termed aspirin-dependent acetylation, raises the possibility that other protein modification events could contribute to the pharmacological effects of aspirin. The first experiments to demonstrate this used radiolabeled aspirin (H^3, C^{14}) to show that human serum albumin is acetylated both *in vitro* and *in vivo* and that a variety of other proteins including immunoglobulins, enzymes, and histones are acetylated *in vitro*.[19,20] This was shortly followed by *in vivo* demonstration of protein-actylation in mouse tissue using similar techniques.[21,22] Additional studies demonstrated that the acetylation occurs on the ε-amine side-chains of lysine residues on fibrinogen.[23] More recently, mass spectrometry was used to identify aspirin-dependent acetylation of cysteine side-chains of lens γ-crystallins *in vitro*, and this acetylation prevents cataract-promoting carbamylation of the same residues.[24] Finally, specific antibodies were used to identify acetylation on lysine 382 of p53 resulting from aspirin treatment of MDA-MB-231 cells.[25] Aspirin treatment increased p53 nuclear localization and

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expression of the target gene p21[CIP1], although acetylation of lysine 382 was not unambiguously identified as the mechanism. Although these radioactivity-based techniques have allowed for the characterization of aspirin-dependent acetylation events, they cannot be used to visualize and identify the entire spectrum of acetate modifications in a complex proteome. The Bhat and Hagen laboratories addressed this limitation using an antiacetyl−lysine antibody that allowed for the visualization of increased cellular lysine-acetylation upon aspirin treatment and enabled the enrichment and identification of 33 of these proteins using mass spectrometry.[26] However, this approach necessarily suffers from contamination by endogenous lysine-acetylated proteins and no identification of other acetylation events (e.g., serine and cysteine).

Given the crucial role of protein-acetylation in almost every area of biology, we expect that understanding aspirin-dependent acetylation will be uniquely fruitful for uncovering other cellular pathways affected by aspirin. However, research into this area has languished since the 1960s and 70s due to a lack of tools that could enable the robust and specific visualization and identification of these aspirin-dependent modification events. Bioorthogonal reactions, such as the Cu(I)-catalyzed [3 + 2] azide−alkyne cycloaddition (CuAAC), have been used in a variety of contexts to overcome the limitations of traditional biological technologies (e.g., antibodies).[27−29] In fact, alkyne-bearing probes have been applied to investigate protein glycosylation, acetylation, methylation, lipidation, other post-translational modifications, and covalent small molecule inhibitors.[30−41] Herein, we describe the synthesis and characterization of the CuAAC compatible aspirin-analogue containing an alkyne (AspAlk). AspAlk allowed for the robust fluorescent visualization of aspirin-dependent acetylation events in a variety of cells. In addition, the treatment of cells with AspAlk in combination with CuAAC resulted in the identification of 120 potentially aspirin−acetylated proteins, representing a range of biological functions.

RESULTS AND DISCUSSION

To generate our chemical reporter of aspirin-dependent acetylation, we reasoned that it should structurally mimic aspirin as closely as possible. The smallest structural perturbation would incorporate a 3-butynoic ester onto salicylic acid to generate the corresponding alkyne-containing aspirin analogue. However, we found that activation of 3-butynoic acid for subsequent ester formation [e.g., *N,N′*-dicyclohexylcarbodiimide (DCC)] resulted in decomposition of the intermediate. Therefore, the symmetric anhydride of 4-pentynoic acid was synthesized by DCC mediated dehydration as previously described[42] and subsequently reacted with salicylic acid to yield AspAlk (Figure 1A). A small panel of cell-lines (HCT-15, PC-3, Cos-7, H1299, HEK293, HeLa, MCF7, and NIH3T3) were treated with AspAlk (1 mM) for 6 h. In-gel fluorescence, following CuAAC with with a previously reported azide-containing rhodamine fluorescent dye (az-rho),[41] enabled robust visualization of a large variety of proteins (Figure 1B). Notably, the pattern of labeled proteins was largely consistent between the different cell types. However, certain cell-types (PC-3, HeLa, and MCF7) had somewhat lower levels, and interestingly, HeLa and NIH3T3 cells have unique protein-targets that are qualitatively not present at high levels in the other cell-lines. Importantly, the pattern of visualized proteins does not simply correspond to protein abundance as judged by Coomassie blue staining (Figure S1 in Supporting Information), suggesting that modification occurs on specific protein−substrates that are expressed in most cell-lines.

