

UbMES and UbFluor: Novel Probes For RBR E3 Ubiquitin Ligase PARKIN

Sungjin Park¹, Peter K. Foote², David T. Krist², Sarah E. Rice¹, Alexander V. Statsyuk,^{2,3}

¹Department of Cell and Molecular Biology, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611, USA; ²Chemistry of Life Processes Institute, Department of Chemistry, Northwestern University, Silverman Hall, 2145 Sheridan Road, Evanston, Illinois 60208, United States;

³Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 3455 Cullen Blvd., Houston, TX 77204-5037.

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Abstract

Ring-Between-Ring (RBR) E3 ligases have been implicated in autoimmune disorders and neurodegenerative diseases. The functions of many RBR E3s are poorly defined and their regulation is complex, involving posttranslational modifications and allosteric regulation with other protein partners. The functional complexity of RBRs, coupled with the complexity of the native ubiquitination reaction that requires ATP, E1, and E2 enzymes, makes it difficult to study these ligases for basic research and therapeutic purposes. To address this challenge, we developed novel chemical probes, ubiquitin C-terminal fluorescein thioesters UbMES and UbFluor, to qualitatively and quantitatively assess the activity of the RBR E3 ligase PARKIN in a simple experimental set up and in real-time using fluorescence polarization. We first confirmed that PARKIN does not require an E2 enzyme for substrate ubiquitination,

lysine selection, and polyubiquitin chain formation. Next, we confirmed that UbFluor quantitatively detects naturally-occurring activation states of PARKIN caused by Ser⁶⁵-phosphorylation (pPARKIN) and phosphorylated ubiquitin (pUb). Secondly, we showed that both pUb and the ubiquitin-accepting substrate contribute to maximal pPARKIN ubiquitin conjugation turnover. pUb enhances transthiolation step, while the substrate clears the pPARKIN~Ub thioester intermediate. Lastly, we established that UbFluor can quantify activation or inhibition of PARKIN by structural mutations. These results demonstrate the feasibility of using UbFluor for quantitative studies of the biochemistry of RBR E3s and for high-throughput screening of small molecule activators or inhibitors of PARKIN and other RBR E3 ligases.

Introduction

Parkinson's disease (PD) is the second most common type of neurodegenerative disease, and is characterized by defects in mitochondrial function. Ubiquitin ligase PARKIN and PTEN-induced putative Ser/Thr kinase 1 (PINK1) reconstitute a major pathway to eliminate dysfunctional mitochondria and are thought to protect dopaminergic neurons from oxidative stress (1). PINK1 acts upstream of PARKIN and is continuously imported to the mitochondria where it is rapidly degraded. Upon mitochondrial damage PINK1 degradation is inhibited, and PINK1 accumulates at the mitochondrial outer membrane (MOM) (2-4). There PINK1 promotes the recruitment of cytoplasmic PARKIN and activation of its ubiquitin ligase activity (4). Active PARKIN causes extensive polyubiquitination of MOM proteins and triggers mitophagy (5). PARKIN ubiquitination is antagonized by deubiquitinases (DUBs), such that a threshold amount of ubiquitination in a given time may be necessary to successfully signal mitophagy (6).

The mechanism of PARKIN activation is complex, but it is known that phosphorylation of Ser⁶⁵ in PARKIN Ubl domain and Ser⁶⁵ in mono- and/or poly ubiquitin chains by PINK1 promote the mitochondrial translocation and activity of PARKIN which in turn stimulate mitophagy (7-22). Dozens of mutations in both PINK1 and PARKIN have been found in patients with autosomal-recessive juvenile Parkinsonism (AR-JP) and have been extensively characterized *in vitro* and in cells (1). In the case of PARKIN these mutations are characterized by diverse translocation and biochemical phenotypes (1,23-33). It is currently thought that pharmacological activators of PINK1 and/or PARKIN may provide potential therapy to treat Parkinson's disease. For example it

was shown that overexpression of PARKIN in rat and mouse PD models is neuroprotective, supporting this hypothesis (34-37). However, while pharmacological activators of PINK1 are known (38), activators of ubiquitin ligase PARKIN are not known in the literature, and attempts to develop them were not successful (39). To develop such probes, it is essential to understand Parkin enzymatic mechanisms.

