A robust and economical pulse-chase protocol to measure the turnover of HaloTag fusion proteins

Ronald A. Merrill, Jianing Song, Rikki A. Kephart, Annette J. Klomp, Claire E. Noack, and Stefan Strack

From the Department of Neuroscience and Pharmacology, and Iowa Neuroscience Institute, University of Iowa, Iowa City, Iowa 52242

Edited by George N. DeMartino

The self-labeling protein HaloTag has been used extensively to determine the localization and turnover of proteins of interest at the single-cell level. To this end, halogen-substituted alkanes attached to diverse fluorophores are commercially available that allow specific, irreversible labeling of HaloTag fusion proteins; however, measurement of protein of interest half-life by pulse-chase of HaloTag ligands is not widely employed because residual unbound ligand continues to label newly synthesized HaloTag fusions even after extensive washing. Excess unlabeled HaloTag ligand can be used as a blocker of undesired labeling, but this is not economical. In this study, we screened several inexpensive, low-molecular-weight haloalkanes as blocking agents in pulse-chase labeling experiments with the cell-permeable tetramethylrhodamine HaloTag ligand. We identified 7-bromoheptanol as a high-affinity, low-toxicity HaloTag-blocking agent that permits protein turnover measurements at both the cell population (by blotting) and single-cell (by imaging) levels. We show that the HaloTag pulse-chase approach is a nontoxic alternative to inhibition of protein synthesis with cycloheximide and extend protein turnover assays to long-lived proteins.

The ability to label proteins in living cells has revolutionized our understanding of many cellular processes. Several different self-labeling proteins have been developed, including SNAP-tag (1) and HaloTag (2), that allow irreversible covalent labeling with a variety of commercially available reporter-labeled ligands, including fluorescent dyes. These tags are usually expressed fused to a specific protein of interest (POI), allowing labeling and monitoring of the POI’s localization in living cells (2) and in intact animals (3). There is growing interest in the use of self-labeling proteins for super-resolution microscopy (4), and a recent publication showed that HaloTag fusions have a 9-fold higher fluorescent signal and greater photostability compared with SNAP-tag fusions (5).

HaloTag is an engineered haloalkane hydrolase in which His-272 in the substrate-binding pocket is mutated to Phe. This mutation blocks hydrolysis and, instead, covalently attaches the HaloTag ligand to Asp-106 (2). Commercially available ligands feature a variety of reporters that enable a wide array of assays, including protein purification and cell imaging. For instance, HaloTag has been used to study the turnover of connexin36 at gap junctions (6) and, more recently, the requirement of CHMP2A as a regulator of autophagosome function (7). These studies highlight the utility of HaloTag (8).

Direct labeling of HaloTag POIs in living cells has proven useful to monitor their location and determine the rate of protein turnover. However, in pulse-chase experiments, it has been observed that, even after removal of the TMR HaloTag ligand and rinsing with new medium, cells still contain unbound HaloTag ligand that will continue to label newly synthesized proteins (9). The addition of another HaloTag ligand at a higher concentration (1000-fold) was necessary to outcompete the residual HaloTag ligand (9). Presumably because of the cost of labeled HaloTag ligands, only one pulse-chase study involving single-cell imaging has been reported so far (6).

Here we identify an inexpensive, readily available HaloTag ligand that can be used as a blocker to prevent further labeling of newly synthesized HaloTag fusion proteins, enabling economical HaloTag pulse-chase experiments. We report both blotting and imaging-based protein turnover assays and show that the low toxicity of the blocker enables long-term analysis of stable proteins that cannot be studied with protein synthesis inhibitors.

Results

Cell population protein turnover assays to evaluate HaloTag blocking agents

Using the structure of the reactive chloroalkane linker from commercial HaloTag ligands (Promega) as a search template (Fig. 1A), we identified a list of haloalkanes that may also interact with HaloTag. This list was filtered by solubility and predicted cell permeability profiles, and four haloalkanes were obtained from chemical vendors for further characterization: 6-chlorohexanol (6CHO), 6-chlorohexanoic acid (6CHA), 5-chlorovaleric acid (5CVA) and 7-bromoheptanol (7BRO; Fig. 1A). These chemicals were dissolved in DMSO and diluted in medium to perform toxicity studies in both COS-1 cells and...
primary cortical cultures from rat embryos. The four compounds were well-tolerated up to at least 1 mM (Fig. 1, B and C). An LD₅₀ could not be determined for 5CVA, which was non-toxic at the highest concentration tested.

