Rapid and direct control of target protein levels with VHL-recruiting dTAG molecules

Behnam Nabet1,2,10✉, Fleur M. Ferguson1,2,10, Bo Kyung A. Seong3,4, Miljan Kuljanin5, Alan L. Leggett1, Mikaela L. Mohardt1, Amanda Robichaud3, Amy S. Conway3, Dennis L. Buckley6,9, Joseph D. Mancias5, James E. Bradner6,7,9, Kimberly Stegmaier3,4,8 & Nathanael S. Gray1,2✉

Chemical biology strategies for directly perturbing protein homeostasis including the degradation tag (dTAG) system provide temporal advantages over genetic approaches and improved selectivity over small molecule inhibitors. We describe dTAGV-1, an exclusively selective VHL-recruiting dTAG molecule, to rapidly degrade FKBP12F36V-tagged proteins. dTAGV-1 overcomes a limitation of previously reported CRBN-recruiting dTAG molecules to degrade recalcitrant oncogenes, supports combination degrader studies and facilitates investigations of protein function in cells and mice.
Modulating protein abundance with small molecule degraders is a powerful approach for investigating functional consequences of rapid and direct protein loss, without alteration of corresponding mRNA levels. Degraders including heterobifunctional degraders (also known as PROteolysis-Targeting Chimera or PROTACs) and non-chimeric molecular glue, co-opt an E3 ubiquitin ligase to induce rapid and reversible proteasome-mediated degradation. Achieving immediate target protein loss with degraders provides a crucial advantage over genetic knockout or knockdown approaches, which require a significant delay to achieve an impactful protein reduction. However, degrader development is hindered by a reliance on target-specific chemical matter, which is unavailable for the majority of the proteome. To address this challenge, several strategies aimed at the direct control of cellular protein levels have been recently developed, including methods that use small molecules, nanobodies, or antibodies.

We previously described a versatile approach known as the degradation tag (dTAG) system to rapidly deplete any tagged target protein in cells and in mice. The dTAG system is a dual component platform requiring the expression of FKBP12F36V in-frame with a gene-of-interest and treatment with a heterobifunctional dTAG molecule (dTAG-1) that engages FKBP12F36V and cerebromediated degradation, exemplified by FKBP12F36V-fusions in a pancreatic ductal adenocarcinoma (PDAC) model. This interaction leads to exclusive degradation of the FKBP12F36V-targeted protein. Studies degrading diverse targets including oncoproteins, transcription factors, chromatin regulators, and kinases illustrate the utility of the dTAG system for drug target validation and discovery. Despite this broad applicability, we observed context-specific and protein-specific differences in the effectiveness of dTAG-1 for inducing target protein degradation.

Here, we report the synthesis, characterization and utility of a second generation, in vivo-compatible dTAG molecule that recruits the von Hippel-Lindau (VHL) E3 ligase complex, dTAGV-1 (Fig. 1a, b). We demonstrate that dTAGV-1 degrades fusion proteins recalcitrant to CRBN-mediated degradation, exemplified by EWS/FLI, a driver of Ewing sarcoma. Collectively, this study describes an important extension to the dTAG platform, towards a second generation, in vivo-compatible dTAG molecule that recruits EWS/FLI, a driver of Ewing sarcoma. Collectively, this study describes an important extension to the dTAG platform, towards a second generation, in vivo-compatible dTAG molecule that recruits EWS/FLI, a driver of Ewing sarcoma.

Results
dTAGV-1 is a potent, selective, in vivo-compatible degrader. To identify a VHL-recruiting dTAG molecule, we synthesized ortho-AP1867-conjugated analogs with varying VHL-binding ligands and linker composition and screened for cellular activity in 293FT FKBP12W-Nluc and FKBP12F36V-Nluc dual luciferase systems. We previously described the synthesis, characterization and utility of a second generation, in vivo-compatible dTAG molecule that recruits the von Hippel-Lindau (VHL) E3 ligase complex, dTAGV-1 (Fig. 1a, b). We demonstrated that dTAGV-1 degrades fusion proteins recalcitrant to CRBN-mediated degradation, exemplified by EWS/FLI, a driver of Ewing sarcoma. Collectively, this study describes an important extension to the dTAG platform, towards a second generation, in vivo-compatible dTAG molecule that recruits EWS/FLI, a driver of Ewing sarcoma.