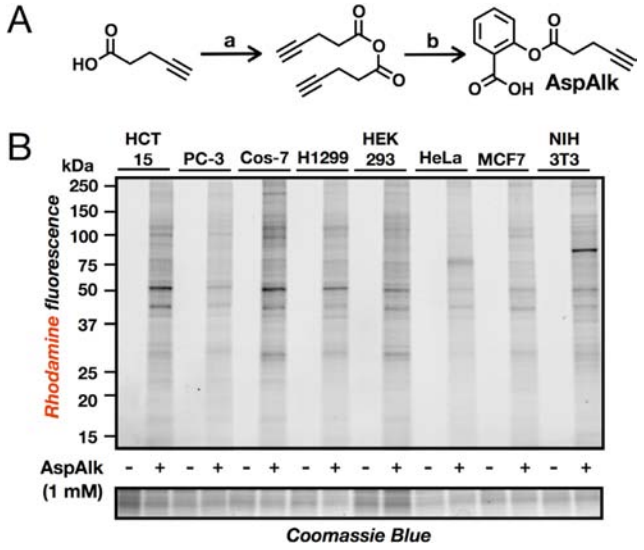

Figure 1. AspAlk is a chemical reporter of aspirin-dependent protein modification. (A) Reagents: (a) *N,N′*-dicyclohexcylcarbo-diimide (DCC), CH_2Cl_2, 16 h; (b) salicylic acid, pyridine, 16 h, 57% over two steps. (B) The indicated cell-lines were incubated with AspAlk (1 mM) for 6 h before CuAAC with Az-Rho and analysis by in-gel fluorescent scanning. Coomassie-blue staining shows protein loading.

Because aspirin has the largest effect on the incidence of gastrointestinal cancers, we next characterized AspAlk labeling in HCT-15 colorectal-cancer cells. These cells were treated with different concentrations of AspAlk for 6 h, before washing, lysis, and reaction of the soluble protein fraction with az-rho using CuAAC.[41] In-gel fluorescent scanning showed dose-dependent labeling of a variety of proteins in as little as 50 to 100 μM (Figure 2A), which represents a notable increase in sensitivity over a published immunoblotting method.[18,26] Importantly, the range of aspirin concentrations used (100 to 300 μM) is achievable in the plasma of patients treated with a short analgesic dose (600 mg) and others undergoing chronic

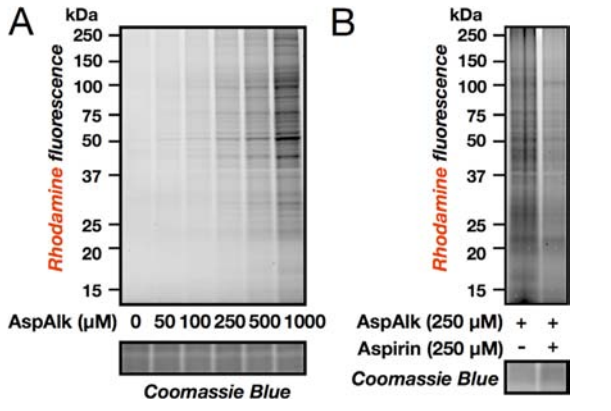

Figure 2. Characterization of AspAlk in HCT-15 colorectal cancer cells. (A) HCT-15 cells were treated with the indicated concentrations of AspAlk for 6 h before reaction with az-rho under CuAAC conditions and visualization by in-gel fluorescence scanning. (B) HCT-15 cells were treated with either AspAlk (250 μM) or AspAlk and aspirin (both @ 250 μM) for 2 h before CuAAC and visualization by in-gel fluorescence scanning. Coomassie-blue staining shows protein loading.

