

1 **A Small-Molecule Activity-Based Probe for Monitoring**
2 **Ubiquitin C-terminal Hydrolase L1 (UCHL1) Activity in Live Cells and**
3 **Zebrafish Embryos**

4
5
6 **Paul P. Geurink,^{*a‡} Raymond Kooij,^{a‡} Aysegul Sapmaz,^{a‡} Sijia Liu,^{a‡} Bo-Tao Xin,^a**
7 **George M. C. Janssen,^b Peter A. van Veelen,^b Peter ten Dijke,^a and Huib Ovaa^{*a}**

8
9 ^a Oncode Institute & Department of Cell and Chemical Biology, Leiden University Medical
10 Center, Eindhovenweg 20, 2333 ZC, Leiden, The Netherlands

11 ^b Center for Proteomics and Metabolomics, Leiden University Medical Center, Albinusdreef 2,
12 2333 ZC, Leiden, The Netherlands

13 [‡] Equal contribution

14 ^{*} Corresponding authors: p.p.geurink@lumc.nl, h.ovaa@lumc.nl

15
16
17 **ABSTRACT:** Many reagents have been emerged to study the function of specific enzymes *in*
18 *vitro*. On the other hand, target specific reagents are scarce or need improvement allowing
19 investigations of the function of individual enzymes in a cellular context. We here report the
20 development of a target-selective fluorescent small-molecule activity-based DUB probe that is
21 active in live cells and whole animals. The probe labels active Ubiquitin Carboxy-terminal
22 Hydrolase L1 (UCHL1), also known as neuron-specific protein PGP9.5 (PGP9.5) and
23 parkinson disease 5 (PARK5), a DUB active in neurons that constitutes 1-2% of total brain
24 protein. UCHL1 variants have been linked with the neurodegenerative disorders Parkinson's
25 and Alzheimer's disease. In addition, high levels of UCHL1 also correlate often with cancer
26 and especially metastasis. The function of UCHL1 or its role in cancer and neurodegenerative
27 disease is poorly understood and few UCHL1 specific research tools exist. We show that the
28 reagents reported here are specific for UCHL1 over all other DUBs detectable by competitive
29 activity-based protein profiling and by mass spectrometry. Our probe, which contains a
30 cyanimide reactive moiety, binds to the active-site cysteine residue of UCHL1 irreversibly in
31 an activity-dependent manner. Its use is demonstrated by labelling of UCHL1 both *in vitro* and
32 in cells. We furthermore show that this probe can report UCHL1 activity during the
33 development of zebrafish embryos.

1 INTRODUCTION

2 The Ubiquitin system relies to a great extent on cysteine catalysis. Ubiquitin is a small protein
3 that consists of 76 amino acids that can modify target proteins through lysine residues although
4 it is also occasionally found to modify N-termini as well as cysteine and threonine residues.¹⁻³
5 Addition of ubiquitin is catalyzed by E1 (2), E2 (~40) and E3 (>600) enzymes in an ATP-
6 dependent conjugation reaction by specific combinations of E1, E2 and E3 enzymes and it is
7 reversed by any of ~100 deubiquitylating enzymes (DUBs) in humans.^{4, 5} The enzyme
8 Ubiquitin Carboxy-terminal Hydrolase L1 (UCHL1), also known as neuron-specific protein
9 PGP9.5 (PGP9.5) and parkinson disease 5 (PARK5), is a small protease that is thought to
10 remove ubiquitin from small substrates and it belongs to the small family of Ubiquitin C-
11 terminal Hydrolases (UCHs).⁶

12 It is clear that UCHL1 can cleave ubiquitin and that mutation and reduced activity of this
13 enzyme have been associated with neurodegenerative diseases, including Parkinson's and
14 Alzheimer's disease.⁷⁻¹² High UCHL1 levels correlate with malignancy and metastasis in many
15 cancers^{13, 14} and have also been attributed to cellular stress, although the molecular mechanism
16 of all these processes is unclear.

17 We earlier observed extreme levels of UCHL1 activity in lysates from prostate and lung cancer
18 cells using a ubiquitin-derived activity-based probe that targets all cysteine DUBs.¹⁵ We
19 reasoned that a good cell-permeable activity-based probe that targets UCHL1 specifically
20 amongst other cysteine DUBs would be a highly valuable tool to understand its function in
21 malignant transformation and its role in the development of neurodegenerative diseases.

22 UCHL1, like many DUBs, is a cysteine protease, a class of enzymes considered extremely
23 difficult to inhibit with small molecules as this class of enzymes is associated with unspecific
24 reaction with cysteine alkylating agents and with redox-cycling artifacts in assays.¹⁶ In
25 addition, DUBs intrinsically bind ubiquitin through a protein-protein interaction, which is by
26 definition difficult to interfere with using small molecules. Many DUBs, including UCHL1,
27 are inactive without a substrate and substrate binding aligns the catalytic triad for cleavage.¹⁷

28 Nevertheless, recently significant successes have been booked in the development of reversible
29 and irreversible selective small-molecule inhibitors of the DUB USP7.¹⁸⁻²³ We have recently
30 reported the development of a selective covalent small-molecule inhibitor of the DUB ovarian
31 tumor (OTU) protease OTUB2 using a covalent fragment approach and parallel X-ray
32 crystallography.²⁴ We reasoned that such covalent molecules are a good inroad for the further
33 elaboration of specific activity-based probes (ABPs) also inspired by earlier work from the
34 Tate lab that recently reported a small-molecule broadly acting DUB probe.²⁵ We were pleased

1 to find a good starting point in patent literature²⁶ that we used in our studies for the design of
2 fluorescent ABPs. We here report the development of a fluorescent small-molecule ABP that
3 can report UCHL1 activity in human cells and in zebrafish embryos.

4 5 **RESULTS AND DISCUSSION**

6 The development of a small-molecule-based DUB ABP starts with the identification of an
7 appropriate DUB-selective small-molecule covalent binder. We reasoned that an ideal
8 compound needed to meet two criteria: 1) it binds covalently to the active-site cysteine residue
9 of a DUB and 2) it can easily be modified by chemical synthesis. Our attention was drawn to
10 a collection of (*S*)-1-cyanopyrrolidine-3-carboxamide-based compounds reported to inhibit
11 UCHL1 activity with submicromolar affinity.²⁶ These compounds are equipped with a
12 cyanimide moiety that is known to react with thiols to form an isothioureia covalent adduct
13 (Figure 1A) and thought to react reversibly.²⁷ Despite the expected reversible nature we
14 decided to investigate this compound as a potential probe starting point.

15 16 **Characterizing UCHL1 cyanimide inhibitors.**

17 In order to gain insight into the mode of action and DUB selectivity of these inhibitors we
18 synthesized and characterized one compound (compound **6RK73**, Figure 1B) that in our hands
19 inhibits UCHL1 with an IC₅₀ of 0.23 μM after 30 minutes of incubation in a biochemical
20 activity assay using fluorogenic Ub-Rho-morpholine²⁸ substrate (for preparation see
21 Supporting Information) in the presence of 2 mM cysteine. Beneficially, **6RK73** proved to be
22 almost unreactive towards the closest DUB family members UCHL3 and UCHL5 (Figure 1C).
23 Selectivity for UCHL1 was further confirmed by IC₅₀ determination against a panel of other
24 cysteine DUBs (including USP7, USP30 and USP16) and the non-DUB cysteine protease
25 papain, showing over 50-fold difference in IC₅₀ value (Figure 1C and Supporting Information
26 Table S1). We next performed a jump dilution experiment²⁹ in which 100× final assay
27 concentration of UCHL1 was treated with 10 μM of **6RK73** followed by 100 times dilution
28 into substrate-containing buffer and direct fluorescence read-out (Figure 1C, D). Only after 30
29 minutes a negligible increase in fluorescence signal could be detected which indicates that the
30 inhibitor acts basically irreversible. The formation of a stable covalent complex between
31 UCHL1 and a single **6RK73** molecule was confirmed in an experiment where UCHL1 was
32 incubated with **6RK73** and the reaction followed by LC-MS analysis (see Supporting
33 Information). Next, we investigated whether the compound would inhibit UCHL1 in live cells.

1 HEK293T cells were treated with 5 μ M **6RK73** or the commercially available active-site
2 directed reversible UCHL1 inhibitor LDN-57444³⁰ for 24h, followed by cell lysis and treatment
3 with the fluorescent broad-spectrum DUB probe Rhodamine-Ubiquitin-propargylamide (Rh-
4 Ub-PA) to label all residual cysteine-DUB activity.^{31, 32} The samples were denatured, resolved
5 by SDS-PAGE and scanned for Rhodamine fluorescence (Figure 1E). Each band represents an
6 active DUB that reacted with the probe and the ability of a compound to inhibit a DUB is
7 reflected by disappearance of its corresponding band. Indeed, the band belonging to UCHL1³³
8 disappears upon treatment with **6RK73**, whereas all other bands remain unchanged, indicating
9 that **6RK73** selectively inhibits UCHL1 in the presence of other DUBs in cells. In comparison,
10 UCHL1 is hardly inhibited by LDN-57444 in this experiment, despite their comparable IC₅₀
11 values (0.88 μ M for LDN-57444), which might be attributed to the fast-reversible nature of
12 this inhibitor.³⁰

13

14 **From inhibitor to probe.**

15 Given the high inhibitory potency and UCHL1 selectivity both *in vitro* and in cells and the fact
16 that it forms an irreversible covalent bond we envisioned that this type of cyanimide-containing
17 molecules can serve as an ideal starting point for the construction of small-molecule selective
18 DUB ABPs. This would require the instalment of a reporter group (e.g. fluorescent label) onto
19 the molecule. Upon close inspection of **6RK73** however, we realized that this molecule does
20 not provide an appropriate site for modification. We therefore generated azide **8RK64** to which
21 then several reporter groups were coupled using the copper(I)-catalyzed azide alkyne
22 cycloaddition (CuAAC) or ‘click reaction’. The compounds and their synthesis routes are
23 shown in Scheme 1. Compound **2** was synthesized from 4-piperidinone (**1**) in four steps
24 according to a reported procedure.²⁶ The Fmoc-protected piperidine amine was liberated with
25 DBU and coupled to 2-azidoacetic acid resulting in compound **3**. Next, the Boc protecting
26 group was removed from the pyrrolidine amine, followed by a reaction with cyanogen bromide
27 to install the cyanimide moiety resulting in **8RK64**. Treatment of UCHL1 with this compound
28 followed by IC₅₀ determination and LC-MS analysis gave results comparable to those for
29 **6RK73** (Figure 2A, B, Supporting Information), which indicates that **8RK64** also functions as
30 a UCHL1 covalent inhibitor. With an IC₅₀ value of 0.32 μ M towards UCHL1 and 216 μ M and
31 >1 mM towards UCHL3 and UCHL5 respectively (Figure 2A, Supporting Information Table
32 S1), this compound also retained its UCHL1 selectivity. In addition, **8RK64**, like **6RK73**, also
33 inhibits UCHL1 activity in cells as shown in a DUB profiling experiment in HEK293T cells

1 using a Cy5-Ub-PA probe (Figure 2C). Notably, **8RK64** could potentially be used as ‘2-step
2 ABP’ by taking advantage of its azide moiety.³⁴

3

4 **Installation of a dye preserves inhibitory properties.**

5 As it was unclear what the effect of coupling a bulky fluorescent group would have on the
6 UCHL1 inhibition profiles and cell permeability we decided to test three commonly used
7 fluorophores. BodipyFL-alkyne, BodipyTMR-alkyne³⁵ and Rhodamine110-alkyne (for
8 preparation see Supporting Information) were coupled using copper(I)-mediated click
9 chemistry to the azide of **8RK64**, resulting in compounds **8RK59**, **9RK15** and **9RK87**
10 (Scheme 1). These ‘one-step’ ABPs can potentially be used for visualization of UCHL1 activity
11 without the need for additional bio-orthogonal chemistry procedures. IC₅₀ determination of
12 these probes against UCHL1 revealed that the instalment of the dyes affected the inhibitory
13 potency only marginally (Figure 3A and Supporting Information Table S1). Rhodamine110
14 probe **9RK87** is almost as potent as its azide precursor **8RK64** with IC₅₀ values of 0.44 μM
15 and 0.32 μM respectively. Instalment of BodipyTMR (**9RK15**) on the other hand, resulted in
16 a 10-fold potency decrease, although the data points could not be fitted properly to a dose-
17 response function. The less bulky BodipyFL-ABP **8RK59**, although not as potent as **8RK64**,
18 showed a very acceptable inhibition of UCHL1 with an IC₅₀ close to 1 μM. The ability of
19 **8RK59** to form a covalent complex with UCHL1 was confirmed in an LC-MS experiment as
20 described above (Supporting Information).

