Chapter 6

Design of a System for Monitoring Ubiquitination Activities of E2 Enzymes Using Engineered RING Finger Proteins

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Abstract

Ubiquitination is a sequential cascade consisting of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. It controls numerous processes such as protein degradation, DNA repair, and signal transduction pathways. E2 enzymes are associated with a variety of diseases such as leukemia, breast cancer, lung cancer, and colorectal cancer. To date, the monitoring of E2 activity for cancer diagnosis is challenging due to its intricate cascade reaction. To surmount this hurdle, we have recently developed a novel strategy for monitoring E2 activities. Here, we describe the concise machinery of ubiquitination with artificial RING finger proteins (ARFs) functioning as E3 enzymes. This machinery enables the simplified monitoring of E2 activities. Furthermore, our system combines a signal accumulation ion-sensitive field-effect transistor biosensor with ARFs, allowing for real-time monitoring of the pathological conditions of cancer cells. The present methodology may lead to novel diagnostic techniques for cancers.

Key words Ubiquitination, Cancer diagnosis, E2, E3, RING finger, ARF

1 Introduction

Protein ubiquitination is a posttranslational modification that controls a variety of cellular processes such as protein degradation, DNA repair, and signal transduction [1–3]. Ubiquitination is a cascade reaction consisting of three steps involving ubiquitin (Ub)-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3) enzymes [2, 4]. E3 transfers activated Ub from E2 enzymes to the substrate lysines [5]. There are three major classes of E3 ubiquitination ligases in eukaryotes known as RING E3, Ubox E3, and HECT E3. RING E3 possesses a RING finger and a substrate-binding domain (SBD) responsible for recognizing a particular substrate. RING fingers comprised approximately 50 amino acids and bind two zinc atoms with eight ligands of Cys and/or His in a unique cross-brace arrangement [6, 7]. E2 and E3 activities are associated with various cancers such as leukemia [8], breast cancer [9], lung cancer [10], and colorectal cancer [11]. For example,

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seven in absentia homolog 1 (SIAH1) belongs to RING E3 and cooperates with UbcH8 of E2. It induces the ubiquitination of the fusion proteins AML1-ETO [acute myeloid leukemia (*RUNX1*)-eight twenty-one (*MTG8*)] and PML-RARα (promyelocytic leukemia-retinoic acid receptor α), which is related to leukemogenesis [12–14].

Ubiquitinated fusion proteins in cells are recruited in the 26S proteasome degradation pathway. Thus, we believe that the detection of E2 activity should enable the monitoring of tumor development and the capturing of pathological conditions of cancers. E2 activities are estimated in terms of the amount of additional Ub that is transferred from E2 conjugates to substrates. However, the capturing of E2 activities for cancer diagnosis is challenging due to the intricate enzymatic cascade reaction of which E2 is a part. Similar to SIAH1, one of the E2s transfers Ub to several different types of substrates via its partner E3. It is difficult to evaluate E2 activity based on the amounts of additional Ub transferred to all these substrates. Furthermore, the intermediate complex of the E2-Ub conjugate and E3 may occur in the Ub-transferring system [15]. To overcome this hurdle, we have recently developed a novel strategy for monitoring E2 activities. In this chapter, we describe the molecular design of artificial E3 enzymes derived from RING and the resulting concise machinery of ubiquitination. This method allowed for the simplified detection of E2 activities, leading to the monitoring of pathological conditions of cancer cells following treatment with the anticancer drug bortezomib. Thus, the present methodology may open up new directions in cancer diagnostic techniques.

2 Materials

Prepare all samples with special grade chemicals and Milli-Q water (18 MΩ·cm). Use HPLC-grade chemicals for peptide purification. Prepare and store all samples at room temperature unless otherwise noted.

2.1 Synthesis and Purification of Peptide

1. Synthesis: Automated peptide synthesizer.
2. Purification: Reverse-phase HPLC equipped with a C18 column.

2.2 Refolding

1. Solution A: 20 mM Tris–HCl, pH 6.8, 50 mM NaCl, 1 mM dithiothreitol (DTT), and 50 μM $ZnCl_2$.
2. Dialysis cassette: Slide-A-Lyzer dialysis cassette.