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identifying targetable dependencies in Ewing sarcoma. Ewing sarcoma is driven by the translocation of EWSR1 and members of the ETS transcription factors, most commonly FLI, giving rise to a fusion transcription factor oncprotein that activates an aberrant transcriptional program. Currently, there are a shortage of model systems and direct-acting agents that allow modulation of EWS/FLI levels or activity with precise kinetic control. To evaluate the effects of EWS/FLI degradation in Ewing sarcoma, we selected EWS502 cells, which are highly dependent on EWS/FLI for proliferation (Supplementary Fig. 4a). Through permutation-based FDR estimation are provided in Supplementary Data 2. Data are from n = 4 biologically independent samples and are representative of n = 3 independent experiments. d Protein abundance after treatment of PATU-8902 LACZ-FKBP12F36V clone with 500 nM dTAGV-1 for 4 h compared to DMSO treatment. Volcano plots depict fold change abundance relative to DMSO versus P value derived from a two-tailed Student’s t-test. Fold change values and significance designations derived from a two-tailed Student’s t-test and a permutation-based FDR estimation are provided in Supplementary Data 2. Data are from n = 3 biologically independent samples. e Immunoblot analysis of PATU-8902 LACZ-FKBP12F36V clone co-treated with DMSO, THAL-SNS-032, and/or dTAGV-1 as indicated for 24 h. Data is representative of n = 3 independent experiments. Source data for c and e are provided as a Source Data file.

Fig. 1 dTAGV-1 is an exclusively selective degrader of FKBP12F36V-tagged proteins. a Schematic depiction of the dTAG system using VHL-recruiting dTAG molecules. VHL-recruiting dTAG molecules promote ternary complex formation between the FKBP12F36V-tagged target protein and E3 ubiquitin ligase complex, inducing target protein ubiquitination and degradation. b Chemical structures of dTAGV-1 and dTAGV-1-NEG. c DMSO-normalized ratio of LACZ-FKBP12F36V clone with 500 nM dTAGV-1 for 4 h compared to DMSO treatment. Volcano plots depict fold change abundance relative to DMSO versus P value derived from a two-tailed Student’s t-test. Fold change values and significance designations derived from a two-tailed Student’s t-test and a permutation-based FDR estimation are provided in Supplementary Data 2. Data are from n = 3 biologically independent samples. e Immunoblot analysis of PATU-8902 LACZ-FKBP12F36V clone co-treated with DMSO, THAL-SNS-032, and/or dTAGV-1 as indicated for 24 h. Data is representative of n = 3 independent experiments. Source data for c and e are provided as a Source Data file.
dTAGV-1 is an in vivo-compatible degrader of FKBP12F36V-tagged proteins. a Immunoblot analysis of PATU-8902 FKBP12F36V, KRASG12V, KRAS−/− clone treated with DMSO, dTAGV-1, or dTAGV-1-NEG for the indicated time-course. b Immunoblot analysis of 293TWT FKBP12F36V, KRASG12V or 293TVHL−/− FKBP12F36V, KRASG12V cells treated with DMSO or the indicated dTAG molecules for 24 h. Data in a, b are representative of n = 3 independent experiments. c DMSO-normalized antiproliferation of PATU-8902 LACZ-FKBP12F36V or FKBP12F36V-KRASG12V; KRAS−/− clones treated with dTAGV-1 or dTAGV-1-NEG for 120 h. Cells were cultured as ultra-low adherent 3D-spheroid suspensions. Data are presented as mean ± s.d. of n = 4 biologically independent samples and are representative of n = 3 independent experiments. d Bioluminescent imaging to evaluate degradation of luciferase-FKBP12F36V in mice was performed daily as follows: day 0 to establish baseline signal, day 1-3 to monitor luciferase-FKBP12F36V signal 28 h after vehicle or dTAG molecule treatment (T), day 4 to monitor duration of luciferase-FKBP12F36V signal 28 h after third and final vehicle or dTAG molecule treatment. Total flux for each mouse is depicted. Data are presented from vehicle (n = 5 biologically independent mice at day 0–4), dTAG-13 (n = 5 biologically independent mice at day 0–3; n = 4 biologically independent mice at day 4) or dTAGV-1 (n = 5 biologically independent mice at day 0–4) treated mice. P values are derived from a two-tailed Welch’s t-test (*P < 0.05, **P < 0.01) and are provided as a Source Data file. Source data for a–d are provided as a Source Data file.