21

22 **ABPs can visualize UCHL1 activity and the covalent linkage is thermally reversed.**

23 We next set out to investigate whether the probes can be used to label and visualize UCHL1
24 activity after SDS-PAGE and fluorescence gel scanning similar to the Rh-Ub-PA probe. To
25 our surprise for none of the three small-molecule probes a clear band corresponding to probe-
26 labelled UCHL1 could be detected after incubation with purified recombinant human UCHL1.
27 We reasoned that the isothiourea bond between UCHL1 and probe, which is stable under the
28 conditions used for inhibition and LC-MS experiments (*vide supra*), might be susceptible to
29 the conditions used for protein denaturation, e.g. boiling in the presence of ~300 mM β-
30 mercaptoethanol. Indeed, when the same samples were resolved by SDS-PAGE under non-
31 denaturing conditions (no boiling and absence of β-mercaptoethanol) a clear band appeared
32 that corresponds to probe-labelled UCHL1 for all three probes (Figure 3B). We also
33 investigated if the ABP-UCHL1 bond would survive when β-mercaptoethanol is replaced by
34 tris(2-carboxyethyl)phosphine) (TCEP), both of which are used to create a reducing

1 environment. Figure 3B clearly shows that the ABP-UCHL1 bands remain intact in the
2 presence of 50 mM TCEP and show a better-resolved profile (less smearing) compared to the
3 non-reducing samples. The Rh-Ub-PA control samples show that nearly all UCHL1 is labeled
4 and that the formed bond for this probe is stable under denaturing conditions, which
5 corroborates earlier findings.³¹ The bands corresponding to Rh-Ub-PA and **9RK87** bound to
6 UCHL1 (both bearing the same dye and present in equal amounts) are of similar intensity,
7 which indicates that the small-molecule probes bind UCHL1 efficiently and that all UCHL1 is
8 active upon probe engagement.

9

10 **ABPs bind to the active site cysteine residue of UCHL1 and visualize UCHL1 activity in** 11 **various cell lines.**

12 We next assessed the ability of the probes to bind and inhibit UCHL1 in a cell lysate by treating
13 HEK293T cell extracts with 5 μ M of the three fluorescent probes, as well as their azide
14 precursor **8RK64** and inhibitor **6RK73** for 1 hour followed by labelling of all residual DUB
15 activity with Cy5-Ub-PA. The Cy5-labelled Ub probe was used here to circumvent spectral
16 interference with either of the other dyes used in the small-molecule probes. Fluorescent
17 scanning of the gel after SDS-PAGE as well as Western blotting using anti-UCHL1 antibody
18 clearly showed that Rhodamine probe **9RK87** inhibits UCHL1 activity similar to **8RK64** and
19 **6RK73** (Figure 3C). Both Bodipy probes also potently inhibit UCHL1 in a cell lysate, although
20 to a somewhat lesser extent, which could be expected on the basis of their IC₅₀ values. All other
21 bands are unchanged, which demonstrates that all compounds are able to bind UCHL1
22 selectively with respect to other DUBs in a cell lysate.

23 Encouraged by these results we set out to assess the ability of the probes to penetrate the cell
24 membrane and to label active UCHL1 in cells. HEK293T cells were treated with 5 μ M of the
25 probes for 24 hours followed by cell lysis, SDS-PAGE (in the absence of β -mercaptoethanol
26 and boiling) and fluorescence scanning at two wavelengths to detect all fluorescent dyes
27 (Figure 3D). A clear band just above 25 kDa is observed for both Bodipy probes (**8RK59** and
28 **9RK15**), which likely corresponds to ABP-labelled UCHL1 with an expected mass of ~25.5
29 kDa. In addition to this band a few extra bands are visible including one just below UCHL1
30 and one more pronounced band around 55 kDa. Interestingly, hardly any band can be seen for
31 the so-far most potent probe **9RK87**. We attributed this effect to the difference in cell
32 permeability between Bodipy and Rhodamine dyes, with the latter known to be less capable of
33 crossing the cell membrane.³⁶ Indeed, upon further investigation using microscopy in ABP-
34 treated HeLa and HEK293T cells we confirmed that Rhodamine probe **9RK87** is unable to

1 enter these cells, whereas both Bodipy ABPs clearly are (Supporting Information Figure S1).
2 For this reason and because the BodipyFL-ABP proved to be a better inhibitor compared to its
3 BodipyTMR analogue we decided to continue with **8RK59** as the preferred probe for all further
4 experiments.

5 The ability of **8RK59** to label UCHL1 activity in different cell lines was further explored in
6 HEK293T cells and in three cancer cell lines known to express high levels of endogenous
7 UCHL1: non-small cell lung cancer (NSCLC) A549 cells, triple negative breast cancer (TNBC)
8 MDA-MB-436 cells and SKBR7 cells.³⁷ Cells transfected with UCHL1 shRNA knock-down
9 (shUCHL1) or siUCHL1 as well as empty vector control or scrambled oligo (siControl) were
10 treated with 5 μ M of each probe for 24 hours, followed by cell lysis, SDS-PAGE (without
11 boiling and β -mercaptoethanol) and fluorescence scanning (Figure 3E). A clear band appears
12 in the fluorescence scan at the expected height (\sim 25.5 kDa) in all four cell lines and this band
13 is significantly decreased in the UCHL1 knock-down samples, indicating that this band indeed
14 corresponds to ABP-labelled UCHL1.

15 To confirm that **8RK59** binds the active site cysteine residue in UCHL1 we overexpressed
16 Flag-HA-tagged UCHL1 and its C90A catalytic inactive mutant in HEK293T cells and
17 incubated these cells with 5 μ M **8RK59** for 24 hours. Fluorescence scanning and anti-FLAG
18 Western blotting shows that **8RK59** only binds to wild-type UCHL1 but not to catalytically
19 inactive UCHL1, indicating that the probe binding site is the active site cysteine (Figure 3F).

20

21 **Determination of DUB selectivity and potential off-targets of the ABP.**

22 As mentioned before, besides the band corresponding to ABP-labelled UCHL1 a few other
23 bands appeared on gel (Figure 3D) but based on the DUB profiling results (Figure 3C) these
24 bands can most likely not be attributed to other DUBs. In order to gain more insight into
25 potential off-targets we performed pull-down experiments coupled to mass spectrometry to
26 identify the proteins binding to our probe. We started with a '2-step ABP' approach in which
27 HEK293T cells were incubated with azide-containing compound **8RK64** or DMSO control,
28 followed by a post-lysis click reaction with biotin-alkyne³⁸ and subsequent pull-down with
29 neutravidin-coated beads (Supporting Information Figure S2A, B). Samples were run (1 cm)
30 on a SDS-PAGE gel, lanes were cut into two pieces and the proteins were subjected to trypsin
31 digestion and analyzed by LC-MSMS. As expected, the most enriched protein identified from
32 this experiment was UCHL1 (Supporting Information Figure S2C). Only one additional protein
33 was also highly enriched, a protein deglycase named DJ-1 (PARK7) with a molecular weight
34 of 20 kDa, which most likely corresponds to the band just below UCHL1 in Figure 3D. This

1 enzyme also harbors an active site cysteine residue which could potentially bind to our probe.
2 Indeed, incubation of UCHL1 and PARK7 knock-down cells with **8RK59**, followed by anti-
3 UCHL1 and anti-PARK7 Western blotting, revealed that PARK7 also reacts with **8RK59** and
4 that the gel band just below UCHL1 corresponds to PARK7 (Supporting Information Figure
5 S2D).

6 In addition to UCHL1 and PARK7, a few other bands can be seen on gel, yet we only identified
7 these two enzymes in the 2-step ABP approach. We therefore performed a 1-step pull-down
8 experiment where we used two biotinylated versions of **8RK64**: compound **11RK72** where
9 biotin is directly linked to the inhibitor and compound **11RK73** with a PEG spacer in between.
10 Both compounds show high inhibitory potential towards UCHL1 (Figure 4A) and form a
11 covalent bond with UCHL1 (Supporting Information). HEK293T cell lysate was incubated
12 with both biotin-ABPs, followed by pull-down with neutravidin-coated beads and subjected to
13 full proteome LC-MSMS analysis (Figure 4B, Supporting Information Figure S2E). Efficient
14 UCHL1 pull-down was confirmed for both biotinylated probes but not the DMSO and biotin-
15 alkyne-treated control samples by Western blotting using anti-UCHL1 antibody (Figure 4C).
16 From the LC-MSMS data, the relative protein abundances were calculated in the pull-down
17 samples and compared to control samples. The list of identified proteins was ranked for total
18 abundance to identify the highest enriched proteins (Supporting Information). Inspection of the
19 list of all enzymes related to Ub (Ub-like proteins, DUBs, E1, E2 and E3 ligases) further
20 substantiates the specificity of the probes for UCHL1 within the Ub system as shown in Figure
21 4D. Only a few of these enzymes were identified in the pull-down experiment with at least
22 150-fold lower abundance compared to UCHL1. The first other DUB on the list is UCHL3, the
23 closest UCHL1 family member, and one of the most abundant DUBs in cells, which could
24 explain this result.

25 The abundances of the top-10 highest ranked proteins are shown in Figure 4E. In line with the
26 results obtained with the 2-step approach, the highest ranked proteins are UCHL1 and PARK7.
27 PARK7 shows a slightly higher abundance here, which contradicts our previous results from
28 the in-cell labeling and 2-step pull-down experiments and might be attributed to the use of a
29 different (biotinylated) version of the ABP or a peptide ionization difference during LC-MSMS
30 measurements. The next highest ranked group of proteins, albeit at much lower abundance
31 levels, includes two amidases NIT1 and NIT2, both harboring an active-site cysteine residue,
32 the isochorismatase domain-containing protein 2 (ISOC2) and glutamine amidotransferase-like
33 class-1 domain-containing protein 3B (GATD3B). Overall, the shorter (**11RK72**) and longer

1 (11RK73) biotin probes give similar results, so the distance between probe and biotin does not
2 seem to influence the binding nor the pull-down efficiency.

3 Upon comparison of the pull-down data (Figure 4) with the fluorescent probe labeling (Figure
4 3) we were unable to assign all bands to proteins. The majority of most abundant proteins in
5 the pull-down experiment have a molecular weight between 20 and 35 kDa. Especially the
6 pronounced band around 55 kDa in Figure 3D remains elusive. In a final attempt to assign this
7 band we resolved the pull-down protein sample from the 1-step labeling experiment by SDS-
8 PAGE. All proteins were visualized by silver staining after which the bands were excised and
9 analyzed by LC-MSMS (Supporting Information Figure S2F). Again, UCHL1 and PARK7
10 were clearly the main proteins identified from the bands at ~25 kDa. The proteins
11 corresponding to the other bands were less clear but the main candidates were GAPDH at ~40
12 kDa and Elongation factor 1 α , tubulin or glutathione reductase (GSR) at ~60 kDa. Whether or
13 not these proteins actually bind to the probe or that these results are due to their high expression
14 levels remains elusive. Based on the result that we identified UCHL1 as the major probe target
15 in three individual experiments and that we found PARK7 as the only major off-target, we
16 reasoned that **8RK59** could well be used for in-cell and *in vivo* labelling of UCHL1 activity.