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aspirin-treatment of rheumatoid arthritis.[43−45] While the concentrations of aspirin in tissues such as the colon are unknown, even higher concentrations of aspirin (2.5 to 10 mM) are routinely used in phenotypic cell-based experiments.[46,47] To qualitatively determine if AspAlk treatment resulted in the acetylation of the same proteins that are modified by aspirin, HCT-15 cells were treated with AspAlk (250 μM) in the presence of an equal concentration of aspirin (250 μM) for 2 h before CuAAC with az-rho and analysis by in-gel fluorescence. Co-treatment with aspirin resulted in an approximately 50% reduction in labeling signal (Figure 2B), suggesting that AspAlk largely modifies the same proteins as aspirin.

A significant property of many chemical reporters is the ability to visualize labeling dynamics in a pulse-labeling experiment. To visualize the rate of AspAlk labeling, HCT-15 cells were incubated with AspAlk (1 mM) for different amounts of time. In-gel fluorescent scanning, after lysis and reaction with az-rho by CuAAC, demonstrated robust labeling in as little as 30 min and near-maximal signal in 4 h (Figure 3A), potentially

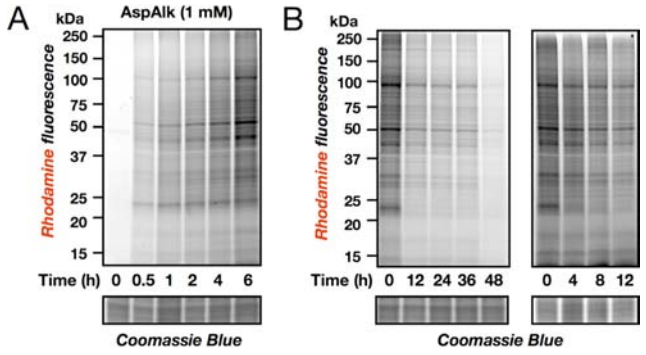

Figure 3. Kinetic analysis of AspAlk labeling. (A) HCT-15 cells were treated with AspAlk (1 mM) for the indicated lengths of time before reaction with az-rho under CuAAC conditions and visualization by in-gel fluorescence scanning. (B) HCT-15 cells were treated with AspAlk (1 mM) for 2 h before fresh media containing aspirin (1 mM) was added. Cells were collected after the indicated lengths of time, subjected to CuAAC, and visualization by in-gel fluorescence scanning. Coomassie-blue staining shows protein loading.

representing an improvement compared to previous Western-blotting based reports, where 8 to 12 h treatments were used.[18,26] Next, to determine how long aspirin-dependent modifications persist in cells, HCT-15 cells were treated with AspAlk (1 mM) for 2 h. At this time, the cells were washed with PBS and media containing aspirin (1 mM) was added. Cell were then harvested at different times, lysed, and subjected to CuAAC with az-rho. Analysis by in-gel fluorescence scanning showed that significant chemical-reporter signal was lost within the first 12 h while some signal persisted for 48 h (Figure 3B). To examine this in more detail, we treated HCT-15 cells as above, followed by harvesting after shorter lengths of time (Figure 3B). As in the previous pulse-chase, more than half of the fluorescent signal was progressively lost within the first 12 h. This fraction of signal is consistent with other chemical reporters of enzymatically reversible protein acylation pathways;[49] however, the persistence of the remaining signal suggests that at a percentage of AspAlk modifications are not removed by protein deacetylases.

Finally, to we used AspAlk to identify proteins that are potential substrates of aspirin-dependent acetylation. HCT-15 cells were treated in triplicate with AspAlk (1 mM) or DMSO vehicle for 2 h and lysed under denaturing conditions (4% SDS). CuAAC was then performed with an azido-biotin affinity tag (azido-PEG3-biotin), followed by incubation with streptavidin beads, washing, and on-bead trypsinolysis. Eluted peptides were then concentrated, identified by LC−MS/MS, and quantified by spectral counting. Proteins were considered aspirin-dependent acetylation substrates using the following criteria: (1) proteins must have been identified by at least one unique peptide (not necessarily overlapping) in each experimental replicate, (2) a total of at least three spectral counts in the AspAlk-treated samples, (3) the average spectral counts for each identified protein in the AspAlk-treated sample must have been at least 3-times greater than the corresponding average counts in the DMSO negative control, and (4) a p-value of at most 0.05 (t test). Proteins meeting these conditions were then rank-ordered based on enrichment-ratio and the average number of spectral counts in the AspAlk-treated sample (Supporting Information, Table S1). We identified 120 proteins with diverse cellular functions, which importantly contained eight proteins previously known to be acetylated by aspirin (Figure 4A).[19,20,26,50] In our list of identified proteins were