2.3 Western Blotting

1. Ubiquitination buffer A: 20 mM Tris–HCl, pH 6.8, 5 mM Mg-ATP, 1 mM DTT, 20 U/mL inorganic pyrophosphatase (IPP), 50 μM $ZnCl_2$, 2.5 μM randomly biotinylated Ub, 0.1 μM

human recombinant E1 (His_6-tagged), and 2.5 μg human recombinant E2 (His_6-tagged) (E1, E2, and Ub available from Enzo Life Sciences, Farmingdale, NY, USA).

2. SDS sample buffer: 65.8 mM Tris–HCl, pH 6.8, 26.3% glycerol, 2.1% SDS, and 0.01% bromophenol blue.
3. Streptavidin-horseradish peroxidase solution: Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA).
4. Western blotting detection reagent for chemiluminescence: ECL.
5. Luminescent Image Analyzer.

2.4 E2 Activity Monitoring via Biosensor

1. Ubiquitination buffer B: 1.5 mM Tris–HCl, pH 6.8, 375 μM Mg-ATP, 75 μM DTT, 1.5 U/mL IPP, 4.0 μM $ZnCl_2$, 3.0 μM Ub, 9.0 nM human recombinant E1, and human recombinant E2.
2. Culture of NB4 cells: RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin under the conditions of 5% CO_2 at 37 °C.
3. Bortezomib (marketed as Velcade): Funakoshi Co., Ltd.
4. Biosensor: AMIS-101 Micro Bioactive Analyzer (Bio-X Inc., Kyoto, Japan).

3 Methods

3.1 Molecular Design of Artificial RING Fingers (ARFs)

Here we describe the protocol for creating ARFs as artificial E3 enzymes to conveniently measure E2 activities during ubiquitination [16, 17].

1. For engineering ARFs, obtain the amino acid sequences of the E3 RINGs of interest from a public database, e.g., Simple Modular Architecture Research Tool (SMART). As an example, Fig. 1a shows the selection of EL5 and SIAH1. These belong to the RING E3 and compose a RING finger and an SBD.
2. Extract the amino acid sequence of the active α-helical region (DMWLGSHST for EL5; PKLTC for SIAH1) (*see* **Note 1**).
3. When designing ARFs, the PHD finger of Williams–Beuren syndrome transcription factor (WSTF) is needed as the scaffold [18]. Prepare the amino acid sequence of the WSTF PHD finger as shown in Fig. 1b (*see* **Note 2**).
4. Delete the flexible long loop of the PHD finger, and instead transplant the α-helical region as the active site of the RING finger into the structure of the PHD finger (Fig. 1b). This is "α-helical region substitution" method [17] (*see* **Note 3**).