PARKIN is a Ring-Between-Ring (RBR) E3 ligase, which forms an obligatory RBR E3~Ub thioester intermediate prior to the transfer of the ubiquitin onto the acceptor lysine (40-43). PARKIN is composed of six distinct domains: an N-terminal ubiquitin-like domain (Ubl), a unique PARKIN-specific domain (UPD, also referred to as RING0), RING1, in-between-RING (IBR), repressor element (REP), and catalytic RING2 domains (Figure S1E) (43-45). RING1 domain of PARKIN is thought to recruit E2~Ub thioester (46), while the RING2 domain of PARKIN harbours the catalytic cysteine (40). Mutation of this catalytic cysteine (C431F) is found in some Parkinson disease patients (47). Recently it was shown RING2 domain of RBR E3 HHARI also harbours a weak Ub binding site important for E2~Ub/HHARI transthioylation (48), while RING1-IBR-Ubl domains of PARKIN form another ubiquitin binding surface important for PARKIN activity.

A shared feature of PARKIN and several other RBR ligases is autoinhibition, which is relieved by binding partners and/or by phosphorylation (49-53). In autoinhibited PARKIN, the E2~Ub binding site on RING1 is blocked by the REP domain, while the catalytic cysteine on RING2 is blocked by the UPD domain (Figure S1E) (46,54,55). Phosphorylation of PARKIN (pPARKIN) and pUb binding to PARKIN/pPARKIN disrupt these autoinhibitory conformations and

activate PARKIN. Interestingly, while the activation roles of PINK1 and pUb have been investigated, the role of protein substrates in PARKIN catalytic turnover have escaped attention. PARKIN substrates can provide acceptor lysines that clear PARKIN~Ub thioester, thus facilitating PARKIN enzyme turnover (56).

Most importantly, there is a lack of methods to a robust and simple quantifying of PARKIN and other RBR E3s activity which is needed for both biochemical studies and small molecule screens. Typical *in vitro* ubiquitination assays use a reconstituted native cascade composed of at least 5 components: E1, E2, E3, ubiquitin, ATP, and a substrate, if the one is used. In this setting, the assay is operationally complex, expensive, and there are many enzyme intermediates that makes it difficult to conduct biochemical studies. Coupled with the complex autoregulatory mechanisms that govern the function of PARKIN and other RBR E3s, the complexity of native ubiquitination assays is a major bottleneck in assessing the activity of RBR E3 enzymes.

Recently developed electrophilic activity-based probes such as UbVME (55) and electrophilic E2~Ub thioester mimics (57) substantially reduce the complexity of PARKIN and other catalytic cysteine containing E3 ligase assays. However, these probes are stoichiometric suicide inhibitors, and therefore do not report on catalytic turnover, lack high throughput capabilities, and rely on western blotting for quantitation. Absolute Quantification Mass Spectrometry (AQUA-MS) enables quantification of polyubiquitin chain formation by PARKIN over multiple rounds of Ub conjugation *in vitro*.(8) While this method offers exquisite sensitivity, it is not amenable to high-throughput screening, and it requires considerable expertise and

instrumentation beyond typical laboratory operations.

To begin addressing these challenges, we previously demonstrated that a ubiquitin C-terminal thioester probe (ubiquitin mercaptoethanesulfonate, UbMES) and its fluorescent analogue UbFluor could bypass the need for E1, ATP, and E2 enzymes, thereby simplifying assessment of HECT E3 ligase activity *in vitro* (Figure 1) (58). Since the resulting system bypasses the need for ATP, E1, and E2 enzymes, we called it bypassing system or ByS. We reasoned that the same system could be useful for RBRs, as RBR ligases also form an obligate E3~Ub thioester prior to ligation. However, two unique features of RBR E3 ligases require consideration. First, unlike HECT ligases, RBRs are cysteine-rich. For example, human PARKIN has 20 surface-exposed cysteines that could potentially undergo non-specific transthioylation with Ub-MES (46). Secondly, PARKIN and other RBR ligases have complex, multistep activation mechanisms, and it was not clear at the outset of this work whether Ub-MES and its analogues could recapitulate these mechanisms. For example, it was not clear if UbMES that lacks E2 enzyme, could sense activating mutations that disrupt REP:RING1 interface and open the E2 enzyme binding site.

In this work, we report that E2-independent ByS probes can be directly and specifically charged to the catalytic cysteine of PARKIN or pPARKIN, resulting in native-like autoubiquitination, substrate ubiquitination, and polyUb chain formation.