We next tested the efficacy of these compounds as HaloTag blockers. Using HaloTag pulse-chase followed by Western blot analysis, we determined the protein turnover of B’/H9252, a regulatory subunit of protein phosphatase 2A (PP2A), whose proteasomal degradation we examined previously using the protein synthesis inhibitor cycloheximide (CHX) (10). B’/H9252 with a C-terminal HaloTag (B’/H9252-Halo) was expressed in COS-1 cells by transient transfection. B’/H9252-Halo was pulse-labeled with 50 nM cell-permeable TMR-conjugated HaloTag ligand for 30 min, which results in maximal labeling, as determined in prior experiments. The medium was removed and replaced with medium containing increasing concentrations of the four unlabelled haloalkanes for 8 h. Total cell lysates were subjected to SDS-PAGE and fluorescence imaging to detect residual TMR-labeled B’/H9252-Halo; the same blots were then immunoblotted for total B’/H9252-Halo for normalization. As reported previously for a different POI (9), replacing the medium without blocker resulted in very little decrease in TMR-labeled B’/H9252-Halo over the 8-h chase period (Fig. 2A), implying retention of free ligand in the cell that continues to label newly synthesized HaloTag. Addition of any of the four haloalkanes at the highest concentrations reduced the amount of TMR-labeled B’/H9252-Halo protein, indicating that they are indeed HaloTag ligands. 7BRO was by far the most effective blocker, with an IC₅₀ close to the concentration of the TMR label (52 nM; Fig. 2, A, B, and D).

To test the four HaloTag blockers on a different POI, we turned to doublecortin (DCX), a microtubule-binding protein critical for normal brain development (11). Based on pilot experiments indicating a longer half-life in COS-1 cells, turnover of doublecortin with a C-terminal HaloTag (DCX-Halo) was assayed after a 24-h chase period. Again, 7BRO was an effective haloalkane blocking agent, with an IC₅₀ of 118 nM for DCX-Halo (Fig. 2, C and D). The remaining blockers were ineffective at blocking continued labeling of DCX-Halo by residual TMR ligand. These data indicate that 7BRO is a nontoxic, high-affinity blocker of HaloTag labeling by the TMR ligand, likely acting as a competitor at the substrate-binding site.

**In vitro binding of HaloTag ligands**

With such varied effectiveness of the four haloalkanes tested, we asked whether this was due to differences in binding to the HaloTag or perhaps bioavailability in cells. Therefore, we expressed biotinylated recombinant HaloTag protein in *Escherichia coli* to perform in vitro competition binding assays between the TMR ligand (2 nM) and increasing concentrations of the four haloalkanes. 7BRO was highly effective at competing with the TMR ligand, with an IC₅₀ of approximately four times the TMR ligand concentration; 6CHA was partially effective, whereas 6CHO and 5CVA were ineffective, even at 1000-fold higher concentration (Fig. 3, A and B). To test whether the ineffective haloalkanes can bind to HaloTag at all, we pre-treated HaloTag protein (1 nM) with the blockers at 100 μM for 30 min prior to addition of 2 nM TMR ligand. If the binding of the haloalkanes to the HaloTag protein is irreversible, then this...
Figure 2. Low-molecular-weight haloalkanes display variable potency in preventing labeling by residual HaloTag ligand. A, representative blots of total COS-1 cell lysates expressing B'β-Halo protein. The HaloTag was labeled with TMR ligand (50 nM, 30 min), followed by replacement of the medium with the designated concentrations of the four haloalkanes (0.1, 1, 10, 100, 300, 1000, and 3000 μM). TMR-labeled B'β was detected by fluorescence imaging, whereas total B'β was detected subsequently on the same blot by immunoblotting for B'β and IR imaging. The position of the 116- and 97-kDa molecular mass markers is indicated next to each blot. B, the TMR-labeled/total B'β ratio is graphed for the indicated chemicals at the indicated concentrations and normalized to the no-chase condition. Plotted are means ± S.E. from five individual experiments and single exponential curve fits with offsets. C, quantification of DCX-Halo labeling with TMR ligand followed by haloalkane chase (0.1, 1, 10, and 100 μM) in COS-1 cells as in A and B. Plotted are means ± S.E. from five individual experiments and single exponential curve fits with offsets. D, IC₅₀ values are listed as means ± 95% CI (n = 5). For DCX, the IC₅₀ of three haloalkanes could not be determined (ND).