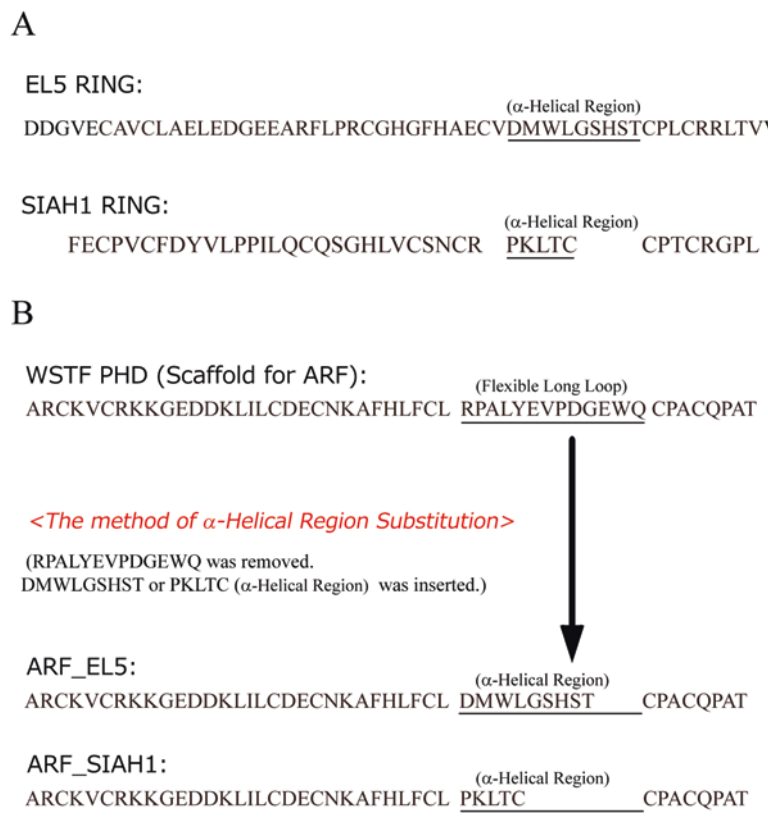

Fig. 1 Schematic for engineering artificial RING fingers (ARFs). (**a**) Amino acid sequences of the RING fingers of EL5 and SIAH1. The α-helical regions for controlling specific E2-binding capabilities are indicated with underlining. (**b**) The method of α-helical region substitution for engineering ARF. The α-helical regions of RINGs are transplanted into the sequence of WSTF PHD. ARF_EL5 and ARF_SIAH1 are designed based on EL5 and SIAH1, respectively. ARFs are chimeric molecules between two different domains

5. The insertion of the only active sites allows for the downsizing of E3s into the small molecule ARFs. For example, SIAH1 functions as a stable homodimer of 564 residues [19]. The molecular size of ARF_SIAH1 (approximately 50 mer) is less than one-tenth that of SIAH1 (Fig. 2).
6. Synthesize ARFs by the standard F-moc solid-phase method on an automated peptide synthesizer based on their amino acid sequences.
7. Purify using HPLC system after cleavage of ARF peptides by the standard method with trifluoroacetic acid.
8. Dissolve the ARF peptide in 1 mL of 8 M guanidine-HCl until it is completely denatured. Dialyze against solution A containing zinc ions overnight at 4 °C using a dialysis cassette,

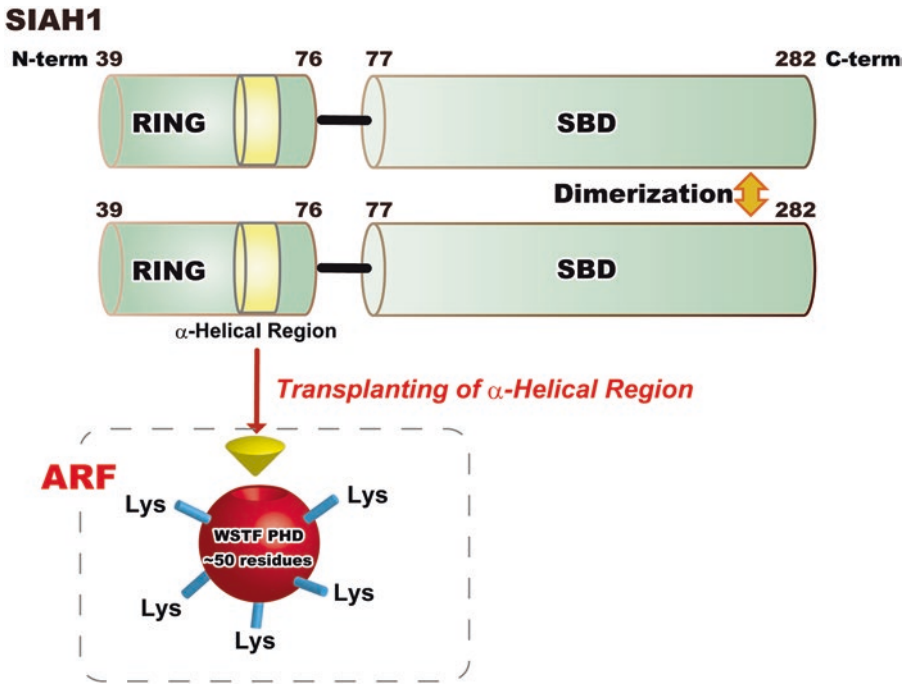

Fig. 2 The grafting design downsizes E3 into a small molecule ARF. SIAH1 has a RING and substrate-binding domain (SBD). It is a stable homodimer of 564 residues. ARF is created by transplanting the α-helical region into an approximately 50 mer peptide. Lys residues of ARF are possible acceptors for the ubiquitin transfer

according to the manufacturer's instructions. This procedure leads to the refolding of the ARF structure.

9. Confirm the formation of the ARF structure by experiments on the chemical modification of Cys residues and by analysis of the circular dichroism spectrum [16, 17] (*see* **Note 4**).