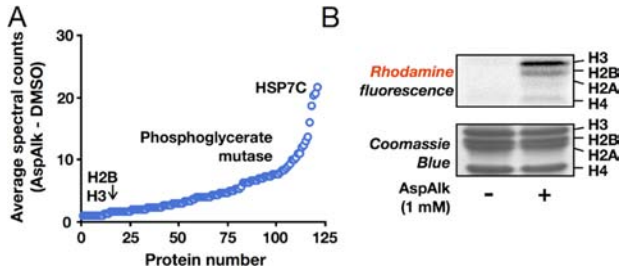

Figure 4. Identification of AspAlk-labeled proteins. (A) HCT-15 cells were treated with AspAlk (1 mM) for 2 h before reaction with azido-biotin under CuAAC conditions, on-bead trypsinolysis, and proteomic identification by LC−MS/MS. Representative proteins are indicated by name. (B) Histones were enriched from HCT-15 cells treated with either AspAlk (1 mM) or DMSO vehicle for 6 h, followed by CuAAC with az-rho and in-gel fluorescence. Coomassie-blue staining shows protein loading.

several of the core histone proteins, including H2B and H3. Given the crucial role of histone acetylation in transcriptional regulation,[51,52] we next confirmed the aspirin-dependent modification of these proteins. Accordingly, histones were enriched from HCT-15 cells treated with either AspAlk (1 mM) or DMSO. After reaction with az-rho under CuAAC conditions, AspAlk-dependent fluorescent signal was readily detected on all three histones identified by proteomics (Figure 4B).

■ CONCLUSIONS

Aspirin-dependent acetylation and subsequent inhibition of cyclooxygenase enzymes (Cox-1 and -2) is well established as one molecular mechanism contributing to cardioprotection and chemoprevention.[8,14−17,53,54] Notably, aspirin is known to acetylate a variety of additional proteins, giving it the potential to simultaneously affect multiple other cellular pathways that could also contribute to the effects of chronic aspirin-treatment. However, understanding the molecular consequences of aspirin-dependent acetylation has been hampered by a lack of tools to visualize and identify protein targets. To enable the robust visualization and identification of these modifications,

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we have developed an aspirin chemical-reporter (AspAlk) that transfers an alkyne functionality to proteins that can be subsequently detected using bio-orthogonal chemistries such as CuAAC. Treatment of a small panel of mammalian cell-lines with AspAlk, followed by CuAAC with an azide-bearing fluorophore (az-rho), enabled the visualization of a range of proteins by in-gel fluorescence scanning. Interestingly, the qualitative pattern of these labeled-proteins was similar in most cell-lines, suggesting that the major targets of aspirin-dependent acetylation may be broadly expressed proteins.

To test the limitations of AspAlk, we next focused on HCT-15 colorectal-cancer cells and aspirin use has the most dramatic effect on gastrointestinal cancers. AspAlk displayed dose-dependent labeling of proteins up to a 1 mM concentration. This labeling could be competed by the equimolar addition of aspirin at levels consistent with other chemical reporters.[37] As noted above, aspirin concentrations in the plasma of patients receiving an analgesic dose (600 to 650 mg) can reach 100 to 300 μM.[43−45] However, to our knowledge, the concentrations of aspirin in the gastrointestinal tract have not been precisely measured, and millimolar concentrations (2.5 to 10 mM) of aspirin have been routinely used in cell-culture experiments in the past.[46,47] Additionally, other studies have found that repetitive administration of aspirin results in a disproportionately large increase in serum levels.[55] Therefore, although 1 mM AspAlk is somewhat higher than the observed plasma concentrations, we utilized it to maximize our labeling efficiency for AspAlk characterization in subsequent experiments. In cells treated with a concentration of 1 mM AspAlk, protein-labeling could be visualized in as little as 30 min, a large improvement over previously reported studies using antiacetyl lysine antibodies, which treated cells for 8 or 12 h with aspirin.[18,26] Crucially, our short labeling-times correspond well to the measured stability of aspirin in the plasma of human patients.[43,45] A pulse-chase experiment showed that AspAlk-dependent modifications are largely turned over in 12 h, a length of time consistent with the enzymatic removal of other chemical reporters of protein acylation.[49] However some in-gel fluorescent signal persisted over 48 h and could represent protein degradation and/or dilution through cell division.