Figure 3. In vitro competition between TMR HaloTag ligand and haloalkane binding to recombinant HaloTag. A and B, bacterially expressed HaloTag protein (1 nM) with a C-terminal biotinylated AviTag was combined with premixed TMR HaloTag ligand (2 nM) and the indicated concentrations of haloalkanes (0.1, 0.5, 1, 2, 10, 50, 100, 500, and 1000 μM) for 30 min, followed by SDS-PAGE and fluorescence detection of TMR and biotin (total, via IR-labeled streptavidin) on the same blot. A, representative blots. The upward mobility shift of the total protein is indicative of covalent labeling by the TMR HaloTag ligand. B, TMR-labeled/total HaloTag ratio normalized to no block is plotted as means ± S.E. with curve fits from four independent experiments. C, pretreatment of the four haloalkanes (100 μM, 30 min) prior to addition of the TMR ligand (2 nM) reduced (5CHA) or eliminated TMR labeling of recombinant HaloTag protein (1 nM). D, TMR-labeled/total HaloTag ratio normalized to no preblock is plotted as means ± 95% CI from four independent experiments.
pretreatment should prevent TMR labeling. With pretreatment, all four haloalkanes were able to block TMR labeling of HaloTag but to variable extents. Although preincubation with 7BRO, 6CHO, and 6CHA prevented TMR labeling completely, 5CHA reduced labeling by only one-third (Fig. 3, C and D). These data indicate that all four haloalkanes are capable of blocking the HaloTag protein but that only 7BRO approaches the affinity of the TMR ligand.

HaloTag pulse-chase with 7-bromoheptanol allows measuring the turnover of long-lived proteins

We previously identified Kelch-like 15 (KLHL15) as an E3 ubiquitin ligase adaptor protein that mediates proteasomal degradation of the B′/H9252 (PPP2R5B) subunit of PP2A and used the protein synthesis inhibitor CHX to determine turnover rates (10). Confounding the use of global protein synthesis inhibitors for turnover assays are cellular toxicity and secondary effects that affect the stability of the POI and thus mask its true half-life (12). These secondary effects include degradation of deubiquitinases and even the POI’s E3 ubiquitin ligase itself (13). Initially, we compared the B′/H9252-Halo turnover rate with either a control GFP-tagged protein or GFP-KLHL15 in cotransfected COS-1 cells, pulse-labeling with TMR ligand (50 nM and 7BRO (10 μM) for up to 8 h. As expected (10), KLHL15 promoted B′/H9252 degradation, reducing the t1/2 of B′/H9252 by more than 2-fold (Fig. 4, A–C). Without 7BRO, the apparent t1/2 for B′/H9252 increased by 2- to 3-fold under the two transfection conditions, highlighting the necessity of an effective blocker (Fig. 4, A and B). Of note, the half-lives of B′/H9252 with (2.8 h) and without KLHL15 overexpression (5.8 h) determined by HaloTag ligand pulse and 7BRO chase are very similar to those obtained by inhibiting protein synthesis in HEK293 cells (2.5 h and ~8 h, respectively) (10). This agreement is despite the fact that the E3 ligase KLHL15 significantly degrades over time in CHX (10), whereas KLHL15 protein levels remain constant under these pulse-chase conditions (Fig. 4A).

We next assayed turnover of the long-lived DCX protein in COS-1 cells to compare the effectiveness of CHX chase and HaloTag pulse-chase protocols. As with B′/H9252, the apparent t1/2 of DCX-Halo without 7BRO chase was more than twice the t1/2 when it was included (12 h; Fig. 5, A and B). Over the 24-h time course, no change was seen in total DCX-Halo or total protein (ERK1/2 loading control; Fig. 5A). In contrast, when we inhibited protein synthesis with increasing doses of CHX (10–100 μg/ml), the apparent degradation of DCX protein at higher CHX concentrations paralleled the loss of total protein over the 24-h time course (Ponceau S; Fig. 5C). Thus, CHX toxicity precluded estimation of DCX protein degradation rates in COS-1 cells (Fig. 5D), suggesting that HaloTag pulse-chase is the method of choice for measuring the turnover of long-lived proteins.

HaloTag pulse-chase measures protein turnover in individual primary neurons

After demonstrating the effectiveness of 7BRO as an effective HaloTag blocker for cell population analysis by SDS-PAGE, we next examined the use of the blocker for imaging-based assays.