17

18 **Probing UCHL1 activity in cells with 8RK59.**

19 To assess the application of **8RK59** in live cells, we used inverted fluorescent microscopy to
20 image the **8RK59** signal in MDA-MB-436 and A549 cells after a 16-hour treatment with
21 **8RK59**. Results showed that **8RK59** could penetrate and label the cells (Figure 5A). Compared
22 to the control group, the BodipyFL signal was significantly decreased in UCHL1 knock-down
23 MDA-MB-436 cells and similar results were observed for A549 cells (Supporting Information
24 Figure S3). After imaging, MDA-MB-436 cells were lysed and followed with SDS-PAGE,
25 fluorescence gel scanning and immunoblotting. A decreased UCHL1 signal was detected in
26 MDA-MB-436 UCHL1 knock-down cells by **8RK59** and by antibody stain (Figure 5B). To
27 further validate whether we can visualize UCHL1 specific activity inside cells, control and
28 UCHL1 depleted A549 cells were pre-incubated with **8RK59** probe for 16 hours and stained
29 with UCHL1 antibody (Figure 5C). We observed changes in the distribution of the probe inside
30 the cells. In the control cells **8RK59** accumulated in both UCHL1-positive and negative
31 subcellular compartments while in the UCHL1 knock-down cells the **8RK59** signal was largely
32 decreased in the UCHL1-positive compartments, implying that UCHL1 binds to **8RK59** probe.
33 In agreement with the gel-based labeling data shown in Figure 3 and the proteomics data shown
34 in Figure 4 (and Supporting Information), we still observed some background subcellular

1 localization of **8RK59** in UCHL1 knock-down cells, which may be the result of PARK7
2 staining. Taken all together, this result shows that the cellular distribution of **8RK59** probe
3 changes upon depletion of UCHL1 and it can therefore be used to monitor UCHL1 activity in
4 cells.

5

6 **Probing UCHL1 activity in zebrafish embryos with 8RK59.**

7 To investigate the application of **8RK59** in tracking UCHL1 activity in an *in vivo* model, we
8 chose the zebrafish (*Danio rerio*) due to their high genetic homology to humans and the
9 transparency of their embryos.³⁹ Firstly, we treated zebrafish embryos with **8RK59** and
10 recorded fluorescent images during the development of embryos from 1 to 7 days post
11 fertilization (dpf). Results showed **8RK59** mainly labeled the nose, eye and brain of the
12 zebrafish embryos (Figure 6A). Interestingly, all these organs are enriched in nerve cells and
13 highly express *Uchl1* mRNA.⁴⁰ To validate that the labelling of **8RK59** in zebrafish embryos
14 is specific to UCHL1 protein, we fixed the **8RK59** labelled embryos and performed IF staining
15 with UCHL1 antibody. Results demonstrated both the **8RK59** and UCHL1 antibody label
16 similar organs of zebrafish embryos (Figure 6B). To assess whether **8RK59** could detect the
17 UCHL1 activity changes in zebrafish embryos, we pretreated the zebrafish embryos with
18 UCHL1 activity inhibitor **6RK73** from 1 to 3 dpf, and then labelled the embryos with **8RK59**
19 from 4 to 6 dpf. We found that increasing concentrations of **6RK73**-pretreated zebrafish
20 embryos resulted in significantly lower **8RK59** signal labelling (Figure 6C). In addition, the
21 lysate of **6RK73**-pretreated zebrafish embryos showed decreased UCHL1 signal in
22 fluorescence scans of a corresponding SDS-PAGE gel (Supporting Information Figure S4).
23 These *in vivo* experiments indicate that **8RK59** can visualize and track UCHL1 activity during
24 the development of zebrafish embryos.

25

26 **CONCLUSIONS**

27 One of the key challenges within DUB research is the creation of activity-based probes that
28 target a single DUB type and at the same time are able to cross the cell membrane, in order to
29 study these enzymes inside living cells or even living organisms.⁴¹ It has recently been shown
30 by us and others that Ub-based tools (such as ABPs) can be made sub-type specific by
31 engineering the amino acid sequence in Ub,^{32, 42, 43} however these ABPs are not cell-permeable,
32 although the use of cell-penetrating peptides has recently been applied to deliver Ub ABPs into
33 cells.⁴⁴ ABPs based on small-molecule inhibitors on the other hand are often cell-permeable
34 and can be tuned chemically to become selective,^{45, 46} although such ABPs for DUBs have been

1 lacking so far. We here provide evidence for the first fluorescent small-molecule target specific
2 DUB ABP (**8RK59**) that hits UCHL1 activity *in vitro*, in cells and *in vivo*. We based our design
3 on a cyanimide-containing inhibitor and show, in contrast to what has been reported in
4 literature,²⁷ that cyanimides can act as (near to) irreversible binders. Whether the irreversible
5 bond formation results from the chemical nature of the cyanimide used here or from its binding
6 mode within the UCHL1 active site and whether this property can be extended to other DUBs,
7 remains to be investigated. Instalment of a fluorescent group onto a small-molecule inhibitor
8 can have a detrimental effect on its inhibitory properties. Our data show that the installation of
9 a Rhodamine fluorophore hardly, and a BodipyFL fluorophore only marginally effected the
10 inhibitory potency towards UCHL1, whereas our Ub-ABP experiments confirmed the
11 preservation of their selectivity for UCHL1 among other cysteine DUBs. From these two
12 probes Rhodamine-tagged **9RK87** showed better *in vitro* characteristics, e.g. lower IC₅₀ value
13 and more potent in cell lysate, but unfortunately proved to be unable to cross the cell membrane.
14 As such, this probe could be preferred for *in vitro* experiments and might be optimized for in-
15 cell use by chemically improving the cell-penetrating properties of Rhodamine.²⁸
16 Small-molecule inhibitors or probes almost inevitably result in unspecific interactors and this
17 is not different for our compounds. We have considerably invested in the identification of
18 potential off-targets of our probes by means of a proteomics approach. The data generated in
19 this effort are not only useful for our own study but also provide valuable information for others
20 working on this type of cyanimide-containing compounds. The proteomics data is in line with
21 the Ub-probe experiments, confirming that these compounds are UCHL1 specific within the
22 Ub system and to enzymes of the closely related Ub-like systems (e.g. Nedd8, SUMO, etc.).
23 We indeed found a few potential off-targets, the main one being the protein and nucleotide
24 deglycase PARK7. These cyanimide compounds may therefore provide a good starting point
25 for small-molecule probes targeting PARK7, which, in spite of its important enzymatic
26 function in protein and DNA repair in virtually any cell, have not been developed yet. Based
27 on our data we expect that the potency and selectivity of the probe can be further improved by
28 means of chemical alterations of the inhibitor. A better knowledge on the structural
29 determinants of the interactions between probe and UCHL1 will be of great value for this,
30 unfortunately despite several crystallization attempts we were unable to obtain appropriately
31 diffracting crystals. During preparation of our manuscript Flaherty and co-workers⁴⁷ reported
32 on a related (*S*)-1-cyanopyrrolidine-2-carboxamide-based UCHL1 inhibitor and they applied
33 NMR and molecular modeling to gain insight in the interactions between inhibitor and UCHL1,
34 which could provide useful information to further optimize our probes. In addition, they

1 modified their inhibitor with an alkyne moiety, which, unlike our molecules, resulted in a
2 decrease in potency towards UCHL1 and selectivity with respect to UCHL3. This 2-step probe
3 was then used to identify off-targets in KMS11 cells but remarkably none of their identified
4 proteins show overlap with our list.

5 In conclusion, we have developed a fluorescent small-molecule activity-based probe that labels
6 UCHL1 activity *in vitro*, in cells and *in vivo*. It is the first example of a ‘1-step’ DUB-selective,
7 cell-permeable ABP and therefore serves as a unique addition to the ‘Ub toolbox’,
8 concomitantly addressing two of the outstanding challenges within this field. Our results show
9 that the probe works in several different cell lines and we therefore foresee a potential wide
10 application of the probe in studying UCHL1 activity related to neurodegenerative disorders
11 and cancer. In fact, we recently showed that **6RK73** decreases UCHL1 activity and thereby
12 inhibits TGF β /SMAD2 and SMAD3 signaling and breast cancer migration and extravasation.⁴⁸
13 We are convinced that the here reported strategy of small-molecule cyanimide-based probes
14 can be expanded to other cysteine proteases and specifically DUBs. With the rising importance
15 of the Ub system as source of practical drug targets we believe that these ABP tools will fill an
16 unmet need allowing us to study active DUBs in their native environment in live cells or
17 animals and as such aid in the development of future therapeutics that target diseases associated
18 with ubiquitination.

19

20 **METHODS**

21

22 **IC₅₀ determination.** The *in vitro* enzyme inhibition assays were performed in “non-binding
23 surface flat bottom low flange” black 384-well plates (Corning) at room temperature in a buffer
24 containing 50 mM Tris-HCl, 100 mM NaCl, pH 7.6, 2.0 mM cysteine, 1 mg/mL 3-[(3-
25 cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and 0.5 mg/mL γ -
26 globulins from bovine blood (BGG) in triplicate. Each well had a final volume of 20.4 μ L. All
27 dispensing steps involving buffered solutions were performed on a Biotek MultiFlowFX
28 dispenser. The compounds were dissolved in DMSO as 10 mM, 1 mM and 0.1 mM stock
29 solutions and appropriate volumes were transferred from these stocks to the empty plate using
30 a Labcyte Echo550 acoustic dispenser and accompanying dose-response software to obtain a
31 12 point serial dilution (3 replicates) of 0.05 to 200 μ M. A DMSO back-fill was performed to
32 obtain equal volumes of DMSO (400 μ L) in each well. 10 mM *N*-ethylmaleimide (NEM) was
33 used a positive control (100% inhibition) and DMSO as negative control (0% inhibition). 10
34 μ L buffer was added and the plate was vigorously shaken for 20 sec. Next, 5 μ L of a 4 \times final

1 concentration enzymes stock was added followed by incubation for 30 min. 5 μ L of the
2 substrate (Ub-Rho-morpholine (final concentration 400 nM) or Cbz-PheArg-AMC (final
3 concentration 10 μ M) in the case of Papain) and the increase in fluorescence intensity over
4 time was recorded using a BMG Labtech CLARIOstar or PHERAstar plate reader (excitation
5 487 nm, emission 535 nm). The initial enzyme velocities were calculated from the slopes,
6 normalized to the positive and negative controls and plotted against the inhibitor concentrations
7 (in M) using the built-in equation “[inhibitor] vs. response – Variable slope (four parameters),
8 least squares fit” with constraints “Bottom = 0” and “Top = 100” in GraphPad Prism 7 software
9 to obtain the IC₅₀ values.

10

11 **Jump dilution assay.** All assays were performed in triplicate. The assay was performed in a
12 buffer containing 50 mM Tris-HCl, 100 mM NaCl, pH 7.6, 2.0 mM cysteine, 1 mg/mL 3-[(3-
13 cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and 0.5 mg/mL γ -
14 globulins from bovine blood (BGG). The final concentrations used were: 3 nM UCHL1, 400
15 nM Ub-Rho-morpholine, 10 μ M or 0.1 μ M or a jump dilution of 10 μ M to 0.1 μ M inhibitor.
16 Samples of 20 μ L containing 300 nM UCHL1 and 20 μ M inhibitor (2% DMSO), 2% DMSO
17 or 20 mM *N*-ethylmaleimide (NEM) were incubated for 30 min. at room temperature. 5 μ L of
18 each sample was then diluted into a 500 μ L solution containing 400 nM Ub-Rho-morpholine.
19 After a brief mixing 20 μ L of each of these solutions was quickly transferred to a “non-binding
20 surface flat bottom low flange” black 384-well plate (Corning) and the increase in fluorescence
21 over time was recorded using a BMG Labtech CLARIOstar plate reader (excitation 487 nm,
22 emission 535 nm). As a control, samples were taken along in which 40 μ L of a 20 μ M and 0.2
23 μ M inhibitor solution in buffer (2% DMSO) were added to 20 μ L of a 12 nM UCHL1 solution.
24 After 30 min. incubation 20 μ L of a 1.6 μ M Ub-Rho-morpholine solution was added after
25 which 20 μ L of each solution was transferred to the same 384 well plate mentioned above and
26 the increase in fluorescent intensity was measured concomitantly. Fluorescent intensities were
27 plotted against time using GraphPad Prism 7.

28

29 **Covalent complex formation mass spectrometry analysis.** Samples of 1.4 μ M UCHL1 in 70
30 μ L buffer containing 50 mM Tris-HCl, 100 mM NaCl, pH 7.6, 2.0 mM cysteine and 1 mg/mL
31 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) were prepared.
32 These samples were treated with 1 μ L DMSO or 1 μ L of a 10 mM inhibitor/probe stock
33 solution in DMSO (140 μ M final concentration) and incubated for 30 min. at room temperature.