3.2 Unique Ubiquitination Machinery with ARF

ARF ubiquitinates itself without a substrate, although a substrate in traditional ubiquitination is inevitably needed for the Ub transfer. The PHD finger serves as a scaffold for ARF, and its Lys residues are possible acceptors for the Ub transfer (*see* **Note 5**) [20]. Figure 3a shows the novel ubiquitination reaction with ARF. ARF binds to the E2-Ub thioester conjugate and directly transfers activated Ub from E2 to itself. The engineered ARF has specific E2-binding capabilities and also assumes the role of substrate. Thus, ARF is an artificial multifunctional molecule with an auto-ubiquitination reaction. E2 activities can be measured based on ARF reactivity, which is equivalent to the amount of Ub transferred to the ARF.

The procedures to perform in vitro substrate-independent ubiquitination with ARF are as follows:

(Conduct all experiments at room temperature unless otherwise noted).

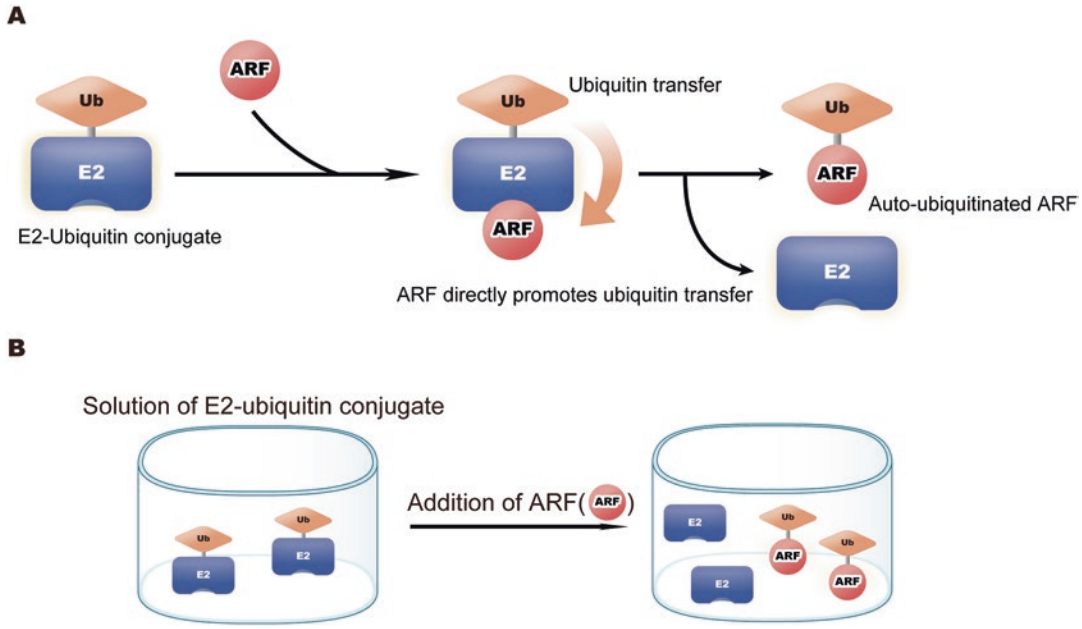

Fig. 3 Novel concise ubiquitination machinery with ARF. (**a**) ARF directly catalyzes ubiquitin (Ub) transfer into itself. ARF is a multifunctional molecule that possesses specific E2-binding and auto-ubiquitination abilities without a substrate. (**b**) Addition of ARF into the solution of E2–Ub conjugates leads to the accumulation of ARF–Ub conjugates and free thiol group (-SH) of E2s

3.2.1 Detection of E2 Activities Using Western Blotting

1. Prepare 45 μL of ubiquitination buffer A, consisting of E1, E2, ATP, and randomly biotinylated Ub. There are approximately 30 E2s in humans [21, 22]. Select the particular E2s of interest.
2. Add 5 μL of ARF (approximately 100 μM), obtained from the above procedure (Subheading 3.1), into 45 μL of ubiquitination buffer A, leading to the accumulation of ARF–Ub conjugates as shown in Fig. 3b. The development of poly- or mono-ubiquitination of ARF depends on the amino acid sequence of the α-helical region that is transplanted by the α-helical region substitution method. For example, ARF_EL5 and ARF_SIAH1 preferentially promote poly-ubiquitination and mono-ubiquitination, respectively. ARF_EL5 and ARF_SIAH1 exhibit similar specific E2-binding capabilities to those of original EL5 and SIAH1, respectively (*see* **Note 6**).
3. Gently mix ubiquitination buffer, and then incubate at 37 °C for 60 min.
4. Add nonreducing SDS sample buffer into the mixture to quench the ARF reaction.
5. Separate using 10–20% SDS-PAGE and transfer to PVDF membrane. Next, for the detection of biotinylated Ub, react the membrane with the streptavidin-horseradish peroxidase