We further adapted the E2-independent ByS to create UbFluor, a *quantitative* probe for assessment of PARKIN activity *in vitro* and for high-throughput screening to identify PARKIN activator compounds (Figure 1, Scheme 1). UbFluor is a fluorescent thioester that

features a fluorescein thiol that is attached to the C-terminus of the ubiquitin via a thioester bond. Effectively, UbFluor mimics an E2~Ub thioester, except E2 enzyme is replaced with fluorescein. When UbFluor is charged to PARKIN's catalytic cysteine, it releases fluorescein thiol much like Ub~E2 releases E2 enzyme upon transthioylation. The fluorescein release can be detected by fluorescence polarization (FP). Thus, fluorescence-based detection is built-in to the transthioylation reaction, enabling real-time monitoring of PARKIN catalytic turnover, while eliminating any need for extra reagent transfer steps or SDS-PAGE analysis. UbFluor can be used to analyze transthioylation under single turnover reaction conditions (excess PARKIN), similar to electrophilic Ub-VS/E2-Ub probes or an E2~Ub discharge assay, or under multiple turnover reaction conditions (excess UbFluor) similar to the native ubiquitination assays. Using UbFluor, we verified PARKIN activation by Ser⁶⁵ phosphorylation and by pUb binding, demonstrating that the ByS is sensitive to the native autoregulation mechanisms governing PARKIN activity. We also discovered that the PARKIN substrate Miro1 and pUb cooperatively increase the ubiquitin conjugation rate of pPARKIN. Thus, while most work in the area has focused on the roles of PINK1 and pUb on parkin activation, our results suggest that PARKIN substrates such as MIRO can also enhance PARKIN catalytic turnover.

Lastly, we demonstrated that UbFluor can detect unnatural activation or inactivation of PARKIN by point mutations that disrupt either the REP:RING1 domain or the UPD:RING2 domain interface, or activation of PARKIN that lacks Ubl and UPD domains all together, further validating the biochemical relevance of the UbFluor probe toward PARKIN mechanisms. These results validate

UbFluor as a probe to study the biochemistry of PARKIN and other RBR E3 ligases and as a high-throughput screening tool to identify small molecules that target these enzymes.

Results

A chemically activated ubiquitin thioester recapitulates PARKIN function in a simplified two-component reaction.

We first examined how well the E2-independent ByS reaction recapitulates PARKIN activity in the native cascade. To test if the UbMES-based ByS recapitulates Ser⁶⁵ phosphorylation-dependent activation, we prepared human PARKIN that was phosphorylated by *Tribolium castaneum* PINK1 (TcPINK1) on Ser⁶⁵ (pPARKIN, Figure S1A and B) (8) and ubiquitin mercaptoethanesulfonate thioester (UbMES) (59) as previously described (Figure S1C). Miro1 (Mitochondrial Rho 1) is a calcium-sensitive regulator of mitochondrial dynamics on the outer mitochondrial membrane.(60) Miro1 is a known substrate that is modified by pPARKIN both in cells and in *in vitro* assays.(5,14,61) We prepared full-length human Miro1 (hMiro1) as well as an N-terminally truncated construct (MiroS) that is expressed more robustly and can be purified in larger amounts than hMiro1 (MiroS; hMiro1 residues 177-592 with C-terminal 6-His tag, 51.6 kDa, Figure S1D).(62)

We examined pPARKIN autoubiquitination, MiroS substrate ubiquitination, and polyubiquitin chain formation using UbMES (Figure 2). In the presence of UbMES, pPARKIN ubiquitinated both itself and MiroS (Figure 2A lanes 4 and 6, respectively). In spite of having ~28 surface lysines (Figure S1D),

MiroS was not modified by UbMES in the absence of pPARKIN (Figure 2A, Lane 5). To show that the observed ubiquitination requires UbMES thioester functionality, we conducted similar experiments with hydrolysed UbMES (ubiquitin) indicated as Hyd. (Figure 2A). Importantly, both pPARKIN autoubiquitination and MiroS substrate ubiquitination by UbMES were strictly dependent on the catalytic cysteine (Cys⁴³¹) of pPARKIN. Therefore, despite the fact that PARKIN is extremely cysteine-rich, UbMES is selective for the catalytic site cysteine. (Figure 2A, Lane 7).(46,62)