1 Samples were then 3× diluted with water and analyzed by mass spectrometry by injecting 1
2 μL on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion
3 source in positive mode (capillary voltage 1.2 kV, desolvation gas flow 900 L/hour, T = 60 °C)
4 with a resolution R = 26,000. Samples were run using 2 mobile phases: A = 0.1% formic acid
5 in water and B = 0.1% formic acid in CH₃CN on a Waters Acquity UPLC Protein BEH C4
6 column, 300 Å, 1.7 μm (2.1 × 50 mm); flow rate = 0.5 mL/min, runtime = 14.00 min, column
7 T = 60 °C, mass detection 200-2500 Da. Gradient: 2 – 100% B. Data processing was performed
8 using Waters MassLynx Mass Spectrometry Software 4.1 and ion peaks were deconvoluted
9 using the built-in MaxEnt1 function.

10

11 **Probe labeling of purified recombinant UCHL1.** The assay was performed in a buffer
12 containing 50 mM Tris-HCl, 100 mM NaCl, pH 7.6, 2.0 mM cysteine and 1 mg/mL 3-[(3-
13 cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). A stock solution
14 containing 8 μM UCHL1 and stock solutions containing 20 μM **8RK59**, **9RK15**, **9RK87** and
15 Rho-Ub-PA in buffer were prepared. 50 μL of the UCHL1 stock solution was mixed with 50
16 μL of all probe solutions followed by incubation for 60 min. at 37 °C. Three aliquots of 10 μL
17 of each sample were taken and treated with 1) 5 μL loading buffer with β-mercaptoethanol,
18 followed by 5 min. heating at 95 °C; 2) 5 μL loading buffer with 50 mM TCEP; 3) 5 μL loading
19 buffer. Samples were resolved by SDS-PAGE using a 4-12% Bis-Tris gel (Invitrogen,
20 NuPAGE) with MES SDS running buffer (Novex, NuPAGE) for 45 min. at 190V. Gels were
21 scanned for fluorescence on a GE Typhoon FLA 9500 using a green ($\lambda_{ex/em}$ 473/530 nm) and
22 red ($\lambda_{ex/em}$ 532/570 nm) channel followed by staining with InstantBlue Coomassie protein stain
23 (Expedeon) after which the gel was scanned on a GE Amersham Imager 600.

24

25 **Cell lines and cell culture.** HEK293T, HeLa, A549 and MDA-MB-436 cells were originally
26 obtained from American Type Culture Collection (ATCC) and SKBR7 cells were obtained
27 from Dr. J. Martens (Erasmus University Medical Center, Rotterdam, The Netherlands). Cells
28 were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal
29 bovine serum (FBS) and 100 U/mL penicillin-streptomycin (15140122; Gibco). Stable
30 shUCHL1 A549 and shUCHL1 MDA-MB-436 cell lines were generated by lentiviral infection
31 and the cell lines were continuously cultured under puromycin selection. Four UCHL1 shRNAs
32 were identified and tested, the most effective shRNA (TRCN0000007273; Sigma) for lentiviral

1 infection were used for experiments. All cell lines were regularly tested for absence of
2 mycoplasma and were authenticated.

3

4 **Transfection.** For shRNA expression, lentiviruses were produced by transfecting shRNA-
5 targeting plasmids together with helper plasmids pCMV-VSVG, pMDLg-RRE (gag-pol), and
6 pRSV-REV into HEK293T cells. Cell supernatants were collected 48 hours after transfection
7 and were used to infect cells. To obtain stable shUCHL1 A549 and shUCHL1 MDA-MB-436
8 UCHL1 knock-down cell lines, cells were infected at low confluence (40%) for 12 hours with
9 lentiviruses in the presence of 5 ng/mL Polybrene (Sigma). Cells were subjected to 1 µg/mL
10 puromycin selection 48 hours after infection. Four UCHL1 shRNAs were identified and tested,
11 the most effective UCHL1 shRNA (TRCN0000007273; Sigma) for lentiviral infection was
12 used for the experiments.

13 For siRNA transfection, siRNAs targeting UCHL1 (Set of 4: siGENOME; MQ-004309-00-
14 0002 2 nmol) and PARK7 (Set of 4: siGENOME; MQ-005984-00-0002 2 nmol) were obtained
15 from Dharmacon. Knock-down of UCHL1 and PARK7 in HEK293T cells was performed as
16 follows: for 6-well plate format 200 µL siRNA (500 nM stock) were incubated with 4 µL
17 Dharmafectin reagent 1 (Dharmacon) diluted in 200 µL medium without supplements by
18 shaking for 20 min. at room temperature. The transfection mix was added to cells and cultured
19 at 37 °C and 5 % CO₂. 48 hours after transfection **8RK59** was added to the cells and incubated
20 for 24 hours. Cells were harvested and analysed as described under the section “DUB activity
21 profiling and competition with Ub-PA DUB probes”.

22 For the expression of UCHL1 in HEK293T cells, Flag-HA-UCHL1 construct was obtained
23 from Addgene (22563). Catalytically inactive mutant (C90A) UCHL1 was generated using
24 site-directed mutagenesis. Wild-type and C90A mutant UCHL1 were transfected into
25 HEK293T cells using PEI transfection reagent. 24 hours after transfection **8RK59** was added
26 to the cells and incubated for 24 hours. Cells were harvested and analysed as described under
27 the section “DUB activity profiling and competition with Ub-PA DUB probes”.

28

29 **Immunoblotting.** Cells were lysed in HR lysis buffer (50 mM Tris, 5 mM MgCl₂, 250 mM
30 sucrose and 2 mM DTT, pH 7.4) with protease inhibitor cocktail for 10 min. on ice. The lysates
31 were sonicated using 10 cycles of 30 sec. pulse on, 30 sec. pulse off. The lysates were
32 centrifuged at maximum speed for 20 min. at 4 °C, thereafter protein concentrations were
33 measured using the DC protein assay (500-0111; Bio-Rad) and equal amounts of proteins were

1 used for each condition that was analyzed by immunoblotting with following antibodies:
2 UCHL1 (ab27053; Abcam), Tubulin (2148; Cell Signaling), GAPDH (MAB374; Millipore),
3 Actin (A5441; Sigma-Aldrich).

4

5 **Immunofluorescence staining.** Cells were fixed for 20 min. in 4% paraformaldehyde and then
6 permeabilized in 0.1% Triton-X for 10 min. Non-specific binding was blocked with blocking
7 buffer (1% BSA in 0.1% PBS-Tween) for 30 min. The primary antibody UCHL1 (ab27053;
8 Abcam) was diluted in blocking buffer and added to the cell for 1 hour. After 3 times washing
9 with PBS, the secondary antibody donkey anti rabbit IgG Alexa Fluorescence 555 (Invitrogen
10 #A31572) was added and incubated for 30 min. After 3 times washing with PBS, samples were
11 mounted with VECTASHIELD antifade mounting medium with DAPI (H-1200; Vector
12 Laboratories). Fluorescence images were acquired with TCS SP8 confocal microscope (Leica).
13 Zebrafish embryos were fixed with 4% paraformaldehyde 2 hours at room temperature.
14 Samples were dehydrated with 33%, 66%, 100% methanol in PBS, followed by a rehydration
15 step. Thereafter, the embryos were successively treated with 10 µg/mL proteinase K for 60
16 min. at 37 °C, permeabilized with 0.25% Triton in PBS for 30 min. on ice, and blocked with
17 10% FBS in PBS for 1 hour at room temperature. Embryos were incubated with primary
18 antibody (ab27053; Abcam) for at least 12 hours at 4 °C. After washing with 0.1% Triton in
19 PBS for 3 times 10 min., the samples were incubated with fluorescein-conjugated secondary
20 antibody donkey anti rabbit IgG Alexa Fluorescence 555 (Invitrogen #A31572) for 2 hours at
21 room temperature. After washing with PBS (0.1% Triton), samples were analyzed using a
22 confocal microscope SP5 STED (Leica, Rijswijk, Netherlands).

23

24 **DUB activity profiling and competition with Ub-PA DUB probes.** HEK293T cells were
25 treated with 5 µM final concentration of the indicated compounds for 24 hours. Cells were
26 lysed in HR lysis buffer supplemented with protease inhibitor cocktail (11836145001; Roche).
27 Samples were kept on ice and lysed by sonication (10 cycles of 30 seconds on and 30 seconds
28 off). 25 µg protein extract was labelled with either 1 µM Rh-Ub-PA probe or 0.5 µM Cy5-Ub-
29 PA probe for 30 min. at 37 °C. For the cell lysate incubation, HEK293T cells were lysate as
30 described above. HEK293T cell lysates were preincubated with 5 µM final concentration of
31 compounds for 1 hour, followed by incubation with 0.5 µM Cy5-Ub-PA probe for 30 min. at
32 37 °C. Labelling reactions were terminated with sample buffer and heating to 100 °C for 10
33 min. Samples were size-separated in SDS-PAGE gels. In-gel fluorescence signals were

1 scanned employing the Typhoon FLA 9500 Molecular Imager (GE Healthcare). Images were
2 analyzed using ImageJ software.

3

4 **Probe labelling of endogenous UCHL1 in living cell.** Cell lines were transfected with
5 shRNAs, siRNAs or UCHL1 constructs as described above. 5 μ M final concentration of probes
6 were added to the cell a day before harvesting. Cells were harvested in HR buffer as described
7 above. NuPAGE LDS sample buffer containing 50 mM TCEP was added to cell lysates.
8 Samples were resolved by SDS-PAGE using a 4-12% Bis-Tris gel (Invitrogen, NuPAGE) with
9 MES SDS running buffer (Novex, NuPAGE) for 45 min. at 190V. Gels were scanned for
10 fluorescence on a GE Typhoon FLA 9500 using a green ($\lambda_{ex/em}$ 473/530 nm) and red ($\lambda_{ex/em}$
11 532/570 nm) channel followed by transferring proteins to nitrocellulose membrane
12 (Amersham) and western blot analysis.

13

14 **Proteomics.** For 1-step approach, 4×10^6 HEK293T cells were seeded into 10 cm dishes for
15 each treatment. 48 hours later, HEK293T cells were harvested in lysis buffer containing 50
16 mM HEPES pH 7.3, 150 mM NaCl and 1% NP-40 and 1 \times protease inhibitor cocktail and
17 incubated for 30 min. on ice. Cell lysates were centrifuged at maximum speed for 20 min. The
18 lysates were incubated with 5 μ M final concentration of Biotin-PEG₄-alkyne, **11RK72** or
19 **11RK73** or same volume of DMSO for 1 hour at room temperature. 30 μ L of neutravidin beads
20 slurry (50%) were added to each sample. The samples were then incubated for 2 hours at 4 $^{\circ}$ C.
21 Beads were washed six times in wash buffer containing 50 mM HEPES pH 7.3, 150 mM NaCl
22 and 1% NP-40. After completely removing the washing buffer, NuPAGE LDS sample buffer
23 (containing 7.5% β -mercaptoethanol) was added to the beads followed by 15 min. incubation
24 at 95 $^{\circ}$ C.

25 For 2-step approach, 4×10^6 HEK293T cells were seeded into 10 cm dishes for each treatment.
26 24 hours later, 5 μ M final concentration of **8RK64** or same volume of DMSO was added to
27 the cells. After 24 hours incubation, HEK293T cells were harvested in lysis buffer containing
28 50 mM HEPES pH 7.3, 150 mM NaCl and 1% NP-40 and 1 \times protease inhibitor cocktail and
29 incubated for 30 min. on ice. Cell lysates were centrifuged at maximum speed for 20 min. 1 \times
30 volume of click cocktail (100 mM CuSO₄·5H₂O, 1M sodium ascorbate, 100 mM TBTA
31 (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) ligand, 0.1 M HEPES pH 7.3 and 5 μ M
32 biotin-alkyne) were added to 2 \times volume of cell lysates and incubated for 45 min. 30 μ L of
33 neutravidin beads slurry (50%) were added to each sample. The samples were then incubated

1 for 2 hours at 4 °C. Beads were washed six times in wash buffer containing 50 mM HEPES pH
2 7.3, 150 mM NaCl and 1% NP-40. After completely removing the washing buffer, SDS sample
3 buffer (containing 7.5% β -mercaptoethanol) was added to the beads followed by 15 min.
4 incubation at 95 °C. For MS analysis proteins were run for 1-2 cm on a 4-12% PAGE
5 (NuPAGE Bis-Tris Precast Gel, Life Technologies) and stained with silver (SilverQuest Silver
6 Stain, Life Technologies). The lane was cut into two equal parts, and gel slices subjected to
7 reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using
8 a Proteineer DP digestion robot (Bruker).