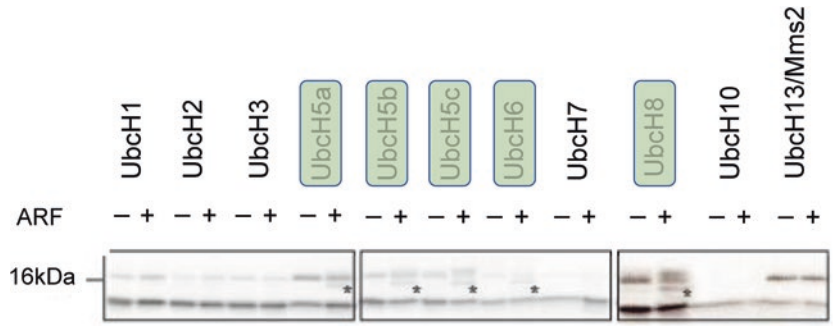

Fig. 4 Substrate-independent ubiquitination reaction with ARF. ARF_SIAH1 was mono-ubiquitinated with UbcH5a, UbcH5b, UbcH5c, UbcH6, or UbcH8, but not with UbcH1, UbcH2, UbcH3, UbcH7, UbcH10, or UbcH13/Mms2. The bands corresponding to the mono-ubiquitination products are identified with stars. Poly-ubiquitination of ARF_SIAH1 was not developed

solution, according to the manufacturer's instructions, and develop by enhanced chemiluminescence of the western blotting detection reagent. Detect the emitted signals on a Luminescent Image Analyzer.

6. As an example, the ubiquitination of ARF_SIAH1 is shown in Fig. 4. ARF_SIAH1 was mono-ubiquitinated in the presence of UbcH5a, UbcH5b, UbcH5c, UbcH6, or UbcH8, but not in the presence of UbcH1, UbcH2, UbcH3, UbcH7, UbcH10, or UbcH13/Mms2. Poly-ubiquitination of ARF_SIAH1 was not promoted if 11 E2s were incubated together. Thus, ARF_SIAH1 has specific E2-binding capabilities and ubiquitinates itself.

3.2.2 E2 Activities Captured Through Polarization Behavior of Electrons in a Semiconductor

A signal accumulation ion-sensitive field-effect transistor biosensor (AMIS sensor) enables the measurement of proton changes in solution [23]. This technique is based on the polarization behavior of electrons in a semiconductor. The system does not require labeling of fluorescent dye molecules, unlike the bioluminescence resonance energy transfer method. The ARF reaction promotes the transfer of Ub from E2–Ub conjugates on the AMIS sensor, yielding ARF–Ub conjugates [16] (Fig. 3b). The aminolytic cleavage of E2–Ub conjugates on the AMIS sensor produces free thiol groups (-SH) of the active-site cysteine of E2s [1], augmenting the free protons in solution. Accordingly, the addition of ARF induces a current that flows through gate channels in the AMIS sensor. This current, which is accumulated and amplified in the sensor, is detected as the AMIS signal, allowing for the high-sensitivity quantitative detection of E2 activities in real time.

1. Prepare ubiquitination buffer B consisting of E1, E2, ATP, and Ub.

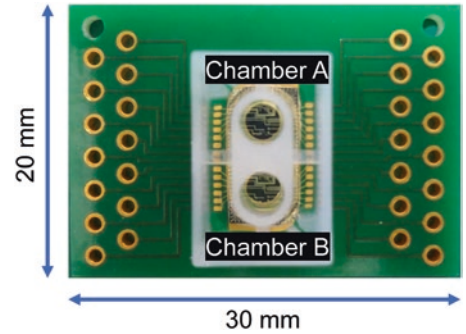

Fig. 5 Signal accumulation ion-sensitive field-effect transistor biosensor (AMIS sensor). The AMIS sensor is a 20 × 30 mm electronic board. It has a two-chambered device (chambers A and B). Each chamber adopts an ultralow volume of 20 μL on the sensor

