The use of activity based protein profiling to study proteasome biology

Paniagua Soriano, Guillem

Chapter 3: An Overview of Direct and Two-Step Activity-Based Proteasome Profiling Strategies

Introduction

Activity-based protein profiling (ABPP) is a chemical proteomics technique used for identification and quantitative comparison of enzymatic activities. ABPP is widely applied to study a broad range of enzyme families *in vitro, in situ* in cell cultures and sometimes also *in vivo* in animal models [1]. ABPP has some advantages compared to other proteomics techniques. Experiments are robust, fast and simple. In case of cell permeable activity-based probes (ABPs), imaging of living cells or in animal models makes its applications broader by allowing localization and dynamic studies of the target enzymes [2, 3].

However, ABPP has also some limitations. The covalent bond between the probe and the target enzyme does not allow the recovering of enzymatic activity in a sample treated with ABP. The ABPP platform makes absolute quantification challenging and only relative quantification is possible by comparing the test samples to control ones. Another possible disadvantage of one step ABPP is that the reporter tag is normally a large moiety, which might affect the probe properties, like cell-permeability, selectivity, affinity or bioavailability. To overcome these problems a new strategy has become increasingly popular in the last years, termed two-step ABPP. In this approach the ABP reporter tag has been replaced by a ligation handle, which will be coupled with the reporter group after the attachment of the probe to its target enzyme. This tactic allows researchers to decide in every condition which reporter group to use depending on the desired method of analysis [4]. The reaction between the ABP ligation handle and the reporter group needs to be fast and selective, with ideally no side reactions.

Bioorthogonal chemistry is suitable for two-step ABPP since it allows the performance of selective chemical modifications in complex biological samples it [5]. The term bioorthogonal stands for a chemoselective reaction that ideally can take place in the aqueous environment of a biological system without any side reaction. Nowadays many bioorthogonal reactions are reported in literature, which differ in tag size, selectivity and biocompatibility as well as in reaction rates, making the choice an important decision [6]. Ideally the tag would be a small biocompatible chemical moiety that is able to perform a selective reaction with a non-bioavailable reagent, which should also be biocompatible. The ideal tag should also have a relative small size minimizing its interference with the

target environment. Alkynes and azide groups have emerged as favorites due their dimensions, just comprising few atoms. The most used bioorthogonal reactions used in proteasome two-step ABPP include the Staudinger-Bertozzi ligation in which an azide-containing ABP is reacted with a phosphine reagent equipped tag, the copper(I)-catalyzed click reaction between an azide and an alkyne, the copper-free strain-promoted azide-alkyne cycloaddition and the inverse-electron-demand Diels-Alder reaction of tetrazine with strained alkenes [7-10]. Each of these bioorthogonal reactions has its intrinsic advantages and disadvantages. The Staudinger-Bertozzi ligation is selective but the use of the large phosphine moiety may give problems especially in native conditions where the ABP is found inside an enzyme pocket. The copper(I)-catalyzed click reaction is a versatile ligation but the need for copper salts does not recommend its application in living cells due to its high toxicity. The copper-free cycloaddition is a fast and efficient ligation method but due to the relatively high reactivity of the tags, often gives off-target reactions. The inverse-electron-demand Diels-Alder reaction is very selective under native conditions probably being the most versatile reaction. The first part of this chapter shows a literature overview of the methodology used in two-step ABPP of the proteasome. The labeling of the different proteasome active sites using the Staudinger-Bertozzi ligation, copper-free cycloaddition and a tandem ligation strategy is described. The second part of this chapter is a case study of the residual activity of the proteasome after ABP labeling in human and mouse cell extracts.