Next, we demonstrated that PARKIN ubiquitination using the ByS is still subject to its native phosphoregulation mechanism (Figure 2B). While both autoubiquitination and MiroS substrate ubiquitination by wild-type PARKIN were strongly activated by TcPINK1 phosphorylation (pPARKIN), mutation of Ser⁶⁵ to alanine (PARKIN^{S65A}) abolished this activation. Both nonphosphorylated wild-type PARKIN and PARKIN^{S65A} treated with TcPINK1 showed only a minimal amount of MiroS ubiquitination with UbMES (Figure 2B, lanes 1-2 and 5-6, respectively). Mutation of the catalytic cysteine Cys⁴³¹ to alanine also abolished PARKIN activity even after TcPINK1 phosphorylation (pPARKIN^{C431A}, Figure 2B, lanes 7-8). pPARKIN ubiquitination of MiroS increased with increasing concentrations of UbMES, but without pPARKIN, MiroS-Ub adduct formation was negligible (Figure 2C). Only after incubating for a 3-fold longer reaction time (120 min) at 80-120 μ M UbMES, a faint band corresponding to the molecular weight of MiroS-Ub adduct was observed (Lane 12-14, Figure 2C). This result shows that our standard reaction conditions (30 μ M UbMES and 40 min reaction time at room temperature) do not cause nonspecific modification of PARKIN, ubiquitin, or the MiroS substrate.

We next examined ubiquitin chain formation by pPARKIN under the ByS reaction. Previous work demonstrated that pPARKIN can form Lys⁴⁸-, Lys⁶³-, Lys⁶-, and Lys¹¹-linked chains *in vitro*.(8) We compared PARKIN ubiquitination using UbMES, Ub(K48R)MES, and Ub(K63R)MES in the ByS reaction. For all of these Ub variants, similar amounts of ubiquitin adducts are formed (Figure 2D). Antibodies against either Lys⁴⁸- or Lys⁶³-linkages detected the corresponding chains, which were absent when Ub(K48R)-MES or Ub(63R)-MES, respectively, were used. MALDI analysis confirmed the presence of both K63- and K48-linkages, similar to the native cascade reaction (Figure 2E). Importantly, this experiment directly shows that the E2 enzyme is dispensable for the formation of polyubiquitin chains by PARKIN under the ByS, consistent with our previous report. (63)

We next compared the sites within full-length hMiro1 that are ubiquitinated by pPARKIN either under ByS or native reaction conditions, using tandem mass spectrometry analysis. We limited hMiro1 modification to ~2-3 ubiquitin molecules in both the ByS and in the native cascade, and found that Lys¹⁵³, Lys³³⁰, and Lys⁵⁷² within hMiro1 were modified in both reactions (Figure S2). These lysines have been previously reported as ubiquitination sites within hMiro1.(5,6,14,64) Because the ByS reaction recapitulates the activity of pPARKIN towards target lysine residues within hMiro1, we can conclude that pPARKIN alone can select target substrate lysines without the help of an E2 enzyme *in vitro*. Testing a larger panel of different substrates could further confirm this finding. Taken together, our results suggest that the E2-independent ByS reaction recapitulates several aspects of pPARKIN's native function: ubiquitin ligation, polyubiquitin chain formation, polyubiquitin chain type

specificity, and selection of substrate lysines.

Lastly, we showed that the reaction conditions of the ByS can be adjusted to approach the timescale of the native ubiquitination reaction, such that similar amounts of the reaction products are formed on a similar timescale (Figure 2F). For these experiments, we used the same amount of ubiquitin species, PARKIN enzyme, and MiroS substrate in each reaction. The native cascade reaction contained 100 nM UBE1, 1 μ M UbCH7, 1 μ M pPARKIN, 30 μ M Ub, 4 mM ATP, and 1 μ M MiroS. The ByS reaction contained 1 μ M PARKIN, 30 μ M UbMES, and 1 μ M MiroS. The similar timescales of MiroS ubiquitination in the ByS using 30 μ M UbMES and in a native cascade reaction with a maximum of 1 μ M UbCH7~Ub certainly do not indicate that UbMES is as good a PARKIN substrate as UbCH7~Ub. However, this result demonstrates that ByS reactions proceed on a very reasonable timescale, providing an experimentally feasible and simplified ubiquitination reaction. Furthermore, the developed ByS system can be used to prepare site-specific mono- or diubiquitinated PARKIN substrates using PARKIN and UbMES (Figure 2F lane 3). This could allow to bypass challenges using regular protein synthesis approaches. Slower timescale of the ByS system and its simplicity (i.e. just two components E3 and UbMES) make it easier to adjust the stoichiometry of reagents.