2. Ubiquitination buffer B (16 μL) was introduced to both chambers A and B of a two-chambered device of the AMIS sensor on an AMIS-101 Analyzer (Fig. 5).
3. Allow the solutions to stand for 10 min to form E2–Ub conjugates.
4. Add 4 μL of solution A into chamber A, and add 4 μL of the 50 μM ARF solution (as obtained from Subheading 3.1) into chamber B. Gently mix the resulting 20 μL mixture in each chamber by pipetting.
5. Acquire the AMIS signals during ubiquitination reactions at 37 °C with an AMIS-101 Analyzer. Set the interval time for collecting real-time signals, typically at 5-s intervals.
6. Obtain the AMIS data for E2 activities by subtracting the AMIS signals of chamber A from those of chamber B (*see* **Note 7**).
7. As an example, Fig. 6 shows the real-time detection of AMIS signals at 37 °C recorded for 600 s at 5-s intervals. ARF_SIAH1 (*see* above Subheading 3.1) was used in the ubiquitination reaction. After the peak of the injection shock, the AMIS signals from E2 UbcH8 activities were detected from approximately 150 s, reaching a plateau at approximately 300 s (*see* **Note 8**). Maintain the device of the AMIS sensor at a constant temperature.

3.2.3 Real-Time Monitoring of E2 Activities Using Cancer Cells with an AMIS Sensor

Bortezomib is a highly selective proteasome inhibitor and induces cell death in various cancers, such as leukemia, multiple myeloma, and breast cancer [24–26]. Treatment with bortezomib reduces cell viability of human acute promyelocytic leukemia-derived NB4 cells, wherein the UbcH8-Ub conjugate leaks out of NB4 cells into the culture supernatant, but the UbcH5 and UbcH6 conjugates do not [8]. The diapedesis of UbcH8-Ub conjugates into the

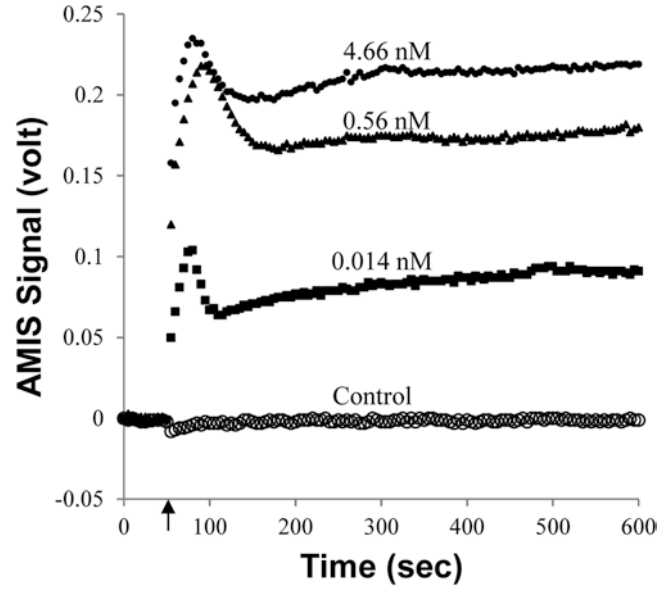

Fig. 6 Real-time monitoring of E2 activities on an AMIS sensor. Addition of ARF_SIAH1 into a solution of E2 UbcH8 causes proton changes, leading to the altering of the electron polarization for the AMIS signals in the sensing layer. The AMIS signals were continuously measured as E2 UbcH8 activities. The representative AMIS signals for 0.014 (filled square), 0.56 (filled triangle), and 4.66 (filled circle) nM E2 UbcH8 were acquired at 5-s intervals, and the AMIS signals in the absence of ubiquitin were used as the control (circle). The arrow indicates the injection time of ARF_SIAH1 on an AMIS sensor

culture supernatant increases in a concentration-dependent manner with drug treatment. The approach described here, combining an AMIS system with the ARF reaction, can be deployed to measure the E2 activity of UbcH8 in crude extracts, such as the culture supernatant of cells [23].