Two-step ABPP overview

In the first report on two-step proteasome ABPP published in 2003, the proteasome subunits could be successfully labeled by means of the Staudinger-Bertozzi ligation [11]. A broad spectrum azide-containing ABP was incubated either in living cells or cell lysates and posteriorly subjected to Staudinger ligation under denaturing conditions. The ability of the probe to cross the cellular membrane *in situ,* efficiently bind and thus inhibit the proteasome is proven by the accumulation of a green fluorescent protein (GFP) fused to an ubiquitin chain, which targets it for degradation via the proteasome. This study shows that the incorporation of an azide group in an ABP has no influence in its selectivity towards proteasome both *in situ* and *in vitro.*

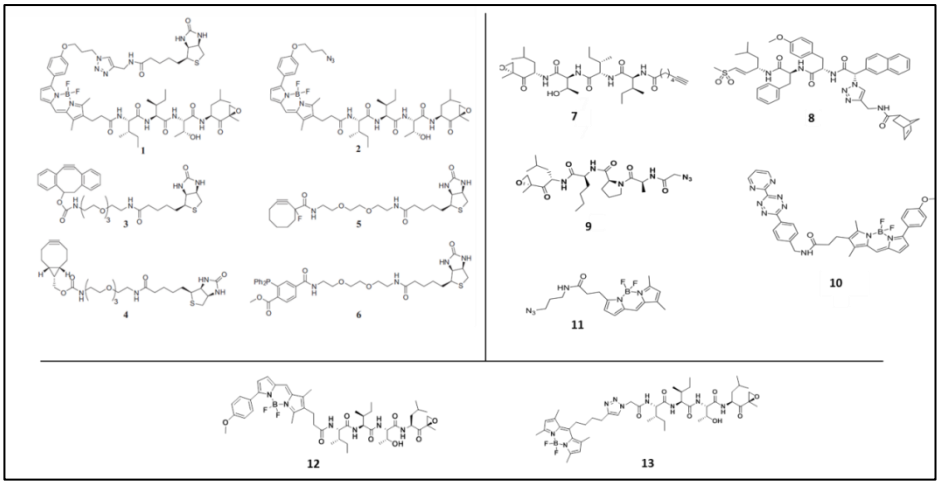

***Figure 1**. ABPs and ligation handles used in the different here presented studies. **1**: biotin-BODIPY-epoxomicin; **2**: azido-BODIPY-epoxomicin; biotin-cyclooctyne derivatives **3:** Dibenzocyclooctynol (DIBO); **4**: Bicyvlenonyne (BCN); **5**: Monofluorinated cyclooctyne (MFCO); **6**: biotin-phosphine; **7**: pan-reactive alkyne-epoxomicin; **8**: β5/β5i selective norbonene-equipped vinyl sulfone; **9**: β1/β1i selective azide-equipped epoxyketone; **10**: Tetrazine-Bodipy-TMR; **11**: Azido-Bodipy; **12**: MVB003 (BODIPY-TMR epoxomicin); **13**: LWA300 (BODIPY-FL epoxomicin).*

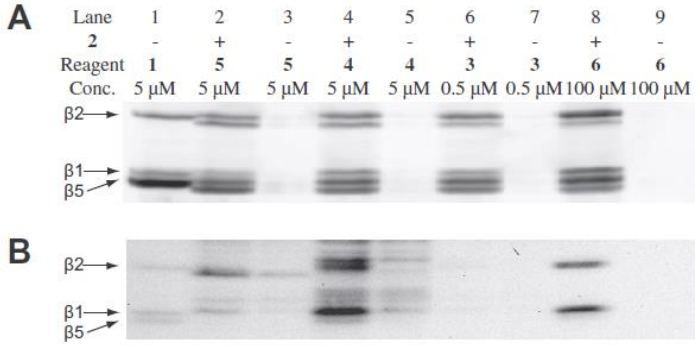

***Figure 2**. [12] A) Fluorescent readout and B) streptavidin blot of labeled cell lysates. HeLa cells were exposed to **2** for 2 hours, excess reagents was removed prior incubation with two-step reagents **3**-**6** for 4 hours. Cells were lysed and resolved by SDS-PAGE.*