Together, this data exemplifies the utility of VHL-recruiting dTAG molecules and provides model systems to evaluate the acute and prolonged consequences of EWS/FLI loss.

Discussion

We report dTAGV-1, a potent and exclusively selective VHL-recruiting degrader of FKBP12F36V-tagged proteins. dTAGV-1 displays improved PK/PD properties and serves as an optimized tool for in vivo applications. Through evaluation of mutant KRAS degradation in PDAC models, we show that either CRBN or VHL can be co-opted to alleviate the aberrant signaling coordinated by this oncoprotein. By contrast, we observed contextual differences in the ability of these E3 ubiquitin ligase complexes to degrade EWS/FLI. This is consistent with our recent report demonstrating effective degradation of a core mediator subunit (MED14) with dTAGV-1 in HCT116 cells, a context in which CRBN-recruiting dTAG molecules were not effective.22. We observed that rapid MED14 degradation abrogated lineage-specifying transcriptional circuits. Together, our studies provide support for use

in NKX2-2 protein levels and modest antiproliferative effects relative to dTAGV-1 (Supplementary Fig. 5b, c). Similar trends in antiproliferation upon FKBP12F36V, KRASG12V degradation were observed upon treatment of PATU-8902 FKBP12F36V, KRASG12V; KRAS−/− cells with dTAG-63 (Supplementary Fig. 5d). These results are consistent with our prior observation that dTAG-7 is less effective across multiple cell lineages than dTAG-13, a CRBN-recruiting dTAG molecule with an all carbon linker.14

Finally, we observed pronounced antiproliferative activity upon degradation of EWS/FLI with dTAGV-1, our data indicates that combination approaches may be necessary to achieve complete loss of viability. Prior work indicates that BET bromodomain inhibitors and degraders may have applications in Ewing Sarcoma33. To investigate potential synergy between direct and indirect repression of the EWS/FLI transcriptional program, we evaluated degradation of EWS/FLI in combination with BET bromodomain degradation. We observed that dBE6, a CRBN-recruiting, pan-BET bromodomain degrader32, synergized strongly with VHL-mediated EWS/FLI degradation (Supplementary Fig. 6a–c).
Fig. 3 EWS/FLI degradation reverses abnormal proteomic signaling and proliferation. a, b Immunoblot analysis of EWS502 FKBP12<sup>F36V</sup>-GFP or FKBP12<sup>F36V</sup>-EWS/FLI<sup>−/−</sup> cells treated with DMSO (a, b), dTAG-13 (a), or dTAG<sup>κ</sup>-1 (b) for 24 h. Data in a, b are representative of n = 3 independent experiments. c Protein abundance after treatment of EWS502 FKBP12<sup>F36V</sup>-EWS/FLI<sup>−/−</sup> cells with 1 μM dTAG<sup>κ</sup>-1 for 6 h compared to DMSO treatment. Volcano plots depict fold change abundance relative to DMSO versus P value derived from a two-tailed Student’s t-test. Fold change values and significance designations derived from a two-tailed Student’s t-test and a permutation-based FDR estimation are provided in Supplementary Data 2. Data are from n = 3 for DMSO and n = 3 for dTAG<sup>κ</sup>-1 biologically independent samples. d Immunoblot analysis of EWS502 FKBP12<sup>F36V</sup>-GFP or FKBP12<sup>F36V</sup>-EWS/FLI<sup>−/−</sup> cells treated with DMSO, 1 μM dTAG<sup>κ</sup>-1 or 1 μM dTAG<sup>κ</sup>-1-NEG for 24 h. Data are representative of n = 3 independent experiments. e GSEA signatures upon assessment of significantly differentially expressed target proteins (FDR < 0.05) after treatment of EWS502 FKBP12<sup>F36V</sup>-EWS/FLI<sup>−/−</sup> cells as described in c. Data are from n = 2 for DMSO and n = 3 for dTAG<sup>κ</sup>-1 biologically independent samples. f Relative growth of EWS502 FKBP12<sup>F36V</sup>-EWS/FLI<sup>−/−</sup> cells treated with DMSO, dTAG<sup>κ</sup>-1, or dTAG<sup>κ</sup>-1-NEG. Y-axis represent luminescence values relative to day 0. Data are presented as mean ± s.d. of n = 8 technical replicates and are representative of n = 3 independent experiments. Source data for a, b, d, and f are provided as a Source Data file.

of dTAG<sup>κ</sup>-1 to overcome the current limitations of the dTAG system, enabling evaluation of the direct effects of fusion proteins recalcitrant to CRBN-recruiting dTAG molecules.