9 Tryptic peptides were extracted from the gel slices, lyophilized, dissolved in 95/3/0.1 v/v/v
10 water/acetonitril/formic acid and subsequently analyzed by on-line C18 nanoHPLC MS/MS
11 with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen,
12 Germany), and a LUMOS mass spectrometer (Thermo). Fractions were injected onto a
13 homemade precolumn (100 μ m \times 15 mm; Reprosil-Pur C18-AQ 3 μ m, Dr. Maisch,
14 Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm \times
15 50 μ m; Reprosil-Pur C18-AQ 3 μ m). The gradient was run from 0% to 50% solvent B
16 (20/80/0.1 water/acetonitrile/formic acid v/v/v) in 20 min. The nano-HPLC column was drawn
17 to a tip of \sim 5 μ m and acted as the electrospray needle of the MS source. The LUMOS mass
18 spectrometer was operated in data-dependent MS/MS (top-10 mode) with collision energy at
19 32 V and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution
20 was 120,000, the scan range 400-1500, at an AGC target of 400,000 @maximum fill time of
21 50 ms. Dynamic exclusion after n=1 with exclusion duration of 10 s. Charge states 2-5 were
22 included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2
23 Da. HCD collision energy was set to 32V. First mass was set to 110 Da. The MS2 scan
24 resolution was 30,000 with an AGC target of 50,000 @maximum fill time of 60 ms.

25 In a post-analysis process, raw data were first converted to peak lists using Proteome
26 Discoverer version 2.2.0.388 (Thermo Electron), and then submitted to the Uniprot Homo
27 sapiens database (67911 entries), using Mascot v. 2.2.04 (www.matrixscience.com) for protein
28 identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and
29 fragment mass, respectively, and trypsin as enzyme. Up to two missed cleavages were allowed,
30 and methionine oxidation was set as a variable modification; carbamidomethyl on Cys was set
31 as a fixed modification. Protein abundance calculation and statistical analysis was performed
32 using Proteome Discoverer software.

33

1 **Zebrafish experiments.** Transgenic zebrafish lines Tg (kdrl: mTurquoise) were raised, staged
2 and maintained according to standard procedures in compliance with the local Institutional
3 Committee for Animal Welfare of the Leiden University. Zebrafish embryos were treated with
4 5 μ M **8RK59** or gradient **6RK73** concentration in the egg water. Fluorescent image acquisition
5 was performed with a Leica SP5 STED confocal microscope (Leica, Rijswijk, Netherlands).
6 The quantification of **8RK59** signal was analyzed by Leica microscope software platform LAS
7 X. 30 zebrafish were treated in each group and 3 representative images were taken and
8 analyzed. Statistical analysis was performed using Graphpad Prism 8 software. Numerical data
9 from triplicates are presented as the mean \pm SD. Two-way analysis of variance (ANOVA) has
10 been used to analyze multiple subjects.

11

12 **ASSOCIATED CONTENT**

13 **Supporting Information**

14 The Supporting Information is available free of charge DOI: ###

15 Detailed synthesis procedures and compound characterization (including Ub-Rho-morpholine
16 and Rhodamine110-alkyne), NMR and LC-MS spectral data, proteomics data, IC₅₀ data and
17 supporting information figures.

18

19 **AUTHOR INFORMATION**

20 **Corresponding Authors**

21 *p.p.geurink@lumc.nl, h.ovaa@lumc.nl

22 **Author Contributions**

23 ‡P.P.G., R.K., A.S. and S.L. contributed equally to this work.

24 **Notes**

25 The authors declare the following competing financial interest(s): H.O. is shareholder of the
26 reagent company UbiQ.

27 **Ethics Statement**

28 Zebrafish were raised, staged and maintained according to standard procedures in compliance
29 with the local Institutional Committee for Animal Welfare of the Leiden University.

30

31 **ACKNOWLEDGEMENTS**

32 We thank Yves Leestemaker for assistance with the DUB activity-based profiling assays and
33 Bjorn van Doodewaerd for LC-MS and HPLC purification assistance. P.t.D. and H.O. are
34 supported by Oncode Institute. S.L. is supported by the China Scholarship Council. This work

1 was supported by the Dutch Organization for Scientific Research NWO VICI grant
2 (724.013.002) to H.O., Investment Grant NWO Medium grant (91116004, partly financed by
3 ZonMw) to P.A.v.V. and Cancer Genomics Centre Netherlands (CGC.NL) to P.t.D.

4

5 REFERENCES

- 6 1. Komander, D.; Rape, M., The ubiquitin code. *Annu. Rev. Biochem.* **2012**, *81*, 203-29.
- 7 2. Cadwell, K.; Coscoy, L., Ubiquitination on nonlysine residues by a viral E3 ubiquitin
8 ligase. *Science* **2005**, *309* (5731), 127-30.
- 9 3. Pao, K. C.; Wood, N. T.; Knebel, A.; Rafie, K.; Stanley, M.; Mabbitt, P. D.;
10 Sundaramoorthy, R.; Hofmann, K.; van Aalten, D. M. F.; Virdee, S., Activity-based E3
11 ligase profiling uncovers an E3 ligase with esterification activity. *Nature* **2018**, *556* (7701),
12 381-385.
- 13 4. Mevissen, T. E. T.; Komander, D., Mechanisms of Deubiquitinase Specificity and
14 Regulation. *Annu. Rev. Biochem.* **2017**; *86*, 159-192.
- 15 5. Behrends, C.; Harper, J. W., Constructing and decoding unconventional ubiquitin chains.
16 *Nat. Struct. Mol. Biol.* **2011**, *18* (5), 520-528.
- 17 6. Wilkinson, K. D.; Lee, K.; Deshpande, S.; Duerksen-hughes, P.; Boss, J. M.; Pohl, J.,
18 The neuron-specific protein PGP-9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science*
19 **1989**, *246* (4930), 670-673.
- 20 7. Leroy, E.; Boyer, R.; Auburger, G.; Leube, B.; Ulm, G.; Mezey, E.; Harta, G.;
21 Brownstein, M. J.; Jonnalagada, S.; Chernova, T.; Dehejia, A.; Lavedan, C.; Gasser,
22 T.; Steinbach, P. J.; Wilkinson, K. D.; Polymeropoulos, M. H., The ubiquitin pathway in
23 Parkinson's disease. *Nature* **1998**, *395* (6701), 451-452.
- 24 8. Maraganore, D. M.; Farrer, M. J.; Hardy, J. A.; Lincoln, S. J.; McDonnell, S. K.; Rocca,
25 W. A., Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in
26 Parkinson's disease. *Neurology* **1999**, *53* (8), 1858-60.
- 27 9. Lunati, A.; Lesage, S.; Brice, A., The genetic landscape of Parkinson's disease. *Revue*
28 *neurologique* **2018**, *174* (9), 628-643.
- 29 10. Bilguvar, K.; Tyagi, N. K.; Ozkara, C.; Tuysuz, B.; Bakircioglu, M.; Choi, M.; Delil,
30 S.; Caglayan, A. O.; Baranoski, J. F.; Erturk, O.; Yalcinkaya, C.; Karacorlu, M.; Dincer,
31 A.; Johnson, M. H.; Mane, S.; Chandra, S. S.; Louvi, A.; Boggon, T. J.; Lifton, R. P.;
32 Horwich, A. L.; Gunel, M., Recessive loss of function of the neuronal ubiquitin hydrolase

- 1 UCHL1 leads to early-onset progressive neurodegeneration. *Proc. Natl. Acad. Sci. U. S.*
2 *A.* **2013**, *110* (9), 3489-94.
- 3 11. Zhang, M.; Cai, F.; Zhang, S.; Zhang, S.; Song, W., Overexpression of ubiquitin
4 carboxyl-terminal hydrolase L1 (UCHL1) delays Alzheimer's progression in vivo. *Sci.*
5 *Rep.* **2014**, *4*, 7298.
- 6 12. Bishop, P.; Rocca, D.; Henley, J. M., Ubiquitin C-terminal hydrolase L1 (UCH-L1):
7 structure, distribution and roles in brain function and dysfunction. *Biochem. J.* **2016**, *473*
8 (16), 2453-62.
- 9 13. Hussain, S.; Foreman, O.; Perkins, S. L.; Witzig, T. E.; Miles, R. R.; van Deursen, J.;
10 Galardy, P. J., The de-ubiquitinase UCH-L1 is an oncogene that drives the development
11 of lymphoma in vivo by deregulating PHLPP1 and Akt signaling. *Leukemia* **2010**, *24* (9),
12 1641-1655.
- 13 14. Hurst-Kennedy, J.; Chin, L. S.; Li, L., Ubiquitin C-terminal hydrolase 11 in tumorigenesis.
14 *Biochem. Res. Int.* **2012**, *2012*, 123706.
- 15 15. de Jong, A.; Merkx, R.; Berlin, I.; Rodenko, B.; Wijdeven, R. H.; El Atmioui, D.;
16 Yalcin, Z.; Robson, C. N.; Neefjes, J. J.; Ovaa, H., Ubiquitin-based probes prepared by
17 total synthesis to profile the activity of deubiquitinating enzymes. *Chembiochem* **2012**, *13*
18 (15), 2251-8.
- 19 16. Harrigan, J. A.; Jacq, X.; Martin, N. M.; Jackson, S. P., Deubiquitylating enzymes and
20 drug discovery: emerging opportunities. *Nat. Rev. Drug Discov.* **2018**, *17* (1), 57-78.
- 21 17. Das, C.; Hoang, Q. Q.; Kreinbring, C. A.; Luchansky, S. J.; Meray, R. K.; Ray, S. S.;
22 Lansbury, P. T.; Ringe, D.; Petsko, G. A., Structural basis for conformational plasticity of
23 the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. *Proc. Natl. Acad. Sci. U.*
24 *S. A.* **2006**, *103* (12), 4675-4680.
- 25 18. Gavory, G.; O'Dowd, C. R.; Helm, M. D.; Flasz, J.; Arkoudis, E.; Dossang, A.; Hughes,
26 C.; Cassidy, E.; McClelland, K.; Odrzywol, E.; Page, N.; Barker, O.; Miel, H.; Harrison,
27 T., Discovery and characterization of highly potent and selective allosteric USP7
28 inhibitors. *Nat. Chem. Biol.* **2018**, *14* (2), 118-125.
- 29 19. O'Dowd, C. R.; Helm, M. D.; Rountree, J. S. S.; Flasz, J. T.; Arkoudis, E.; Miel, H.;
30 Hewitt, P. R.; Jordan, L.; Barker, O.; Hughes, C.; Rozycka, E.; Cassidy, E.; McClelland,
31 K.; Odrzywol, E.; Page, N.; Feutren-Burton, S.; Dvorkin, S.; Gavory, G.; Harrison, T.,
32 Identification and Structure-Guided Development of Pyrimidinone Based USP7
33 Inhibitors. *ACS Med. Chem. Lett.* **2018**, *9* (3), 238-243.