In a later study the copper-free click ligation with three different cyclooctynes (figure 1, compounds **3**, **4** and **5**) was tested for two-step proteasome ABPP and compared to the Staudinger-Bertozzi ligation with compound **6** [12]. The use of the azide-bearing fluorescent ABP **2** allows observation the probe in two different ways, by biotin read-out after biorthogonal ligations and by a shift of the modified proteasome subunits in the

fluorescent image due to the incorporated biorthogonal reagents size (figure 2, lane 1 compared to the rest). Comparing both biorthogonal reactions performance, the Staudinger-Bertozzi ligation turned out to be more specific, although longer reaction times are needed, than the copper-free click cycloadditions due to the high background found and the low signal intensity of the different cyclooctynes (figure 2B). The results obtained for the different ligations *in situ* demonstrate the applicability of these ligation techniques in the labeling of proteasomes in living cells.

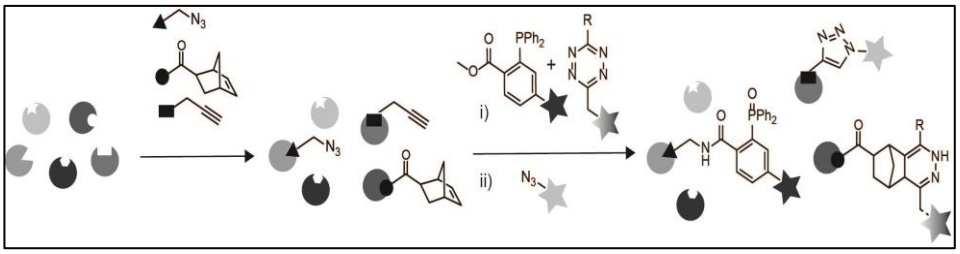

Figure 3. *Schematic workflow of the triple ligation strategy involving a copper(I)-catalyzed click ligation, Staudinger-Bertozzi ligation and tetrazine ligation.*

The use of subunit selective proteasome probes in a two-step proteasome ABPP setup was used to test orthogonality between the different ligation reactions and to develop a strategy, which selectively labels each subunit with a different readout-tag [13]. In figure 3 a schematic workflow of the triple ligation strategy used by Willems and coworkers is shown. The chemical tools used in this strategy are shown in figure 1. The three active proteasome subunits were labeled with a different tag via one of the different ligation reactions. The β5-subunit selective norbornene-tagged ABP **8** together with the β1-selective azide-functionalized ABP **9** were incubated with HEK293T lysate. After exposure to ABP **8** and **9**, panreactive ABP **7** was added, which due to the fact that the β5 and β1 sites were already blocked, could only label the free β2-subunits. This addition sequence allows to selective tag the β5-subunits with a norbornene, the β1-subunits with an azide and the β2-subunits with an alkyne. Next, the cell extracts were incubated with tetrazine **10** and phosphine **6** for 1h. Reagent excess was removed before performing the copper(I)-catalyzed click ligation for an extra hour with azide **11**. The washing step before the click reactions is required to remove both the excess of tetrazine and phosphine, which might react with the cooper catalyst and the azide ABP, respectively. By using two different fluorophores and a biotin tag, the proteasome subunits labeled by the different ligation techniques can be visualized. As it can be seen in figure 4, the triple ligation strategy successfully labels the different proteasome subunits in a selective manner. The results obtained by the separate bioorthogonal reactions (last 3 lanes in fig. 4) are comparable to those of the simultaneous triple ligation, showing the value of the triple ligation strategy.