Employing dTAG<sup>κ</sup>-1 to study EWS/FLI, we demonstrate that VHL-mediated degradation of EWS/FLI rapidly alters downstream target protein expression and leads to pronounced growth defects in Ewing sarcoma cells, providing a powerful model system to investigate immediate consequences of EWS/FLI loss. This data supports that targeting EWS/FLI for degradation with direct-acting heterobifunctional degraders or molecular glues may be a tractable strategy and identifies potential combination strategies with BET bromodomain degraders. Together, the suite of dTAG molecules and paired controls provided in this study will facilitate evaluation of the functional consequences of precise post-translational protein removal for an expanded target pool. The dTAG system enables rapid modulation of protein abundance and serves as a versatile strategy to determine whether targeted degradation is a promising drug development approach for a given target in vitro and in vivo.

Methods

Molecule synthesis. Full details on molecule synthesis are provided in Supplementary Methods and Supplementary Figs. 7–18.
**Cell lines.** The following cell lines were employed in this study: 293T (source: ATCC #CRL-3216, media: DMEM with 10% FBS and 1% Penicillin-streptomycin; Thermofisher Scientific #R70007, media: DMEM with 10% FBS and 1% Penicillin-streptomycin), PATU-8902 (source: DSMZ #ACC-179, media: DMEM with 10% FBS and 1% Penicillin-streptomycin), MV4-11 (source: ATCC #CRL-9591, media: RPMI with 10% FBS and 1% Penicillin-streptomycin) and EWS502 (source: kindly provided by Dr. Stephen L. Lessnick of Nationwide Children’s Hospital and established by Dr. Jonathan A. Fletcher of Harvard Medical School, media: RPMI with 15% FBS and 1% Penicillin-streptomycin-t-Glutamine). Development of engineered cell lines are detailed below. All cell lines were maintained in 37 °C and 5% CO₂ incubators and routinely tested negative for mycoplasma contamination using the MycoAlert kit (Lonza).

**Lentiviral dTAG plasmid construction.** To generate pLEX_305-dTAG-GFP and pLEX_305-dTAG-EWS/FLI plasmids, gateway recombination cloning strategies (Invitrogen) were employed to clone GFP or EWS/FLI into pLEX_305-N-dTAG. In brief, to first generate pDONR221-EWS/FLI, EWS/FLI was cloned into pDONR221 using BP clonase (Invitrogen) after PCR with the following primers containing BP overhangs: Forward-N-E/dTAG, 5'-ggggacccgtgtaaacaagcctgtgcagggcagggataacagtacct-3' and Reverse-N-E/dTAG, 5'-ggggacccgtgtaaagaagcagcctgtgcagggcagggataacagtacctgtaaacaagcctgtgcagggataacagtacct-3'. Second, pENTREGM2 (Addgene #22450) and pDONR221-EWS/FLI were cloned into pLEX_305-N-dTAG using LR clonase (Invitrogen). pLEX_305-dTAG-KRASG12V and pLEX_305-LACZ-dTAG plasmids were also employed in this study.

**Lentiviral CRISPR/Cas9 plasmid construction.** pXPR007-sgGFP and pXPR007-sgKRAS were employed in this study. To generate lentiCRISPR v2-Blast-sgFLI_Ex9, sgFLI_Ex9 (5'-GCGTACAGCGGTGTACGAGAAG-3') was cloned into lentiCRISPR v2-Blast vector (Addgene #83340) using BamH restriction sites. The PAM motif between sgGFP and sgKRAS is present in an intron, enabling cutting of the endogenous locus only with DNA enzyme.

**Development of engineered cell lines.** To generate 293TWT FKBP12F36V, KRASG12V and 293TWT/FLI-/- FKBP12F36V-KRASG12V cells, lentiviral supernatants* were applied to 293TWT and 293TVHL by Dr. Jonathan A. Fletcher of Harvard Medical School, media: RPMI with 15% FBS and 1% Penicillin-streptomycin. The following cell lines were employed as appropriate.