1. Prepare a 1.0 mM stock solution of bortezomib dissolved in DMSO and store at −20 °C.
2. Culture NB4 cells with RPMI 1640 medium [23].
3. After 3 h of growth in FBS-free RPMI-1640 medium, treat NB4 cells (1×10^5 cells/mL) with 0, 10, and 100 nM bortezomib for 48 h at 37 °C.
4. Collect the supernatant of NB4 cells for the following ubiquitination experiments, after centrifugation at $300 \times g$ for 300 s.
5. The supernatant obtained should be diluted fivefold with water (*see* **Note 9**).
6. Inject the diluted samples (16 μL) into a two-chambered device of the AMIS sensor (Fig. 5).

7. Prepare ARF_SIAH1 (*see* above Subheading 3.1) for the ubiquitination reaction.
8. Add 4 μL of solution A into chamber A and 4 μL of the 50 μM ARF solution into chamber B. Gently mix the mixtures (each chamber should contain 20 μL total of its combined solution) by pipetting.
9. Acquire the AMIS signals during the ubiquitination reactions at 37 °C on an AMIS-101 Analyzer.
10. Obtain the AMIS data for the E2 activity of UbcH8 by subtracting the AMIS signals of chamber A from those of chamber B (*see* **Notes 7** and **10**) [23].

Using the Ub machinery with ARF, the rapid and accurate monitoring of E2 activities was conveniently achieved within several minutes. Leukemia is diagnosed by the existence of approximately 2×10^5 cells/mL of blood [27]. With the technique described here, E2 activities leaked out of NB4 cells (cultured NB4 cells, 1×10^5 cells/mL) were successfully detected in the culture supernatant. Thus, the present approach could be applied to the detection of E2 activities in leukemia patients and may prove a convenient method for arranging suitable treatment programs with patients. Different E2 activities are associated with various cancers, such as lung cancer (UbcH10) and breast cancer (UbcH13) [28, 29]. Therefore, the ARF method presented here could be widely applicable to the measuring of E2 activities related to these cancers. We hope that this monitoring system will be used to evaluate responses to treatment with a proteasome inhibitor, as a new companion diagnostic approach.

4 Notes

1. RING fingers have the essential active site of the α-helical region corresponding to the amino acid sequence between the sixth and seventh zinc-binding residues [30–33]. The helical region controls the specific E2–E3 binding capabilities [34].
2. Like the RING finger, the PHD finger adopts a cross-braced zinc-binding arrangement. However, the PHD finger has a randomized flexible long loop rather than the α-helical region. Accordingly, the PHD finger obviously does not have the E2-binding site, and thus it is not associated with the ubiquitination system [35, 36].
3. The substituting region confers specific E2-binding capabilities on the PHD finger. ARF is created as a RING-PHD chimeric molecule. ARF_EL5 and ARF_SIAH1 are designed from EL5 and SIAH1, respectively.

4. The cysteine modification of ARF is performed through treatment with *p*-(hydroxymercuri) benzoic acid, and the released zinc ions are quantified using the metallochromic indicator 4-(2-pyridylazo) resorcinol. The molar ratio of [Zn]/[ARF] should be approximately 2.0. The typical CD spectrum of ARF indicates an ordered secondary structure including the α-helical conformation with double minima occurring at approximately 205 (π-π* transitions) and 225 nm (n-π* transitions) [17].
5. The PHD finger specifically recognizes histone H3 methylated at Lys4 and controls transcriptional regulation in cells [37, 38]. The PHD finger is characterized as a positively charged Lys-rich motif in the nucleus [18].
6. Original EL5 develops poly-ubiquitination on the substrate with UbcH5a but not with UbcH7/8 [31]. Original SIAH1 promotes mono-ubiquitination with UbcH5 or UbcH8 [14, 39].
7. In AMIS measurements, the effects of protons, except for the reaction between ARF and E2s, are completely canceled out by subtracting the AMIS signals of chamber A from those of chamber B.
8. The AMIS signals of E2 activities adopt concentration-dependent behavior and indicate a linear relationship between intensity (volts) and logarithm of concentration of E2s over a wide response range of femtomolar to micromolar concentrations [16].
9. To reduce the viscosity of the sample, it should be diluted with water prior to AMIS measurements.
10. The monitoring system indicated 8.9 pg/100 μL and 30.4 pg/100 μL of UbcH8 activity in the culture supernatant of NB4 cells (1×10^5 cells/mL) following treatment with 10 nM and 100 nM bortezomib, respectively [23].

Acknowledgments

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Author contributions
K.M. designed this study. K.M. and K.S. wrote the main manuscript text and prepared all the figures.

Competing interests
The authors declare no competing financial interests.

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