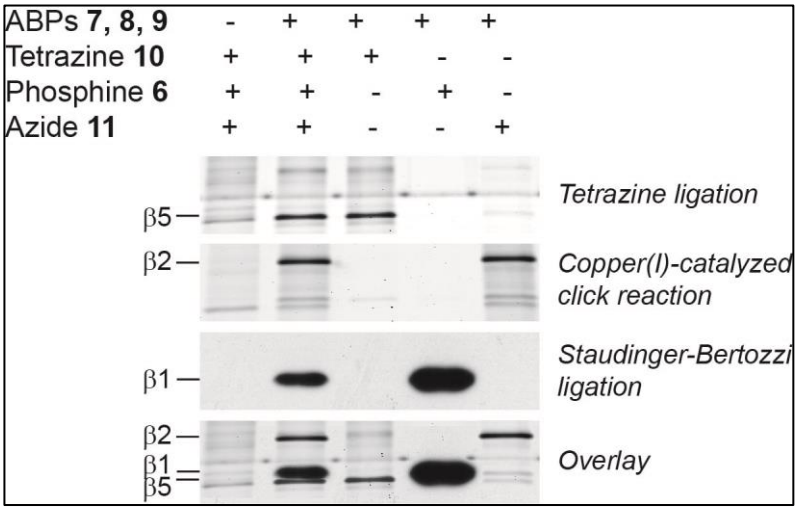

Figure 4*. [13] Anti-biotin blots and in-gel fluorescent imaging to visualize the proteasome labeling patterns in each single reaction and for the simultaneous triple ligation.*

Determination of the unlabeled free proteasome fraction

The second part of this chapter describes the evaluation in human and mouse cell lines of residual proteasome activity after exposure to pan-reactive ABP **12**. Next the optimal labeling conditions for probe **12** in the mouse B-lymphocyte cell line B3/25 were determined. Incubation of B3/25 lysate with varying concentrations of ABP **12** for one hour (figure 5B) shows that above 0.5 µM the plateau phase is observed and that an increase in probe concentration does not result in a rise of signal but in a higher background. Figure 5A shows that although after one hour of incubation the maximum labeling is not reached, an extra exposure of 30 minutes results in only a small increase in signal percentage (around 10%). But again, in this case the signal to background ratio is compromised, indicating that 90 min incubation is not a recommendable labeling time. ABP **12** optimal labeling conditions were determined to be 0.5 µM for one hour-exposure in lysate and 4 hours incubation with 4 µM probe end concentration for *in situ* labeling for both mouse and human cell lines. Figures 5 C&D show that the fluorescent signal of the proteasome-bound ABP **12** is directly proportional to the amount of protein loaded on SDS-PAGE in both lysate and living cells exposures. The high R-square values in both graphs illustrate the applicability of ABPP for relative quantification purposes in mouse samples.