**FKBP12 WT and FKBP12 F36V dual luciferase assay.** Dual luciferase assays were performed using 293FT FKBP12F36V-WT, Nluc and FKBP12F36V-Nluc cells. In brief, cells were plated at 2000 cells per well in 20 µL of appropriate media in 384-well white culture plates (Corning), allowed to adhere overnight, and 100 nL of compounds were added using a Janus Workstation pin tool (PerkinElmer) for 24 h at 37 °C. Next, samples were vortexed thoroughly. Each sample was centrifuged at 14,000 × g for 5 min at room temperature. Protein pellets were resolubilized in 200 mM EPPS buffer and 5 µg/mL of the protein pellet was washed twice with methanol and centrifuged at 14,000 × g for 5 min at room temperature. Protein pellets were resolubilized in 200 mM EPPS buffer and digested overnight with Lys-C (1:100, enzyme:protein ratio) at room temperature. The next day, trypsin (1:100 ratio) was added and incubated at 37 °C for an additional 6 h in a ThermoMixer set to 1000 RPM. To each digested sample, 30% anhydrous acetonitrile was added and 100 µg of peptides were labelled using 200 µg of TMT reagent (TMT-111). Following labeling, a 5% hydroxyamidine solution was used to quench excess TMT reagent. To isolate protein, a loading, a ratio test was performed by pooling 2 µg of each TMT-labeled sample. Samples were pooled and desalted using in-house packed C18 StageTips and analyzed by LC-MS/MS. Normalization factors were calculated from this label check, samples were mixed 1:1 across all TMT channels and desalted using a 100 µg Sep-Pak solid phase extraction cartridge. Eluted pooled peptides were further fractionated with basic pH reverse-phase (SP) HPLC using an Agilent 300 extend C18 column and collected into a 96 deep-well plate. Samples were consolidated into 24 fractions, and 12 nonadajacent fraction were desalted using StageTips prior to analyses using LC-MS/MS. All mass spectrometry data was acquired using an Orbitrap Fusion mass spectrometer in-line with a Proxeon NanoLC-1000 UHPLC system. Peptides were separated using an in-house 100 µm capillary column packed with 40 cm of Accucore 150 resin (2.6 µm, 130 Å) (Thermo Fisher Scientific) using a 180 min LC gradient per fraction. Eluted peptides were acquired using synchronous precursor selection (SPS-MS3) method for

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TMT quantification. Briefly, MS1 spectra were acquired at 120 K resolving power with a maximum of 50 ms in the Orbitrap. MS2 spectra were acquired by selecting the top 10 most abundant features via collision induced dissociation (CID) in the ion trap using an automatic gain control (AGC) of 15 K, quadrupole isolation width of 0.7 m/z and a maximum ion time of 100 ms. For MS3 acquisition, a synchronous precursor selection of ten fragment ions was acquired with an AGC of 150 K for 150 ms and a normalized collision energy of 55.

Quantitative proteomics: MS data analysis. All acquired raw data were converted to mzXML using Raw File Reader (v3.0.77). Spectra were processed using Comet (2019.01.15)34–36, search results were filtered using the LDA function MASS Package in R, and data were processed using an in-house informatics pipeline12–14. Custom code was not developed in this study. Briefly, peptide spectral libraries were first filtered to a peptide false discovery rate (FDR) of less than 1% using linear discriminant analysis employing a target decoy strategy. Spectral searches were performed using a 2020 Uniprot Human database including canonical isoforms (96,788 total entries) fasta formatted database which included custom sequences for LACZ-FKBPI22901 and FKBP125943. EWS/FLI, common contaminants, reversed sequences (Uniprot Human, 2020) and the following parameters: peptide fragment tolerance, fully tryptic peptides, fragment ion tolerance of 0.9 Da and a static modification of TMT (+229.163 Da) on lysine and peptide N-termini, carbamidomethylation of cysteine residues (+57.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was set as a variable modification. Resulting peptides were further filtered to obtain a 1% protein FDR and proteins were collapsed into groups. Reporter ion intensities were adjusted to correct for impurities during synthesis of different TMT reagents according to the manufacturer’s specifications. For quantitation, a total sum signal-to-noise of all report ions of 100 was required for analysis, isolation specificities of the MS2 and MS3 level >0.50 and no missing values (11/11 TMT channels). Agreement, or at: http://graylab.dana-farber.org/probes.html. Source data are provided with this paper.