- 1 20. Turnbull, A. P.; Ioannidis, S.; Krajewski, W. W.; Pinto-Fernandez, A.; Heride, C.;
2 Martin, A. C. L.; Tonkin, L. M.; Townsend, E. C.; Buker, S. M.; Lancia, D. R.;
3 Caravella, J. A.; Toms, A. V.; Charlton, T. M.; Lahdenranta, J.; Wilker, E.; Follows, B.
4 C.; Evans, N. J.; Stead, L.; Alli, C.; Zarayskiy, V. V.; Talbot, A. C.; Buckmelter, A. J.;
5 Wang, M.; McKinnon, C. L.; Saab, F.; McGouran, J. F.; Century, H.; Gersch, M.;
6 Pittman, M. S.; Marshall, C. G.; Raynham, T. M.; Simcox, M.; Stewart, L. M. D.;
7 McLoughlin, S. B.; Escobedo, J. A.; Bair, K. W.; Dinsmore, C. J.; Hammonds, T. R.;
8 Kim, S.; Urbe, S.; Clague, M. J.; Kessler, B. M.; Komander, D., Molecular basis of USP7
9 inhibition by selective small-molecule inhibitors. *Nature* **2017**, *550* (7677), 481-486.
- 10 21. Lamberto, I.; Liu, X.; Seo, H. S.; Schauer, N. J.; Iacob, R. E.; Hu, W.; Das, D.;
11 Mikhailova, T.; Weisberg, E. L.; Engen, J. R.; Anderson, K. C.; Chauhan, D.; Dhe-
12 Paganon, S.; Buhrlage, S. J., Structure-Guided Development of a Potent and Selective
13 Non-covalent Active-Site Inhibitor of USP7. *Cell Chem. Biol.* **2017**, *24* (12), 1490-
14 1500.e11.
- 15 22. Kategaya, L.; Di Lello, P.; Rouge, L.; Pastor, R.; Clark, K. R.; Drummond, J.;
16 Kleinheinz, T.; Lin, E.; Upton, J. P.; Prakash, S.; Heideker, J.; McClelland, M.; Ritorto,
17 M. S.; Alessi, D. R.; Trost, M.; Bainbridge, T. W.; Kwok, M. C. M.; Ma, T. P.; Stiffler,
18 Z.; Brasher, B.; Tang, Y.; Jaishankar, P.; Hearn, B. R.; Renslo, A. R.; Arkin, M. R.;
19 Cohen, F.; Yu, K.; Peale, F.; Gnad, F.; Chang, M. T.; Klijn, C.; Blackwood, E.; Martin,
20 S. E.; Forrest, W. F.; Ernst, J. A.; Ndubaku, C.; Wang, X.; Beresini, M. H.; Tsui, V.;
21 Schwerdtfeger, C.; Blake, R. A.; Murray, J.; Maurer, T.; Wertz, I. E., USP7 small-
22 molecule inhibitors interfere with ubiquitin binding. *Nature* **2017**, *550* (7677), 534-538.
- 23 23. Pozhidaeva, A.; Valles, G.; Wang, F.; Wu, J.; Sterner, D. E.; Nguyen, P.; Weinstock,
24 J.; Kumar, K. G. S.; Kanyo, J.; Wright, D.; Bezsonova, I., USP7-Specific Inhibitors
25 Target and Modify the Enzyme's Active Site via Distinct Chemical Mechanisms. *Cell*
26 *Chem. Biol.* **2017**, *24* (12), 1501-1512.e5.
- 27 24. Resnick, E.; Bradley, A.; Gan, J.; Douangamath, A.; Krojer, T.; Sethi, R.; Geurink, P.
28 P.; Aimon, A.; Amitai, G.; Bellini, D.; Bennett, J.; Fairhead, M.; Fedorov, O.; Gabizon,
29 R.; Gan, J.; Guo, J.; Plotnikov, A.; Reznik, N.; Ruda, G. F.; Diaz-Saez, L.; Straub, V.
30 M.; Szommer, T.; Velupillai, S.; Zaidman, D.; Zhang, Y.; Coker, A. R.; Dowson, C.
31 G.; Barr, H. M.; Wang, C.; Huber, K. V. M.; Brennan, P. E.; Ovaa, H.; von Delft, F.;
32 London, N., Rapid Covalent-Probe Discovery by Electrophile-Fragment Screening. *J. Am.*
33 *Chem. Soc.* **2019**, *141* (22), 8951-8968.

- 1 25. Ward, J. A.; McLellan, L.; Stockley, M.; Gibson, K. R.; Whitlock, G. A.; Knights, C.;
2 Harrigan, J. A.; Jacq, X.; Tate, E. W., Quantitative Chemical Proteomic Profiling of
3 Ubiquitin Specific Proteases in Intact Cancer Cells. *ACS Chem. Biol.* **2016**, *11* (12), 3268-
4 3272.
- 5 26. Jones, A.; Kemp, M. I.; Stockley, M. L.; Gibson, K. R.; Whitlock, G. A.; Madin, A.
6 Novel compounds. **2016**, International patent WO 2016/046530 A1.
- 7 27. Falgueyret, J. P.; Oballa, R. M.; Okamoto, O.; Wesolowski, G.; Aubin, Y.; Rydzewski,
8 R. M.; Prasit, P.; Riendeau, D.; Rodan, S. B.; Percival, M. D., Novel, nonpeptidic
9 cyanamides as potent and reversible inhibitors of human cathepsins K and L. *J. Med.*
10 *Chem.* **2001**, *44* (1), 94-104.
- 11 28. Ub-Rho-morpholine is an improved version of the classically used Ub-Rho110Gly and
12 releases the brighter Rh110-morpholinecarbonyl species upon enzymatic cleavage, see
13 also: Lavis, L. D.; Chao, T. Y.; Raines, R. T., Fluorogenic label for biomolecular imaging.
14 *ACS Chem. Biol.* **2006**, *1* (4), 252-60.
- 15 29. Copeland, R. A.; Basavapathruni, A.; Moyer, M.; Scott, M. P., Impact of enzyme
16 concentration and residence time on apparent activity recovery in jump dilution analysis.
17 *Anal. Biochem.* **2011**, *416* (2), 206-10.
- 18 30. Liu, Y.; Lashuel, H. A.; Choi, S.; Xing, X.; Case, A.; Ni, J.; Yeh, L. A.; Cuny, G. D.;
19 Stein, R. L.; Lansbury, P. T., Jr., Discovery of inhibitors that elucidate the role of UCH-
20 L1 activity in the H1299 lung cancer cell line. *Chem. Biol.* **2003**, *10* (9), 837-46.
- 21 31. Ekkebus, R.; van Kasteren, S. I.; Kulathu, Y.; Scholten, A.; Berlin, I.; Geurink, P. P.;
22 de Jong, A.; Goerdayal, S.; Neeffjes, J.; Heck, A. J.; Komander, D.; Ovaas, H., On terminal
23 alkynes that can react with active-site cysteine nucleophiles in proteases. *J. Am. Chem.*
24 *Soc.* **2013**, *135* (8), 2867-70.
- 25 32. Gjonaj, L.; Sapmaz, A.; Gonzalez-Prieto, R.; Vertegaal, A. C. O.; Flierman, D.; Ovaas,
26 H., USP7: combining tools towards selectivity. *Chem. Commun.* **2019**, *55* (35), 5075-
27 5078.
- 28 33. Altun, M.; Kramer, H. B.; Willems, L. I.; McDermott, J. L.; Leach, C. A.; Goldenberg,
29 S. J.; Kumar, K. G. S.; Konietzny, R.; Fischer, R.; Kogan, E.; Mackeen, M. M.;
30 McGouran, J.; Khoronenkova, S. V.; Parsons, J. L.; Dianov, G. L.; Nicholson, B.;
31 Kessler, B. M., Activity-Based Chemical Proteomics Accelerates Inhibitor Development
32 for Deubiquitylating Enzymes. *Chem. Biol.* **2011**, *18* (11), 1401-1412.
- 33 34. Cravatt, B. F.; Wright, A. T.; Kozarich, J. W., Activity-based protein profiling: from
34 enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* **2008**, *77*, 383-414.

- 1 35. Verdoes, M.; Hillaert, U.; Florea, B. I.; Sae-Heng, M.; Risseeuw, M. D. P.; Filippov,
2 D. V.; van der Marel, G. A.; Overkleeft, H. S., Acetylene functionalized BODIPY dyes
3 and their application in the synthesis of activity based proteasome probes. *Bioorg. Med.*
4 *Chem. Lett.* **2007**, *17* (22), 6169-6171.
- 5 36. Butkevich, A. N.; Mitronova, G. Y.; Sidenstein, S. C.; Klocke, J. L.; Kamin, D.;
6 Meineke, D. N. H.; D'Este, E.; Kraemer, P. T.; Danzl, J. G.; Belov, V. N.; Hell, S. W.,
7 Fluorescent Rhodamines and Fluorogenic Carbopyronines for Super-Resolution STED
8 Microscopy in Living Cells. *Angew. Chem. Int. Ed.* **2016**, *55* (10), 3290-3294.
- 9 37. The human protein atlas (UCHL1): [https://www.proteinatlas.org/ENSG00000154277-](https://www.proteinatlas.org/ENSG00000154277-UCHL1/cell)
10 UCHL1/cell
- 11 38. Meier, J. L.; Mercer, A. C.; Rivera, H.; Burkart, M. D., Synthesis and evaluation of
12 bioorthogonal pantetheine analogues for in vivo protein modification. *J. Am. Chem. Soc.*
13 **2006**, *128* (37), 12174-12184.
- 14 39. Ren, J.; Liu, S.; Cui, C.; Ten Dijke, P., Invasive Behavior of Human Breast Cancer Cells
15 in Embryonic Zebrafish. *J. Vis. Exp.: JoVE* **2017**, (122).
- 16 40. Son, O. L.; Kim, H. T.; Ji, M. H.; Yoo, K. W.; Rhee, M.; Kim, C. H., Cloning and
17 expression analysis of a Parkinson's disease gene, uch-L1, and its promoter in zebrafish.
18 *Biochem. Biophys. Res. Commun.* **2003**, *312* (3), 601-7.
- 19 41. Hewings, D. S.; Flygare, J. A.; Bogoyo, M.; Wertz, I. E., Activity-based probes for the
20 ubiquitin conjugation-deconjugation machinery: new chemistries, new tools, and new
21 insights. *FEBS J.* **2017**, *284* (10), 1555-1576.
- 22 42. Ernst, A.; Avvakumov, G.; Tong, J. F.; Fan, Y. H.; Zhao, Y. L.; Alberts, P.; Persaud,
23 A.; Walker, J. R.; Neculai, A. M.; Neculai, D.; Vorobyov, A.; Garg, P.; Beatty, L.;
24 Chan, P. K.; Juang, Y. C.; Landry, M. C.; Yeh, C.; Zeqiraj, E.; Karamboulas, K.; Allali-
25 Hassani, A.; Vedadi, M.; Tyers, M.; Moffat, J.; Sicheri, F.; Pelletier, L.; Durocher, D.;
26 Raught, B.; Rotin, D.; Yang, J. H.; Moran, M. F.; Dhe-Paganon, S.; Sidhu, S. S., A
27 Strategy for Modulation of Enzymes in the Ubiquitin System. *Science* **2013**, *339* (6119),
28 590-595.
- 29 43. Gjonaj, L.; Sapmaz, A.; Flierman, D.; Janssen, G. J.; van Veelen, P. A.; Ovaas, H.,
30 Development of a DUB-selective fluorogenic substrate. *Chem. Sci.* **2019**, Advance Article.
- 31 44. Gui, W. J.; Ott, C. A.; Yang, K.; Chung, J. S.; Shen, S. Q.; Zhuang, Z. H., Cell-Permeable
32 Activity-Based Ubiquitin Probes Enable Intracellular Profiling of Human Deubiquitinases.
33 *J. Am. Chem. Soc.* **2018**, *140* (39), 12424-12433.

- 1 45. Sanman, L. E.; Bogyo, M., Activity-Based Profiling of Proteases. In *Annu. Rev. Biochem.*,
2 **2014**, *83*, 249-273.
- 3 46. Verdoes, M.; Verhelst, S. H. L., Detection of protease activity in cells and animals.
4 *Biochim. Biophys. Acta, Proteins Proteomics* **2016**, *1864* (1), 130-142.
- 5 47. Krabill, A. D.; Chen, H.; Hussain, S.; Feng, C.; Abdullah, A.; Das, C.; Aryal, U. K.;
6 Post, C. B.; Wendt, M. K.; Galardy, P. J.; Flaherty, D. P., Biochemical and cellular
7 characterization of a cyanopyrrolidine covalent Ubiquitin C-terminal hydrolase L1
8 inhibitor. *Chembiochem* **2019**.
- 9 48. Liu, S.; González Prieto, R.; Zhang, M.; Geurink, P. P.; Kooij, R.; Vasudevan Iyengar, P.;
10 van Dinther, M.; Bos, E.; Zhang, X.; Le Dévédec, S.; van de Water, B.; Koning, R. I.; Zhu,
11 H. J.; Mesker, W.; Vertegaal, A.; Ovaa, H.; Zhang, L.; Martens, J. W. M.; ten Dijke, P.,
12 Deubiquitinase activity profiling identifies UCHL1 as a candidate oncoprotein that
13 promotes TGF β -induced breast cancer metastasis. *Clin. Cancer Res.* **2019**, accepted for
14 publication.