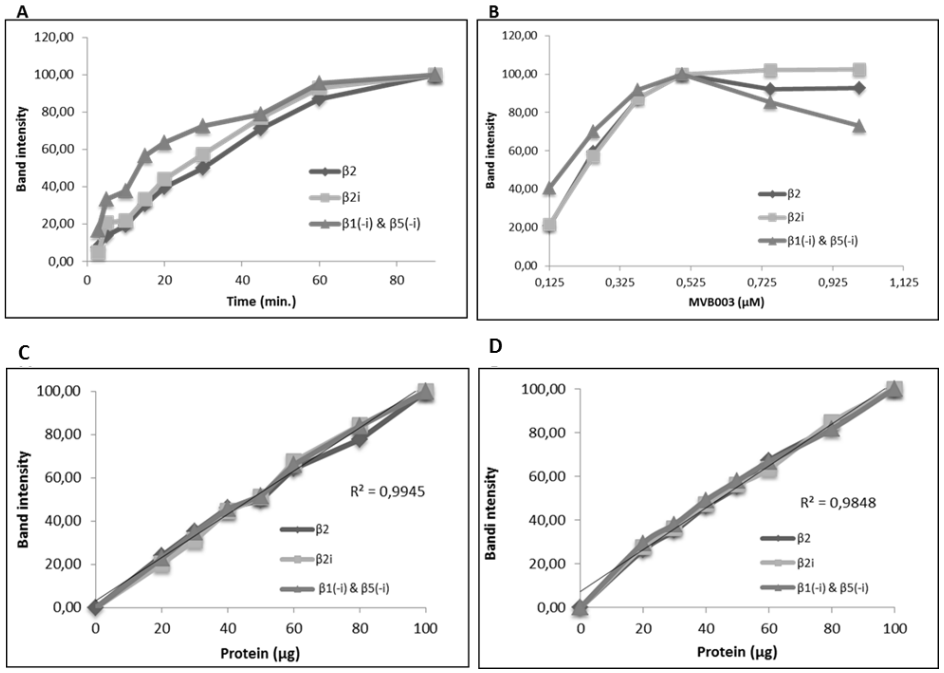

Figure 5. *A) Incubation time of ABP **12** up to 90 minutes in murine B3/25 cell line lysate. B) Lysate was incubated for 1 hour with different probe concentrations. Calibration curves for ABP **12** C) in lysate and D) in living cells.*

In order to remove probe excess when performing the labeling in lysates, samples were loaded on a size-exclusion column prior posterior incubation with ABP **13**, a Cy2-fluorescent analog of ABP **12**. Figure 6 shows that applying the sample on a size-exclusion column directly after addition of ABP **12** allows removal of the unbound ABP (lane D) and that subsequent incubation with probe **13** results in almost full labeling of proteasome subunits (lane C). The light proteasome band (probably β5/5i) visible in the Cy3-fluorescent channel (lanes C and D) indicates that ABP **12** reacts very quickly with the proteasome subunits highlighting the ABP selectivity against proteasome. Incubation of the probe after size-exclusion yields in a small loss of proteasome activity signal compared to its exposure before size-exclusion (lanes A vs. B and E vs. F in figure 6). This small loss (about 10%) was corrected afterwards with the use of coomassie stain as loading control, resulting in a comparable signal for both conditions. No size-exclusion columns were used in the unlabeled proteasome fraction determination. Instead, cells were thoroughly washed with PBS after incubation with the probe prior lysing and posterior exposure to Cy2-fluorescent counter-ABP **13**.

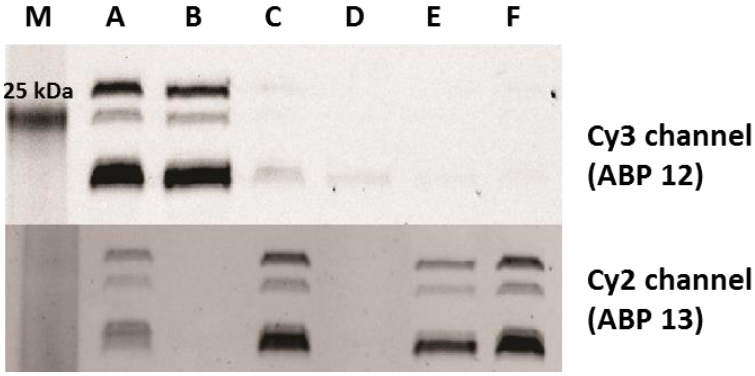

Figure 6. *Gel image showing the different signals for both ABP* ***12*** *(top) and ABP* ***13*** *(bottom) for the free fraction determination.* ***M****: Marker.* ***A****: 1h ABP* ***12****, size exclusion, 1h ABP* ***13****.* ***B****: size exclusion, 1h ABP* ***12****.* ***C****: ABP* ***12*** *short exposure before size exclusion, 1h ABP* ***13****.* ***D****: ABP* ***12*** *short exposure before size exclusion.* ***E****: 1h ABP* ***13****, size exclusion.* ***F****: size exclusion, ABP* ***13****.*