Figure 4. HaloTag ligand pulse with 7BRO chase quantifies accelerated turnover of B′/H9252 by expression of the E3 ligase KLHL15. A, representative blots of a B′-Halo protein turnover time course with coexpressed GFP-KLHL15 or control GFP-tagged protein (Ctrl). COS-1 cells were pulse-labeled with TMR ligand (50 nM, 30 min), followed by chase with medium or medium containing 7BRO (10 μM) for the indicated times. Following SDS-PAGE of total lysates, TMR was detected by fluorescence, and B′ and GFP were detected by immunoblotting and IR imaging of the same blots. The molecular mass markers (in kilodaltons) indicated on the right apply to all blots in the same row. B, TMR-labeled/total B′ ratios normalized to no chase are plotted as means ± S.D. from three independent experiments. Half-lives were determined from the shown monoexponential decay curve fits. C, means ± S.D. of areas under the curve (AUC) for the decay curves of the three individual experiments (****, p < 0.0001).
of protein turnover in individual cells. To this end, we transfected primary rat hippocampal neurons with B’β-Halo or DCX-Halo, labeled cultures with TMR ligand and chased for variables times with medium with or without 7BRO. Cultures were then fixed and immunofluorescently labeled for the transfected proteins (Fig. 6, A and C). As before, chasing with 7BRO increased the apparent rate of B’β and DCX turnover, although less dramatically so for the latter protein (Fig. 6, B and D). Interestingly, turnover of B’β (t1/2 = 2.7 h) and especially DCX (t1/2 = 3.6 h) was faster in neurons than in COS-1 cells (t1/2 = 5.8 h and 12 h, respectively; Fig. 4). Because both B’β and DCX are most highly expressed in the nervous system (11, 14), neurons may be especially adapted to regulate the levels of these proteins when overexpressed. In conclusion, HaloTag pulse-chase with 7BRO is a robust and economical method to measure protein turnover in both easy- and hard-to-transfect cell types.

Discussion

Specific labeling of fusion proteins containing the HaloTag with a variety of reporters has been adopted for a diverse array of applications, including turnover studies (2, 4, 8). However, a limitation of the technology, at least for hydrophobic, cell-permeant HaloTag ligands, is their retention in cells after removal from the medium, where they continue to label newly synthesized POIs. Previous studies utilized an additional HaloTag ligand containing a different functional group at higher concentrations to outcompete the residual labeling ligand (9). Although this technique is effective, it dramatically increases the cost of experiments and precludes widespread adoption of HaloTag technology for protein turnover studies. In this report, we identified an inexpensive (<1 cent/24 well plate), widely available, and high-affinity ligand for the HaloTag that is well-tolerated by cell lines and primary neurons and effectively blocks the continued labeling of new synthesized proteins. Using 7BRO as a blocker, we were able to reliably measure protein turnover of two POIs both by SDS-PAGE analysis and single-neuron imaging.

HaloTag pulse-chase for protein turnover assays using Western blot analysis has significant advantages over commonly used assays in which cells are treated with CHX or similar inhibitors (15). The first is avoiding the toxicity of shutting down global protein synthesis, which is of critical importance for long-lived proteins. Second, total protein levels of the POI and, more importantly, of the degrading machinery responsible for turning over the POI are unchanged by addition of the HaloTag ligand and blocker. Last, it avoids confounding activation of AKT signaling and transcription factor activation in response to CHX treatment, which was reported recently (12). These advantages are shared with other pulse-chase methods, such as labeling with radioactive amino acids. However, HaloTag pulse-chase does not require affinity purification of the POI prior to analysis of label incorporation and is therefore compatible with high-throughput applications. HaloTag is derived from a bacterial enzyme, so it is unlikely that mammalian cells have evolved the machinery for its targeted degradation. Indeed, at least for the PP2A subunit B’β, whose turnover we determined by both CHX chase (10) and HaloTag pulse-chase (Fig. 4), fusion to the ~33-kDa HaloTag does not alter protein stability, although this may have to be verified for other POIs.