1 **FIGURE LEGENDS**

2

3 **Scheme 1. Synthesis of azide-containing UCHL1 inhibitor 8RK64 and fluorescent and**
4 **biotinylated probe derivatives thereof.** ^a Synthetic steps described in literature.²⁶

5

6 **Figure 1.** Biochemical characterization of UCHL1 inhibitor **6RK73**. A) Reaction of a thiol
7 with a cyanamide results in the formation of an isothiurea adduct. B) Structure of UCHL1
8 inhibitor **6RK73**. C) IC₅₀ determination of **6RK73** for UCHL1, UCHL3 and UCHL5. D)
9 Progress curves for UCHL1 proteolytic activity after jump dilution (see also Figure C). DMSO
10 and *N*-ethylmaleimide (NEM) are used as controls. E) Deconvoluted mass spectra of UCHL1
11 before (blue) and after (red) reaction with **6RK73**. F) Fluorescence labelling of remaining DUB
12 activity in HEK293T cells upon treatment with UCHL1 inhibitors LDN-57444 and **6RK73**.

13

14 **Figure 2.** Biochemical characterization of **8RK64**. A) IC₅₀ determination of **8RK64** for
15 UCHL1, UCHL3 and UCHL5. B) Deconvoluted mass spectra of UCHL1 before (blue) and
16 after (red) reaction with **8RK64**. C) Fluorescence labelling of remaining DUB activity in
17 HEK293T cells upon treatment with UCHL1 inhibitors **8RK64** and **6RK73**.

18

19 **Figure 3.** Characterization of the fluorescent UCHL1 probes *in vitro* and in cells. A) IC₅₀
20 determination of **8RK59**, **9RK15** and **9RK87** for UCHL1. B) Labelling of purified
21 recombinant human UCHL1 by the three probes and Rh-Ub-PA. β: β-mercaptoethanol; T:
22 TCEP. C) Fluorescence labelling by Cy5-Ub-PA of remaining DUB activity in HEK293T cell
23 lysate upon treatment with UCHL1 inhibitors and probes. D) Fluorescence scans showing the
24 labelling pattern in HEK293T cells of the three probes. E) Fluorescence labelling of UCHL1
25 activity in HEK293T, A549, MDA-MB-436 and SKBR7 cells with **8RK59**. F) **8RK59** labels
26 overexpressed Flag-HA-UCHL1 wt but not the C90A active site mutant in HEK293T cells.

27

28 **Figure 4.** Proteomics experiments with biotinylated ABP analogs to identify ABP targets. A)
29 IC₅₀ determination of **11RK72** and **11RK73** for UCHL1. B) Schematic representation of pull-

1 down experiment to identify ABP binding proteins. C) Confirmation of UCHL1 pull-down
2 with biotinylated ABP analogs by Western Blot Analysis. Immunoblotting was performed
3 using UCHL1 and Actin antibodies. Actin was used as a loading control and incubated together
4 with UCHL1 antibody in the input sample. D) Abundances of the top-10 highest ranked
5 proteins from the pull-down LC-MSMS experiment averaged over three replicates. E)
6 Abundances of all enzymes related to the Ub(-like) system identified in the pull-down LC-
7 MSMS experiment averaged over three replicates.

8

9 **Figure 5.** Probing UCHL1 activity in cells with **8RK59**. A) Live-cell fluorescence imaging of
10 PLKO and shUCHL1 MDA-MB-436 cells treated with **8RK59**. B) Fluorescence labeling of
11 endogenous UCHL1 in PLKO and shUCHL1 MDA-MB-436 cells treated with **8RK59** in SDS-
12 PAGE gel. Immunoblotting was performed using UCHL1 antibody, and Tubulin was used as
13 a loading control. C) Immunofluorescent staining of UCHL1 in **8RK59** pretreated PLKO and
14 shUCHL1 A549 cells.

15

16 **Figure 6.** Probing UCHL1 activity in zebrafish embryos with **8RK59**. A) Tracking the
17 localization of active UCHL1 with **8RK59** during the development of zebrafish embryos from
18 1 to 7 dpf. B) Immunofluorescent staining of UCHL1 in 6 dpf zebrafish embryo pretreated with
19 **8RK59**. C) Monitoring UCHL1 activity changes in 6 dpf zebrafish embryos with **8RK59**
20 pretreated with UCHL1 inhibitor **6RK73**. Representative images from five groups with zoom
21 in of the brain area are shown in the left panel. The quantification of **8RK59** signal in three
22 **6RK73** treatment groups are shown in the right panel. DMSO and BodipyFL dye were used as
23 controls. **, $P < 0.01$, ***, $P < 0.001$, two-way ANOVA.

24

Scheme 1

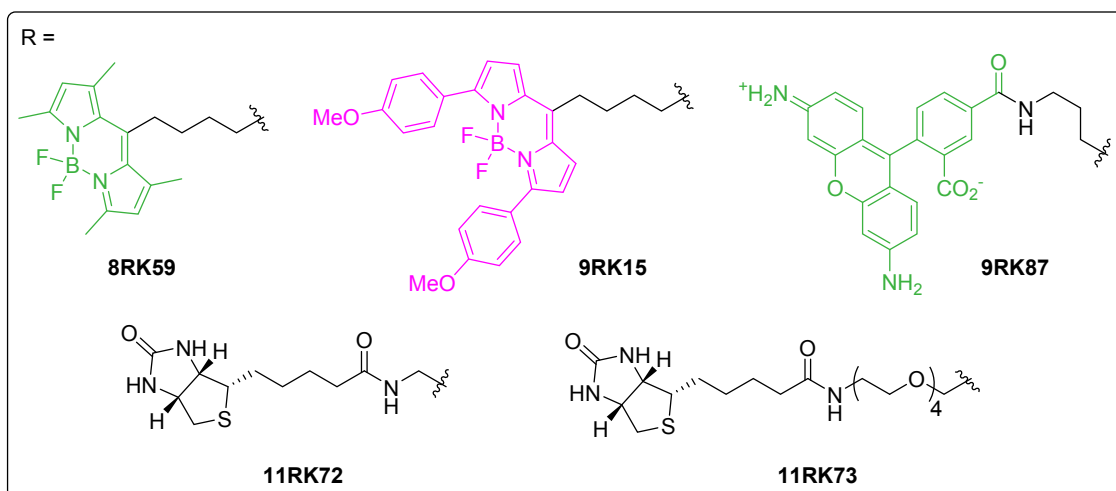
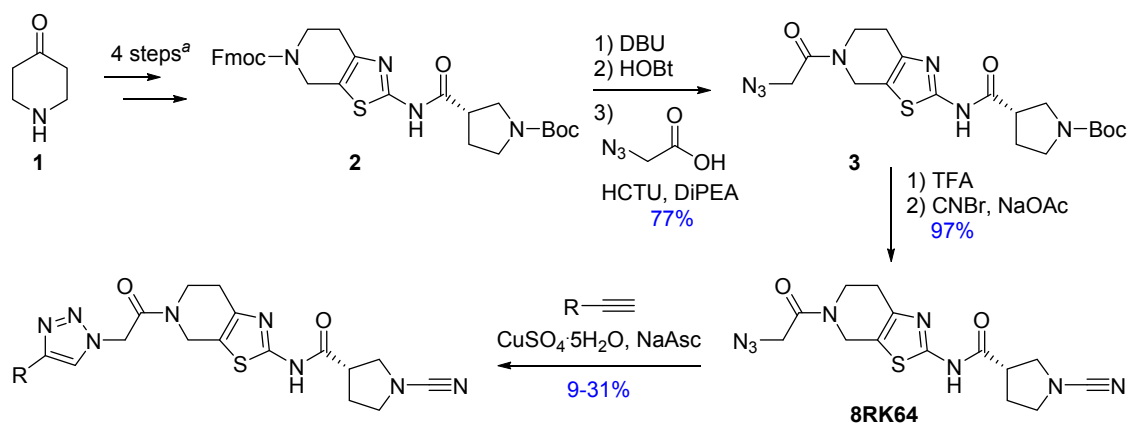