Animal studies: compound formulation. For IP injections, dTAG-13 and dTAG-1 were formulated by dissolving into DMSO and then diluting with 20% solutol (Sigma): 0.9% sterile saline (Molotx) (wv) with the final formulation containing 5% DMSO. Maximal solubility of 35 mg kg\(^{-1}\) and 40 mg kg\(^{-1}\) were observed for dTAG-13 and dTAG-1, respectively. Formulations were stable at room temperature for 7 days. For IV injections, dTAG-13 and dTAG-1 were formulated by dissolving into DMSO and then diluting with 5% solutol (Sigma): 0.9% sterile saline (Molotx) (wv) with the final formulation containing 5% DMSO.

Animal studies: pharmacokinetic (PK) evaluation. All procedures for PK studies were compliant with ethical regulations for animal testing and research, and were approved by and performed in accordance with standards of the Institute Animal Care and Use Committee (IACUC) at Scripps Florida. Housing conditions for mice at Scripps Florida were as follows: lights on 7 AM, lights off = 7 PM, average ambient temperature = 72 °F, and average humidity = 45%. PK was assessed in 8-week-old C57BL/6 male mice (Jackson Laboratory, #000664) with blood collected at 0.08, 0.25, 0.5, 1, 2, 4, 6, and 8 h (2 mg kg\(^{-1}\) dTAG-13 intravenous (IV) tail vein, 10 mg kg\(^{-1}\) dTAG-13 intramuscular (IP) and 1 mg kg\(^{-1}\) dTAG-1 IV tail vein administration). Plasma was gen-erified and centrifuged and plasma concentrations were determined by LC-MS/MS following the mass transition 49600340 AMU. PK parameters were calculated using Phoenix WinNonlin to determine peak plasma concentration (C\(_{\text{max}}\)), oral bioavailability (%F), exposure (AUC), half-life (t\(_{1/2}\)), clearance (CL), and volume of distribution (Vd).

Animal studies: pharmacodynamic (PD) evaluation. All procedures for PD studies were compliant with ethical regulations for animal testing and research, and were approved by and performed in accordance with standards of the IACUC at Dana–Farber Cancer Institute. Housing conditions for mice at Dana–Farber Cancer Institute were as follows: lights on 6 AM, lights off = 6 PM, average ambient temperature = 72 °F, and average humidity = 35–55%. Evaluation of degradation of luciferase in vivo was performed using MV4-11 luc-FKBPI22901 cells and bioluminescent measurements64,65. In brief, 250,000 viable MV4-11 luc-FKBPI22901 cells were transplanted by tail-vein injection in 8-week-old immuno-competent female NOD.Cg-Pkd1<sup>−/−</sup>Xid.Pkd2<sup>−/−</sup>Idd<sup>−/−</sup> (Stl, Nsg; Jackson Laboratory, #005557). Bioluminescent measurements were performed following IP injection of 75 mg kg\(^{-1}\) l-D Luciferin (Promega). Mice were then anesthetized with 2–3% isoflurane, imaged on an IVIS Spectrum (Caliper Life Sciences), and total body bioluminescence was determined using a standardized region of interest. Two and seven days after tail-vein injection, bioluminescent measurements were per-formed to confirm engraftment18. Detectable bioluminescent signal in the cohort was observed at day 7 and was used as the baseline signal for each mouse. For treatments, compounds were formulated as described above, administered via IP injection and bioluminescent measurements were performed daily as described in Fig. 2d, beginning 8 days after tail-vein injection.

Statistical analysis. Information regarding center values, error bars, number of replicates or samples, number of independent experiments, and statistical analyses are described in the corresponding figure legends and data files. Experiments were not blinded nor randomized, and sample sizes were not predetermined using statistical analyses.

Data availability
Mass spectrometry-based proteomics raw data files are provided in Supplementary Data 1 and have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018937. Mass spectrometry-based proteomics processed data files underlying Figs. 1d and 3c and Supplementary Fig. 1f are provided in Supplementary Data 2. The source data underlying Figs. 1c, e, 2a–d, 3a, b, d, f and Supplementary Figs. 1e–c, e, a–d, 5b–d, 6a–c are provided as a Source Data file. Reagents are available upon request, following completion of a material transfer agreement, or at: http://graylab.dana-farber.org/probes.html. Source data are provided with this paper.

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