UbFluor enables real-time monitoring of PARKIN activity by FP.

Encouraged that UbMES specifically recognized the PARKIN catalytic cysteine and recapitulated several essential aspects of PARKIN activity, we designed and

prepared UbFluor, a probe that enables quantitative analysis of PARKIN activity using the ByS. UbFluor preparation is described in the methods section and in previously published procedures (Scheme 1, Figure S3) (65). When UbFluor is charged to PARKIN's catalytic cysteine, it releases fluorescein thiol and this release can be monitored in real time using FP (Fluor-SH, Figure 3A).

First, we tested whether UbFluor releases free Fluor-SH in a pPARKIN-dependent manner. When UbFluor (0.5 μ M) was mixed with beta-mercaptoethanol (BME; Figure 3B, lane 2), cysteamine (Figure S4, lane 2) or pPARKIN (5 μ M, Figure 3B, lane 4), the fluorescent band corresponding to UbFluor (~9 kDa) diminished in intensity while Fluor-SH intensity increased, in a time-dependent manner (Figure 3C, Figure S5). pPARKIN harbouring a C431A mutation failed to release fluorescein from UbFluor after 60 minutes, demonstrating that the release of Fluor-SH from UbFluor requires the catalytic cysteine of PARKIN (Figure 3B, lane 6).

Next, we tested whether the consumption of UbFluor can be measured in real-time using FP (Figure 3D-E). As expected, increasing the amount of pPARKIN in the UbFluor reaction accelerated the rate of FP decrease under excess enzyme conditions (Figure 3D-E). Early time point data (1 - 5 min) were fitted to obtain initial rates of FP decrease at an effectively constant concentration of UbFluor (Figure S6). The initial rate of UbFluor consumption increased linearly with pPARKIN concentration, as expected for a bimolecular reaction. Since UbFluor lacks E2 enzyme we high K_m values can be expected, similar to HECT E3s for which K_m values were ~800 μ M (65). These data demonstrate that UbFluor provides a robust

FP-based assay for real-time monitoring of PARKIN activity.

UbFluor confirms the native activation mechanism of PARKIN by Ser⁶⁵-phosphorylation and pUb.

Changing the ratio of UbFluor to E3 ligase in ByS reactions enables examination of different aspects of the E3 ligase mechanism. In a single turnover (ST) reaction with excess PARKIN (10 fold excess of PARKIN over UbFluor), **the rate of FP decay only reflects the rate of transthiolation reaction between UbFluor and PARKIN.** However, under multiple turnover (MT) conditions (10 fold excess of UbFluor over PARKIN), the UbFluor consumption rate (FP decay) should reflect both transthiolation and isopeptide ligation rates. Under normal MT conditions one molecule of PARKIN can consume >1 molecule of UbFluor. However, if there is a defect in the isopeptide ligation step, PARKIN can only consume one molecule of UbFluor, because the resulting inactive PARKIN~Ub thioester will not be able to react with the second molecule of UbFluor.

We used both ST and MT reaction conditions to examine PARKIN activation by Ser⁶⁵ phosphorylation and by pUb to test whether the UbFluor assay can detect these known PARKIN activation mechanisms, similar to the native reaction. We prepared pUb according to previously established methods (Figure S7).(64) ST experiments were performed at 5 μ M PARKIN and 0.5 μ M UbFluor, while MT experiments were performed at 2 μ M PARKIN and 20 μ M UbFluor. To measure initial velocities during which <20% of available UbFluor was consumed, ST reactions were carried out for 5 minutes, and MT reactions were carried out for 15 minutes.

We titrated 0-25 μ M pUb into ST (Figure 4A) and MT (Figure 4B) UbFluor reactions using both PARKIN and pPARKIN. Titrations are written as PARKIN/pUb or pPARKIN/pUb, with the titrated reaction component after the slash, throughout this work. Without PARKIN or pPARKIN present, the small amount of background UbFluor consumption is not affected by pUb concentrations up to 25 μ M (No Enz/pUb, Figure 4A and B). However, addition of pUb increases the UbFluor consumption rate of both PARKIN and pPARKIN under both ST and MT conditions (Figure 4A and B). The pUb-dependent increase in UbFluor consumption by PARKIN under ST conditions was small but significant (~1.5 fold increase) (Figure S8A-C). This slight activation of PARKIN by pUb was not previously detected using Ub-VS.(64) As expected based on previous results, the pUb-dependent increase in UbFluor consumption by pPARKIN was much larger (~7 fold for ST conditions) (pPARKIN/pUb, Figure 4A and B).(7,64) Non-phosphorylated ubiquitin did not increase UbFluor consumption by pPARKIN (pPARKIN/Ub, Figure 4A and B), emphasizing the importance of Ser⁶⁵ phosphorylation of ubiquitin for activating PARKIN. Together, these experiments confirm that the UbFluor assay can detect the natural Ser⁶⁵ phosphorylation and pUb-dependent activation mechanisms of PARKIN.