Figure 1

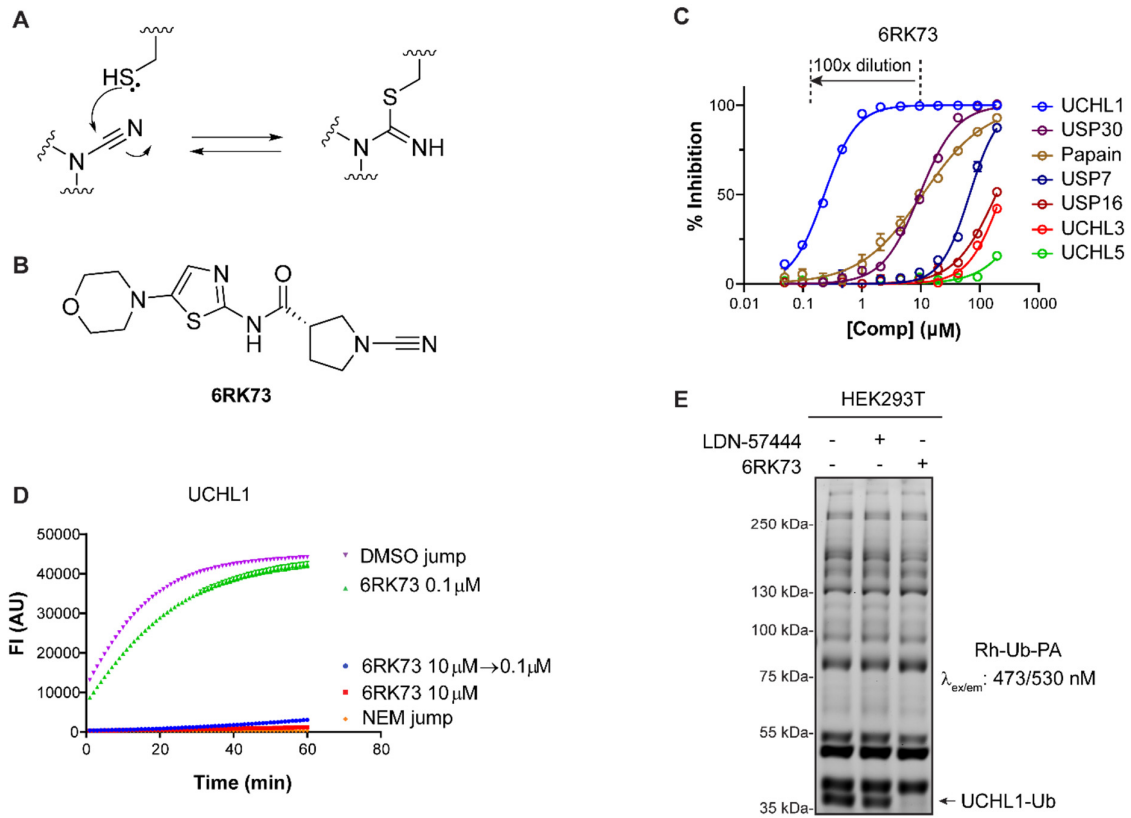


Figure 2

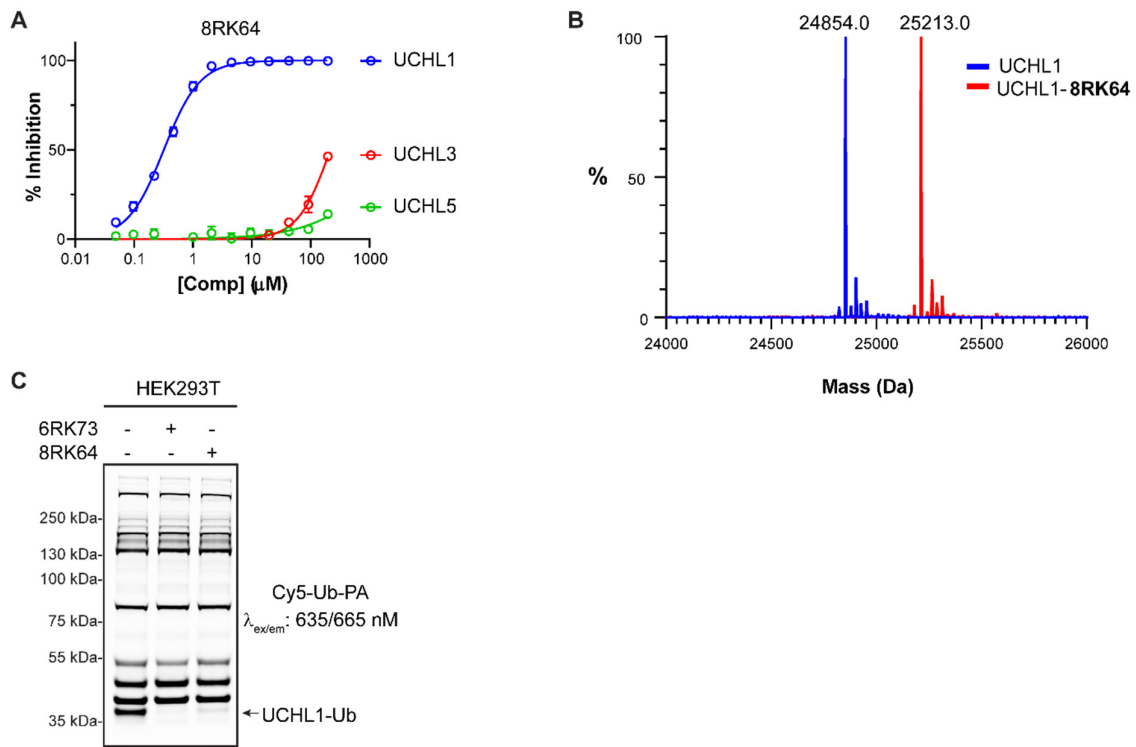


Figure 3

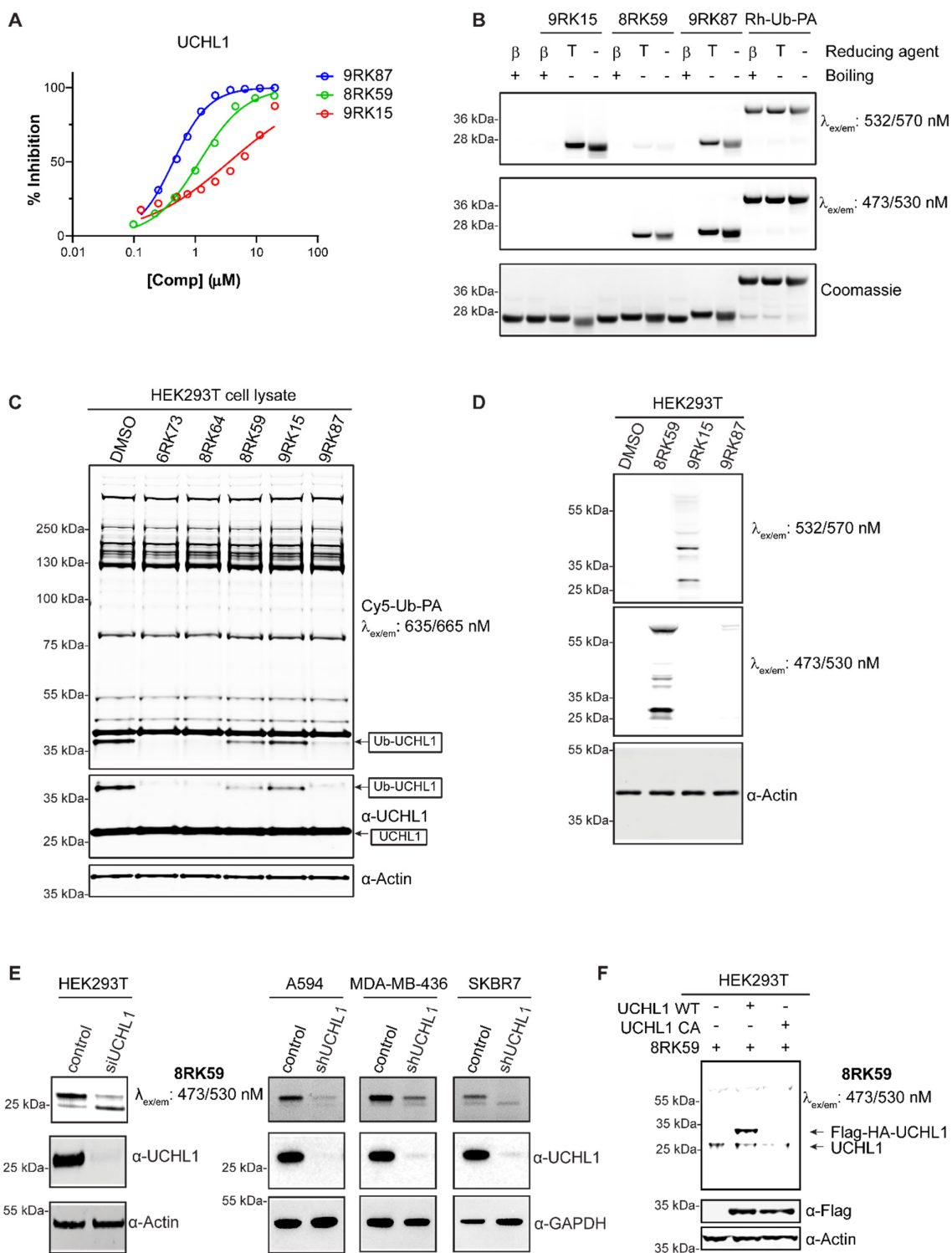


Figure 4

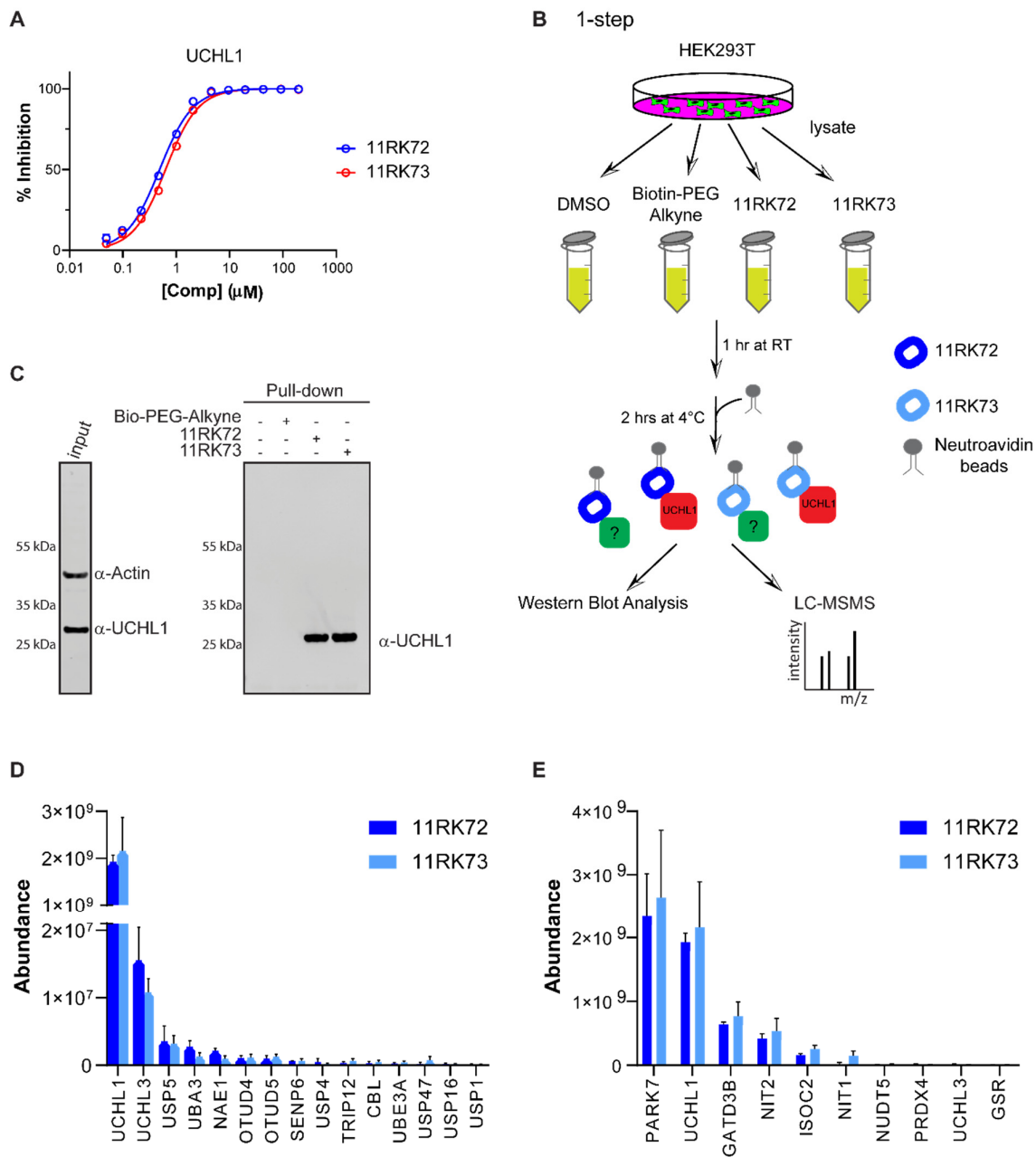


Figure 5

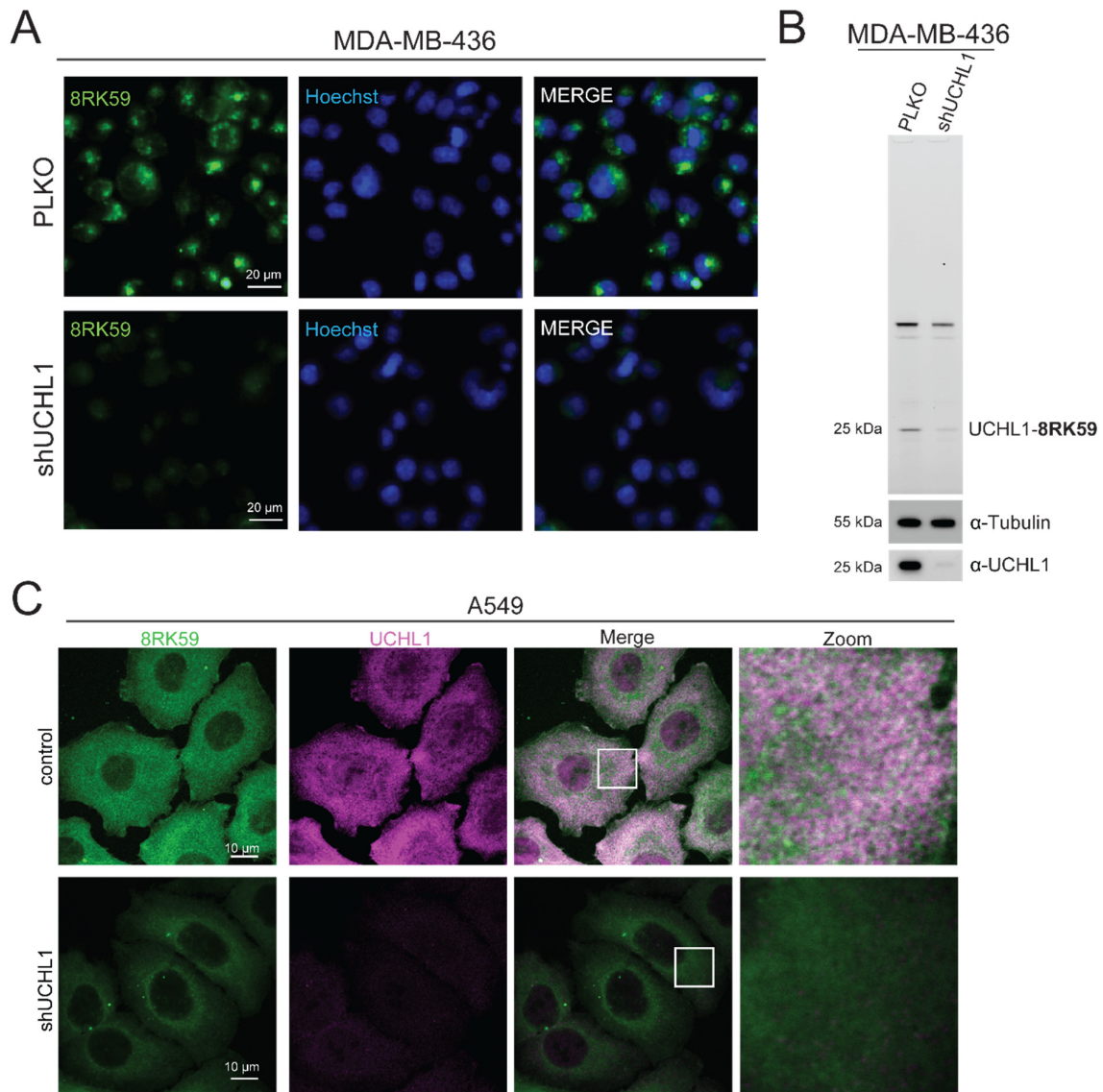


Figure 6