Table 1 shows the unlabeled proteasome subunits percentage obtained in lysate and living cell labeling respectively. Due to the poor separation of the β5/5i/1/1i subunits achieved on gel with both ABPs, these subunits were considered as a single moiety and were quantified together. The overall non-labeled proteasome fraction in the human cell line by probe **12** was found to be around 10% for the different subunits. These residual activity percentages were comparable between *in vivo* and *in vitro* labeling strategies, with the ones found in lysates slightly bigger than those from *in vivo* labeling. Labeling of mouse cells *in situ* yields in a similar free fraction percentage as the one obtained for human living cells (around 5%). Unexpectedly, this fraction increases dramatically (up to 35%) when labeling is performed in mouse cellular extracts (figure 6 lane A; table 1). The large difference between *in vivo* and *in vitro* labeling of mouse samples suggests a systematic error during the *in vitro* experiments performance.

Human

Lysate	Free fraction (%)	Standard deviation (%)
beta2	12	5
beta2i	14	10
beta1+5	7	2

Living cells	Free fraction (%)	Standard deviation (%)
beta2	6	0
beta2i	6	3
beta1+5	4	2

Mouse

Lysate	Free fraction (%)	Standard deviation (%)
beta2	39	8
beta2i	34	16
beta1+5	31	5

Living cells	Free fraction (%)	Standard deviation (%)
beta2	2	1
beta2i	2	0
beta1+5	3	1

***Table 1**. Free fraction percentage for the human (left) and mouse (right) proteasome subunits after 1h incubation with ABP **12** in lysate and for 4h exposure in living cells. The values are the average of 3 replicates.*

Discussion

As it can be appreciated in figure 2A, the used cyclooctynes and phosphine concentrations, or the reaction times, were insufficient for complete ABP labeling, characterized by a shift in the fluorescent gel image. Despite the copper-free cycloadditions were more efficient in terms of reagents concentrations and reaction times compared to the Staudinger-Bertozzi reaction, the ligations with the three cyclooctynes yielded in a much higher background than the one observed with the Staudinger ligation. This suggests that cyclooctyne moieties are able to react with natural biological entities and thus are not truly orthogonal. Further investigations are needed in order to decipher which proteins react with the cyclooctynes and in which manner. The phosphine reagent in the Staudinger-Bertozzi ligation is prone towards oxidation, which is probably the explanation for the high reagent concentration.

The triple ligation strategy [13] shows that it is possible to perform several biorthogonal reactions in one test tube. In this strategy though, a buffer exchange between the tetrazine and the click ligations is needed to prevent undesired side reactions. All three ligation reactions show low or almost no background labeling. Click ligation showed higher background labeling than the other two, but much smaller than the background obtained in the previously discussed study by the cyclooctynes, indicating that although copper-

catalyzed click chemistry is not the optimal reaction for its performance in living cells due to its Cu(I)-induced toxicity, it still is favorable compared to copper-free cycloaddition in terms of background labeling, at least in the case of proteasome two-step ABPP. This triple ligation strategy should be also tested under denaturing conditions where, click ligation is supposed to have a better performance since most of its background labeling is thought to be due to a side reaction between alkyne and reactive cysteine residues, which might be less reactive under denaturing conditions. If the tetrazine ligation is also successful under these conditions, the tandem ligation strategy may have broader applications, allowing researchers to decide at which moment to perform the ligations.