Potential other applications for 7BRO with HaloTag labeling include estimation of the protein synthesis rate in cells in which the HaloTag is integrated into the genomic locus encoding the

Figure 5. HaloTag pulse-chase, but not cycloheximide chase, can be used to measure the half-life of a stable protein. A, representative blots of TMR-labeled (50 nM, 30 min) DCX-Halo protein turnover time course with either medium or medium plus 7BRO (10 μM) chase for up to 24 h. Following SDS-PAGE of total lysates, TMR was detected by fluorescence, and DCX and ERK1/2 (loading control) were detected by immunoblotting and IR imaging of the same blots. B, TMR-labeled/total DCX ratios normalized to no chase are plotted as means ± S.D. from three independent experiments. Half-lives were determined from the shown monoexponential decay curve fits. C, representative blots from an attempt to measure DCX turnover by protein synthesis inhibition with increasing concentrations of CHX for up to 24 h. Blots were stained for total protein with Ponceau S (PonS), followed by immunodetection for DCX. In A and C, molecular mass markers (in kilodaltons) indicated on the right apply to all blots in the row. D, DCX/total protein ratios normalized to the zero time point are plotted as means ± S.D. from three independent experiments. Lines are the nonlinear regression fits and show no DCX turnover.

Discussion
Specific labeling of fusion proteins containing the HaloTag with a variety of reporters has been adopted for a diverse array of applications, including turnover studies (2, 4, 8). However, a limitation of the technology, at least for hydrophobic, cell-permeant HaloTag ligands, is their retention in cells after removal from the medium, where they continue to label newly synthesized POIs. Previous studies utilized an additional HaloTag ligand containing a different functional group at higher concentrations to outcompete the residual labeling ligand (9). Although this technique is effective, it dramatically increases the cost of experiments and precludes widespread adoption of HaloTag technology for protein turnover studies. In this report, we identified an inexpensive (<1 cent/24 well plate), widely available, and high-affinity ligand for the HaloTag that is well-tolerated by cell lines and primary neurons and effectively blocks the continued labeling of new synthesized proteins. Using 7BRO as a blocker, we were able to reliably measure protein turnover of two POIs both by SDS-PAGE analysis and single-neuron imaging.

HaloTag pulse-chase for protein turnover assays using Western blot analysis has significant advantages over commonly used assays in which cells are treated with CHX or similar inhibitors (15). The first is avoiding the toxicity of shutting down global protein synthesis, which is of critical importance for long-lived proteins. Second, total protein levels of the POI and, more importantly, of the degrading machinery responsible for turning over the POI are unchanged by addition of the HaloTag ligand and blocker. Last, it avoids confounding activation of AKT signaling and transcription factor activation in response to CHX treatment, which was reported recently (12). These advantages are shared with other pulse-chase methods, such as labeling with radioactive amino acids. However, HaloTag pulse-chase does not require affinity purification of the POI prior to analysis of label incorporation and is therefore compatible with high-throughput applications. HaloTag is derived from a bacterial enzyme, so it is unlikely that mammalian cells have evolved the machinery for its targeted degradation. Indeed, at least for the PP2A subunit B’β, whose turnover we determined by both CHX chase (10) and HaloTag pulse-chase (Fig. 4), fusion to the ~33-kDa HaloTag does not alter protein stability, although this may have to be verified for other POIs.

Potential other applications for 7BRO with HaloTag labeling include estimation of the protein synthesis rate in cells in which the HaloTag is integrated into the genomic locus encoding the

**Discussion**

Specific labeling of fusion proteins containing the HaloTag with a variety of reporters has been adopted for a diverse array of applications, including turnover studies (2, 4, 8). However, a limitation of the technology, at least for hydrophobic, cell-permeant HaloTag ligands, is their retention in cells after removal from the medium, where they continue to label newly synthesized POIs. Previous studies utilized an additional HaloTag ligand containing a different functional group at higher concentrations to outcompete the residual labeling ligand (9). Although this technique is effective, it dramatically increases the cost of experiments and precludes widespread adoption of HaloTag technology for protein turnover studies. In this report, we identified an inexpensive (<1 cent/24 well plate), widely available, and high-affinity ligand for the HaloTag that is well-tolerated by cell lines and primary neurons and effectively blocks the continued labeling of new synthesized proteins. Using 7BRO as a blocker, we were able to reliably measure protein turnover of two POIs both by SDS-PAGE analysis and single-neuron imaging.