Given that the AspAlk chemical reporter improved the visualization of aspirin-dependent acetylation substrates compared to previous methods, it was used in combination with an azido-biotin tag and on-bead trypsinolysis to identify 120 potential protein targets for modification by aspirin. Again, we used AspAlk at a concentration of 1 mM to robustly identify as many potential protein substrates as possible that could be validated in cell-culture and animal model experiments in the future. Finally, we validated our proteomics data by visualizing the aspirin-dependent acetylation of several core histones, suggesting a possible role for aspirin in transcriptional regulation. In summary, the experiments described here represent the first application of chemical-reporter technology to aspirin-dependent acetylation and a significant improvement in the analysis of this modification. The use of azide-containing fluorescent tags, in combination with CuAAC, allows for the visualization of modification patterns and dynamics not accessible to immunoblotting methods. Furthermore, AspAlk enables the direct identification of potentially acetylated proteins using streptavidin-enrichment and proteomics. We anticipate that this discovery tool will complement antiacetyl lysine antibodies in efforts to functionally characterize aspirin-dependent acetylation, which will be crucial for a complete understanding of the role of aspirin in cardioprotection and chemoprevention.

EXPERIMENTAL PROCEDURES

General Information. All reagents used for chemical synthesis were purchased from Sigma-Aldrich unless otherwise specified and used without further purification. All anhydrous reactions were performed under argon atmosphere. Analytical thin-layer chromatography (TLC) was conducted on EMD Silica Gel 60 F_{254} plates with detection by potassium permanganate ($KMnO_4$), anisaldehyde, or UV. Flash chromatography was performed on 60 Å silica gel (EMD). ^{1}H and ^{13}C spectra were obtained Varian VNMRS-600 at 600 and 125 MHz. Chemical shifts are recorded in ppm (δ) relative to solvent. Coupling constants (J) are reported in Hz.

Synthesis of 2-Acetylphenyl pent-4-ynoate (AspAlk). Pentynoic acid (500 mg, 5.10 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (526 mg, 2.55 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL), and the solution was allowed to stir for 16 h at room temperature under an argon atmosphere. The reaction mixture was then diluted with CH_2Cl_2 (10 mL), and filtered to remove *N*,*N*′-dicylohexylurea. The resulting 4-pentynoic anhydride was dissolved in pyridine (20 mL), and salicylic acid (Alfa Aesar, 211 mg, 1.53 mmol) was added. After it was stirred for 16 h under an argon atmosphere, the reaction mixture was concentrated and subjected to silica gel column chromatography (90:2.5:1, ethyl acetate:methanol:water). The resulting mixture was resuspended in CH_2Cl_2 (5 mL), filtered to removed contaminating 4-pentynoic acid, and concentrated to yield 188 mg, (57% yield) of 2-acetylphenyl pent-4-ynate as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 (dd, J = 7.8, 1.7 Hz, 1H), 7.59 (t, J = 7.8 Hz, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 2.82 (dd, J = 8.3, 6.6 Hz, 2H), 2.60 − 2.54 (m, 2H), 2.30 (t, J = 2.6 Hz, 1H). ^{13}C NMR (125 MHz, MeOH) δ 225.53, 189.97, 185.16, 170.96, 153.86, 151.79, 146.00, 143.87, 102.36, 89.70, 53.25, 33.70. ESI-MS calcd for $C_{12}H_{10}O_4Na$ $[M+Na]^+$ 241.05; found, 240.00.