The low proteasome residual activity percentages and the low standard deviations obtained with ABP **12** shows the use of this probe and of ABPP for quantifications purposes, because although having a small fraction of proteasomes not labeled with the probe, this portion seems to remain constant. The little variations among the different proteasome subunits prove that ABP **12** has broad-spectrum activity towards the proteasome subunits. The large percentage differences found when exposing the probe to mouse cellular extracts compared to living cells suggest the appearance of a systematic error, which is supported by the low standard deviations (table 1). This is reinforced by the fact that the probe has the same optimal labeling conditions in both organisms and also by the high amino acid sequence similarity between the proteasome subunits, thus the values were expected to be similar as those found for human proteasome subunits, same as it is for the *in vivo* labeling. Taking a look at the free fractions found in human proteasomes, the values between *in vivo* and *in vitro* labelings do not differ much, although it seems like the *in vivo* labeling may be slightly more efficient. A plausible reason for this may be the capacity of the ABP to access the active-site threonine. Biological systems, or like in this case machineries, are tightly regulated and proteasomes are not an exception. Although it is known that the 20S core particle itself is already catalytically active with some known substrates, most of its substrates are dependent on the presence of regulatory particles like the 19S cap or the 11S. These regulatory particles trigger a rearrangement of the core particles subunits, which results in the opening of the alpha rings allowing the protein substrates to enter the catalytic core. The binding of these regulatory particles with the 20S proteasome is not very strong and the lysing and sample preparation procedures may be enough to disrupt it. Although the proteasome-directed ABPs are known to diffuse through the 20S particle to its inside to react with the active sites, the rearrangement of the 20S proteasome when regulatory particles are attached might induce holes or cavities permitting a faster diffusion of the probe. Another possible explanation would be that the probe gets degraded before it labels all proteasome subunits, although this option is less likely due to the ABPs high stability in aqueous

solutions. In both cases, pulse labeling should allow the ABP to efficiently reach and bind all different subunits decreasing in this manner the unlabeled proteasome pool.

Conclusion

The one-step ABP **1** and the two-step ABP **2** used by van der Linden and coworkers [12] are a good example of the versatility of ABPP, having in a single probe two different tags, a BODIPY for fluorescent read out, and a biotin moiety which can be used as enrichment tag or for visualization via Strep-HRP blotting. One-step ABPs are useful tools in proteasome enzymology research and may be also applied in the clinic, for example as fluorescent indicators of the proteasome activity in a specific tissue or sample but also of its cellular location using fluorescent microscopes. ABPP can be used to screen and compare proteasome activities in different tissues or organisms (see Chapter 4) but its applications can be extended to the study of the different roles proteasomes have in for example antigen presentation, or in cellular stress responses (see Chapter 5). ABPP could, in theory follow the proteasome half-life, by blocking first all proteasomes with one probe (or perhaps better yet just one subunit due to cellular toxicity) and posterior addition of a second probe which will only bind the non-occupied newly synthetized proteasomes. Being able to follow the localization and activity of different proteasomes in time until their destruction and recycling should in principle be achievable by means of proteasome ABPP.

Experimental procedures

Cell culture and probe treatment

Human cell line Amo1 (plasmacytoma) was grown in RPMI-1640 medium. The mouse cell line B3/25 (myeloma) was grown in IMDM medium. The entire medium was supplemented with 10% fetal calf serum and 0.1 mg/mL of streptomycin and penicillin. Both cell lines were grown at 37ºC with 5% CO_2 in a humid incubator. Both cell lines were purchased from ATCC.
ABP was dissolved in DMSO before use. 1000x stock solution of the desired end concentration of the ABP was added to the cell culture to have the DMSO concentration lower than 1% in the culture media. Treatments were done for different probe concentrations for 4 hours and for different amounts of time with 4 μM probe. Cells were washed couple of times with cold PBS, pelleted and stored at -80ºC until its usage.

Activity-based protein profiling

Cell pellets were lysed in 3 volumes of lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin and 0.2% NP40), kept on ice for 1 hour and further disrupted by 30 seconds sonication. After cold centrifugation at 13.000 g for 10 min, protein concentration was measured with the Qubit Protein Assay on the soluble fraction and kept at -80ºC until use. Equal amounts of protein were incubated with the desired concentrations of ABP for 1 hour at 37ºC, resolved by 12.5% SDS-PAGE and scanned with the ChemiDoc™ MP System with the Cy3 and Cy2 settings. When specified, prior resolution on gel some samples were loaded on a 30 kDa size-exclusion column. The procedure was carried on as suggested by manufacturer. Commassie blue staining was used as loading control. All gel images were analyzed by the Image Lab software (Bio-Rad).

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