HaloTag pulse-chase for protein turnover assays using Western blot analysis has significant advantages over commonly used assays in which cells are treated with CHX or similar inhibitors (15). The first is avoiding the toxicity of shutting down global protein synthesis, which is of critical importance for long-lived proteins. Second, total protein levels of the POI and, more importantly, of the degrading machinery responsible for turning over the POI are unchanged by addition of the HaloTag ligand and blocker. Last, it avoids confounding activation of AKT signaling and transcription factor activation in response to CHX treatment, which was reported recently (12). These advantages are shared with other pulse-chase methods, such as labeling with radioactive amino acids. However, HaloTag pulse-chase does not require affinity purification of the POI prior to analysis of label incorporation and is therefore compatible with high-throughput applications. HaloTag is derived from a bacterial enzyme, so it is unlikely that mammalian cells have evolved the machinery for its targeted degradation. Indeed, at least for the PP2A subunit B’β, whose turnover we determined by both CHX chase (10) and HaloTag pulse-chase (Fig. 4), fusion to the ~33-kDa HaloTag does not alter protein stability, although this may have to be verified for other POIs.

Potential other applications for 7BRO with HaloTag labeling include estimation of the protein synthesis rate in cells in which the HaloTag is integrated into the genomic locus encoding the
endogenous POI. In this scenario, existing POI HaloTag fusion protein is blocked with a low dose of 7BRO, followed by addition of HaloTag ligand for different periods of time at slightly higher concentrations to label only the newly synthesized POI. In summary, we have identified 7BRO as an economical HaloTag-blocking agent that can compete with commercially available HaloTag ligands attached to highly sensitive reporters. HaloTag ligand pulse followed by 7BRO chase is nontoxic and offers advantages over traditional methods of protein turnover determination, especially for long-lived proteins.

Experimental procedures

Reagents and antibodies
The human B′β and DCX complementary DNAs were cloned into the pHTC vector (Promega), also incorporating a HiBiT, myc, and FLAG tag between the N-terminal complementary DNA and the C-terminal HaloTag. The chemical reagents used in this study were procured as follows: 7-bromoheptanol from Alfa Aesar (catalog no. H54762), 6-chlorohexanoic acid from Ark Pharm Inc. (catalog no. AK-48239), 6-chlorohexanol from Sigma (catalog no. C45008-5G), 5-chlorovaleric acid from Chem-Impex International Inc. (catalog no. 26912), HaloTag® TMR ligand from Promega (catalog no. G825A), NeuroMag from OZBiosciences (catalog no. NM51000), and cycloheximide from Sigma (catalog no. C1988).

The following commercially sourced antibodies and labels were used: rabbit anti-GFP (Abcam, catalog no. ab290), rabbit anti-B′β serum (14), mouse anti-DDK (FLAG; OriGene, catalog no. TA50011), rabbit anti-ERK1/2 (Santa Cruz Biotechnology; catalog no. sc94), mouse anti-doublecortin (Sant Cruz, catalog no. 271390), goat anti-mouse IgG DyLight® 350 conjugate (Invitrogen, catalog no. 62273), goat anti-mouse IRDye® 680RD (Licor, catalog no. 926-68070), goat anti-rabbit IRDye® 800CW (Licor, catalog no. 926-32211), and streptavidin IRDye® 800CW (Licor, catalog no. 926-32230).

Cell culture conditions and lethal dose determination
COS-1 cells were cultured (37 °C and 5% CO₂) in DMEM (Gibco) with 10% (v/v) FBS (Atlanta Biologicals) and 1% (v/v) GlutaMax (Gibco). Primary hippocampal cultures were prepared from E18 rat embryos (16) in Neurobasal complete (Neurobasal medium (Gibco), 2% B27 supplement (Gibco), 10 mM Hepes, and 0.25% GlutaMax), and half of the medium was replaced with new medium twice a week. For the high-concentration doses, reagents were dissolved in DMSO and diluted in complete medium. For the acids, stocks were dissolved in plain medium and neutralized with concentrated HCl before dilution in complete medium. For the lethal dose survival assays, a Cell-Titer-Blue assay was performed after 24-h treatment with the haloalkanes as specified by the manufacturer (Promega, catalog no. G8081).

SDS-PAGE and blotting analysis of protein turnover
COS-1 cells were grown to 70%–80% confluency, seeded at 2.5 × 10⁵ cells/ml on collagen-coated plates, and transfected with HaloTag vectors using Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol for transient transfection of adhered cells. 24–48 h post-transfection, cells were incubated with a final concentration of 50 nM cell-permeant HaloTag® TMR ligand at 37 °C for 30 min for maximum labeling. Then the medium was removed completely and replaced with medium containing different blockers at the indicated
**Pulse-chase labeling HaloTag fusion proteins**