Cell Culture. CHO, COS-7, HEK293, HeLa, and MCF-7 cells were cultured in high glucose DMEM media (CellGro) enriched with 10% fetal bovine serum (FBS, CellGro,). NIH3T3 cells were cultured in high glucose DMEM media (CellGro) with 10% fetal calf serum (FCS, CellGro). H1299 cells were cultured in RPMI 1640 (CellGro) medium enriched with 10% FBS. All cell lines were maintained in a humidified incubator at 37 °C and 5.0% CO_2.

Metabolic Labeling. To cells at 80−85% confluency, high glucose media containing AspAlk (1000 × stock in DMSO), or DMSO vehicle was added as indicated. For competition and chase experiments, the indicated media was supplemented with O-acetyl salicylic acid (Alfa Aesar) at the indicated concentrations.

Preparation of NP-40-Soluble Lysates. The cells were collected by trypsinization and pelleted by centrifugation at 4 °C for 2 min at 2000*g*, followed by washing with PBS (1 mL) two times. Cell pellets were then resuspended and lysed in 75 μL of 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4] with Complete Mini protease inhibitor cocktail (Roche Biosciences) for 15 min, followed by centrifugation at 4 °C for 10 min at 10 000 *g*. The resulting supernatant (soluble cell lysate) was collected and protein concentration was determined via BCA assay (Pierce, ThermoScientific).

Cu(I)-Catalyzed [3 + 2] Azide−Alkyne Cycloaddition (CuAAC). Soluble cell lysate (200 μg) was diluted with cold 1% NP-40 lysis buffer to a concentration of 1 μg/μL. Newly made click chemistry cocktail (12 μL) was added to each sample [az-rhodamine tag (100 μM, 10 mM stock solution in DMSO); tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in water); tris[(1-benzyl-1-*H*-1,2,3-triazol-4-yl)methyl]-amine (TBTA) (100 μM, 10 mM stock solution in DMSO); $CuSO_4$·$5H_2O$ (1 mM, 50 mM freshly prepared stock solution in water) for a total reaction volume of 200 μL. The reaction was gently vortexed and allowed to sit at room temperature for 1 h. Upon completion, 1 mL of ice cold methanol was added to the reaction, and proteins were precipitated at −20 °C for 2 h. The reactions were then centrifuged at

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4 °C for 10 min at 10 000 *g*. The supernatant was removed, the pellet was allowed to air-dry for 5 min, and then 50 μL of 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 50 μL of 2× loading buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β-mercaptoethanol) was then added. The samples were boiled for 5 min at 98 °C, and 40 μg of protein was then loaded per lane for SDS-PAGE separation (Any kD Criterion Gel, Bio-Rad).

Biotin Enrichment and On-Bead Trypsinolysis. HCT-15 cell pellets labeled with AspAlk (1 mM) or DMSO for 2 h were resuspended in 200 μL of H_2O, 60 μL of PMSF in H_2O (250 mM), and 500 μL of 0.05% SDS buffer (0.05% SDS, 10 mM TEA, pH 7.4, 150 mM NaCl) with Complete Mini protease inhibitor cocktail (Roche Biosciences). To this was added 8 μL of Benzonase (Sigma), and the cells were incubated on ice for 30 min. Then, 4% SDS buffer (2000 μL) was added, and the cells were briefly sonicated in a bath sonicator followed by centrifugation (20 000 *g* for 10 min at 15 °C). Soluble protein concentration was normalized by BCA assay (Pierce, ThermoScientific) to 1 mg/mL, and 10 mg of total protein was subjected to the appropriate amount of click chemistry cocktail containing azido-PEG3-biotin (5 mM, Click Chemistry Tools) for 1 h, after which time 10 volumes of ice-cold MeOH were added. Precipitation proceeded for 2 h at −20 °C. Precipitated proteins were centrifuged at 5200*g* for 30 min at 0 °C and washed three times with 40 mL of ice-cold MeOH, with resuspension of the pellet each time. The pellet was then air-dried for 1 h. To capture the biotinylated proteins by streptavidin beads, the air-dried protein pellet was resuspended in 2 mL of resuspension buffer (6 M urea, 2 M thiourea, 10 mM HEPES pH 8.0) by bath sonication. To cap cysteine residues, 100 μL of freshly made TCEP (200 mM stock solution, Thermo) was then added, and the mixture was incubated for 30 min, followed by 40 μL of freshly prepared iodoacetamide (1 M stock solution, Sigma) and incubation for a further 30 min in the dark. Steptavadin beads (250 μL of a 50% slurry per sample, Thermo) were washed 2× with 1 mL of PBS and 1× with 1 mL of resuspension buffer and resuspended in resuspension buffer (200 μL). Each sample was combined with streptavadin beads and incubated on a rotator for 2 h. These mixtures were then transferred to Mini Bio-Spin columns (Bio-Rad) and placed on a vacuum manifold. Captured proteins were then washed 5× with resuspension buffer (10 mL per wash), 5× with 1% SDS in PBS (10 mL per wash), and 30× with PBS (1 mL per wash). Beads were then resuspended in 2 M urea in PBS (1 mL), transferred to screw-top tubes, and pelleted by centrifugation (2000*g* for 2 min). At this time, 800 μL of the supernatant was removed, leaving a volume of 200 μL. To this bead-mixture was added 2 μL of $CaCl_2$ (200 mM stock, 1 mM final concentration) and 2 μL of 1 mg/mL sequence grade trypsin (Promega) and incubated at 37 °C for 18 h. The resulting mixtures of tryptic peptides and beads were transferred to Mini Bio-Spin columns (Bio-Rad) and the eluent was collected by centrifugation (1000*g* for 2 min). Any remaining peptides were eluted by the addition of 100 μL of 2 M urea in PBS followed by centrifugation as immediately above. The tryptic peptides were then applied to C18 spin columns (Pierce) according to manufacturer's instructions, eluted with 70% acetonitrile in H_2O, and concentrated to dryness on a speedvac.