Concentrations. Blockers were made as 10 mM stocks in DMEM before dilution to the final concentration in DMEM plus serum. Then cells were returned to the incubator for the indicated time periods before washing with Dulbecco’s phosphate-buffered saline (Gibco) and harvested in Laemmli sample buffer, followed by sonication to shear genomic DNA. SDS-PAGE was performed to separate HaloTag proteins, and TMR fluorescence was detected by the FLA-7000 Image Reader (Fujifilm) or Sapphire (Azure) using appropriate laser excitation and emission filters. Total cell lysates were also probed for B’β, DCX, FLAG, GFP, and ERK1/2 (loading control) antibodies. Relative TMR labeling was quantified by dividing TMR fluorescence signals by B’β or DCX immunoblotting signals in the same lane and normalizing to the zero time point (set to 1) in ImageJ software (National Institutes of Health). The t½ of B’β and DCX was estimated by the one-phase decay model (nonlinear regression, plateau = 0) using GraphPad Prism software.

**In vitro competition of HaloTag ligands**

HaloTag protein containing a C-terminal AviTag (for biotinylation) was expressed in BL-21 cells (Agilent, catalog no. 200131). Soluble bacterial extract was diluted to 1 nM HaloTag protein (estimated by SDS-PAGE of BSA protein standards) and incubated for 30 min with a premixed combination of haloalkanes at varying concentrations (0.1, 0.5, 1, 2, 10, 50, 100, 500, and 1000 μM) and 2 nM TMR HaloTag ligand. For pretreatment, 100 μM tested haloalkane was incubated for 30 min with 1 nM HaloTag protein before addition of 2 nM TMR ligand. After SDS-PAGE, the fluorescence of TMR and biotin (via Streptavidin-DyLight® 800) was detected on the same blot for ratiometric quantitation.

**Protein turnover assays in primary hippocampal cultures**

Cultures were transfected using 0.25 μg of DNA/0.5 μl of NeuroMag/well at 9 days in vitro. The replacement medium always consisted of half new neurobasal complete and half conditioned medium. Three days after transfection, cultures were incubated with 100 nM TMR HaloTag ligand for at least 45 min prior to removal of the medium and addition of replacement blank medium or 7BRO-containing medium for the designated times.

**Fluorescence microscopy**

Fixed hippocampal cultures were blocked for 30 min with 200 μl of 4% normal donkey serum in TBS containing 0.1% Triton X-100 and incubated overnight at 4 °C with 190 μl of 1:400 dilutions of antibody against the DDK (FLAG) tag. Following washes (2 × 750-μl Tris-buffered saline, 0.1% Triton X-100 (TTBS) rinse, 2 × 750-μl of TTBS wash for 10 min), cultures were incubated for 2 h at room temperature with 190 μl of 1:250 dilution of the DyLight® 350-conjugated secondary antibody. Cultured neurons were viewed on a Leica DM14000B epifluorescence microscope with a ×40 oil objective following washes (2 × 750-μl TTBS rinse, 2 × 750-μl TTBS wash for 10 min, 1 × 750-μl PBS rinse, stored in 500 μl of PBS). A 2.5:1 gain ratio, 3:1 exposure time ratio, and 2:1 exposure intensity ratio for blue and red channels, respectively, remained constant across imaging for all experiments and conditions.

**Imaging-based protein turnover analysis**

Captured wide-field epifluorescence images were analyzed using ImageJ software, measuring the integrated density of neuronal cell bodies in the red (TMR) and blue (FLAG antibody) channels. The ratio of blue intensity minus a control background selection to red intensity minus a control background selection was calculated as a measure of relative TMR labeling of the expressed HaloTag fusion protein.

**Statistical analyses**

The robust outlier removal (ROUT) method of removing outliers was used at Q = 1% prior to normalization of data to the 0-h time point for each experimental condition. Data were analyzed by one-way analysis of variance, followed by Tukey post hoc tests for multiple comparisons. Dose–response data were fitted by a one-phase exponential decay curve using the least squares method. All analyses were carried out using GraphPad Prism software.

**Author contributions**—R. A. M. and S. S. conceptualization; R. A. M., J. S., R. A. K., and S. S. formal analysis; R. A. M., J. S., R. A. K., A. J. K., C. E. N., and S. S. supervision; S. S. funding acquisition; S. S. validation; S. S. visualization; S. S. project administration; S. S. writing-and revising and editing.

**Acknowledgments**—We thank the Carver College of Medicine Genomics Division and Central Microscopy Core for support.

**References**

1. Keppeler, A., Grendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnson, K. (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol.* 21, 86–89

2. Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urb, M., Simpson, D., Mendez, J., Zimmerman, K., Otto, P., Vidiguris, G., et al. (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3, 373–382

3. Masch, J. M., Steffens, H., Fischer, J., Engelhardt, J., Hubrich, J., Keller-Findeisen, J., D’Este, E., Urban, N. T., Grant, S. G. N., Sahl, S. J., Kamin, D., and Hell, S. W. (2018) Robust nanoscopy of a synaptic protein in living mice by organic-fluorophore labeling. *Proc. Natl. Acad. Sci. U.S.A.* 115, E8047–E8056

4. Liss, V., Barlag, B., Nietzschke, M., and Hensel, M. (2015) Self-labeling enzymes as universal tags for fluorescence microscopy, super-resolution microscopy and electron microscopy. *Sci. Rep.* 5, 17740

5. Erdmann, R. S., Baguley, S. W., Richens, J. H., Wissner, R. F., Xi, Z., Allgeyer, E. S., Zhong, S., Thompson, A. D., Lowe, N., Butler, R., Bewersdorff, J., Rothman, J. E., St Johnston, D., Scheparz, A., and Toomre, D. (2019) Labeling strategies matter for super-resolution microscopy: a comparison between HaloTags and SNAP-tags. *Cell Chem. Biol.* 26, 584–592.e6

6. Wang, H. Y., Lin, Y. P., Mitchell, C. K., Ram, S., and O’Brien, J. (2015) Two-color fluorescent analysis of connexin 36 turnover: relationship to functional plasticity. *J. Cell Sci.* 128, 3888–3897

7. Takahashi, J., He, H., Tang, Z., Hattori, T., Liu, Y., Young, M. M., Serfass, J. M., Chen, L., Gebru, M., Chen, C., Wills, C. A., Atkinson, J. M., Chen, H., 16170 J. Biol. Chem. (2019) 294(44) 16164–16171

ASBMB
Abraham, T., and Wang, H. G. (2018) An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. Nat. Commun. 9, 2855 CrossRef Medline

8. England, C. G., Luo, H., and Cai, W. (2015) HaloTag technology: a versatile platform for biomedical applications. Bioconjug. Chem. 26, 975–986 CrossRef Medline

9. Yamaguchi, K., Inoue, S., Ohara, O., and Nagase, T. (2009) Pulse-chase experiment for the analysis of protein stability in cultured mammalian cells by covalent fluorescent labeling of fusion proteins. Methods Mol. Biol. 577, 121–131 CrossRef Medline

10. Oberg, E. A., Nifoussi, S. K., Gingras, A. C., and Strack, S. (2012) Selective proteasomal degradation of the B’β regulatory subunit of protein phosphatase 2A by the E3 ubiquitin ligase adaptor Kelch-like 15. J. Biol. Chem. 287, 43378–43389 CrossRef Medline

11. Gleeson, J. G., Lin, P. T., Flanagan, L. A., and Walsh, C. A. (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23, 257–271 CrossRef Medline

12. Dai, C. L., Shi, J., Chen, Y., Iqbal, K., Liu, F., and Gong, C. X. (2013) Inhibition of protein synthesis alters protein degradation through activation of protein kinase B (AKT). J. Biol. Chem. 288, 23875–23883 CrossRef Medline

13. Yewdell, J. W., Lacsina, J. R., Rechsteiner, M. C., and Nicchitta, C. V. (2011) Out with the old, in with the new? Comparing methods for measuring protein degradation. Cell Biol. Int. 35, 457–462 CrossRef Medline

14. Saraf, A., Virshup, D. M., and Strack, S. (2007) Differential expression of the B’β regulatory subunit of protein phosphatase 2A modulates tyrosine hydroxylase phosphorylation and catecholamine synthesis. J. Biol. Chem. 282, 573–580 CrossRef Medline

15. Kao, S. H., Wang, W. L., Chen, C. Y., Chang, Y. L., Wu, Y. Y., Wang, Y. T., Wang, S. P., Nesvizhskii, A. I., Chen, Y. J., Hong, T. M., and Yang, P. C. (2015) Analysis of protein stability by the cycloheximide chase assay. Bio. Protoc. 5, e1374 Medline

16. Lim, I. A., Merrill, M. A., Chen, Y., and Hell, J. W. (2003) Disruption of the NMDA receptor–PSD-95 interaction in hippocampal neurons with no obvious physiological short-term effect. Neuropharmacology 45, 738–754 CrossRef Medline