LC−MS/MS Analysis. Extracted peptides were desalted on a trap column following separation on a 12 cm/75um reversed phase C18 column (Nikkyo Technos Co., Ltd. Japan). A 3 h gradient increasing from 10% B to 45% B in 3 h (A, 0.1% formic acid; B, acetonitrile/0.1% formic acid) was delivered at 150 nL/min. The liquid chromatography setup (Dionex, Boston, MA, USA) was connected to an Orbitrap XL (Thermo, San Jose, CA, USA) operated in top-5 mode. Acquired tandem MS spectra (CID) were extracted using ProteomeDiscoverer, version 1.3 (Thermo, Bremen, Germany) and queried against the human Uniprot protein database using MASCOT 2.3.02 (Matrixscience, London, UK). Peptides fulfilling a Percolator calculated 1% false discovery rate threshold were reported. All LC−MS/MS analysis were carried out at the Proteomics Resource Center at The Rockefeller University, New York, NY, USA. A total of 810 proteins were identified, with 120 fulfilling our criteria as a "hit." A list of all identified peptides and proteins will be made available in Excel format upon request.

Acid Extraction of Histones. HCT-15 cells were treated with DMSO or 1 mM AspAlk for 6 h. Cells were collected by trypsinization and pelleted by centrifugation at 4 °C for 2 min at 2000*g*, followed by washing with PBS (1 mL) two times. Cell pellets were then resuspended in ice-cold hypotonic lysis buffer [10 mM triethanolamine (TEA), 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM PMSF, pH 7.4 with Complete Mini protease inhibitor cocktail (Roche Biosciences)]. The resuspended cells were homogenized by Dounce homogenizer and lysed in three cycles of freeze−thaw. Intact nuclei were pelleted at 4 °C for 10 min at 10 000 *g* and washed 2× with ice-cold hypotonic lysis buffer. The nuclear pellet was resuspended in 0.4 N H_2SO_4 and agitated overnight on a rotator at 4 °C. Nuclear debris was pelleted at 4 °C for 10 min at 16 000 *g* and the supernatant containing histones was collected and precipitated in ice-cold MeOH in −80 °C overnight. Precipitated histones were collected at 4 °C for 10 min at 16 000 *g* and washed 2× with ice-cold MeOH. The resulting protein pellet was air-dried and resuspended in water. Concentration was determined by BCA Assay and normalized with 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4] with Complete Mini protease inhibitor cocktail] for CuAAC.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures, NMR characterization of AspAlk, and proteomic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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