pUb and substrate enhance pPARKIN catalytic turnover rate by different mechanisms.

Next, we examined how the inclusion of substrate affects the catalytic turnover rate of pPARKIN in the presence of a saturating concentration of pUb (pPARKIN+pUb; 25 μ M pUb in our assays), which is the maximally activated state of

PARKIN in the native reaction that has been previously described.(7) We titrated increasing concentrations of MiroS into pPARKIN+ pUb. While 15 μ M MiroS did not increase the ST rate of pPARKIN+pUb, it did increase the MT rate 2.6 fold (Figure 4 C and D, 4G). In contrast to MiroS addition, adding an additional 15 μ M pUb to a total concentration of 40 μ M did not further increase either the ST or the MT rate of UbFluor turnover by pPARKIN (Figure S9). The simplest interpretation of these results is that the presence of the MiroS substrate does not significantly increase the transthiolation rate but does enhance the ligation rate of pPARKIN+pUb. To determine whether the MiroS-dependent increase in pPARKIN+pUb activity under MT conditions that we observed could be due to lysine-dependent clearance of the Ub thioester, we titrated free lysine into the UbFluor reaction with pPARKIN+pUb in place of MiroS. We note that UbFluor is stable at 100 mM free lysine (Figure 4E and F and Figure S10). Similar to MiroS, addition of free lysine increased the MT rate of pPARKIN+pUb (~2 fold), but not the ST rate (Figure 4E and F). Based on these results it is reasonable to conclude that the MiroS-dependent increase in the MT rate may be due to the increased ligation rate that facilitates the clearance of pPARKIN~Ub thioester, regenerating the free enzyme able to consume another equivalent of UbFluor. Furthermore, the increase in UbFluor consumption of pPARKIN+pUb upon lysine or MiroS titration suggests that pUb may be a poor substrate for the isopeptide ligation step compared to MiroS or lysine.

We further confirmed that pUb activates transthiolation of pPARKIN but is a poor substrate for ligation using the native reaction with fluorescent ubiquitin (FUb). Fluorescent ubiquitin (FUb) is different from UbFluor, as it features a ubiquitin that has a

permanent fluorophore at its N-terminus. This fluorophore is for gel imaging purposes only, and is not transferred or released from FUb at any point. FUb can be activated by E1, and then be transferred onto E2 and then E3 enzyme. We evaluated the effects of pUb on both E2-discharge (ST) and total ubiquitination (MT) activity of pPARKIN (Figure S11). In an E2-discharge assay, the E2~FUb thioester adduct (UbchH7~FUb in our assays) is separately prepared and chased by excess pPARKIN with the indicated amount of pUb, MiroS, or both (Figure S11A). The appearance of a fluorescent band at ~55 kDa (pPARKIN~FUb and Miro-FUb, Figure S11B) and the reduction in the 25 kDa UbchH7~Ub band (Figure S11C), corresponded to transthiolation activity. The total ubiquitination activity of pPARKIN under native cascade conditions was measured after adding pUb, MiroS, or both (Figure S11D). The appearance of fluorescent bands above 10 kDa (Figure S11E) and the reduction in the FUb band at 10 kDa (Figure S11F) were quantified to reveal total Ub turnover. We confirmed that pUb enhances the transthiolation of pPARKIN at least ~4 fold, while MiroS has no effect under these end point assay conditions (Figure S11A, lane 3 and 4). In contrast, we observed that the total ubiquitin consumption for pPARKIN+pUb was ~2 fold lower than pPARKIN+MiroS (Figure S11D, lane 3 and 4). These results indicate that pUb is a strong allosteric activator of pPARKIN transthiolation, but is not a good ubiquitin acceptor compared to MiroS.

We were unable to conclusively determine whether or not MiroS addition further enhanced the pPARKIN+pUb transthiolation rate in the native reaction, because transthiolation reactions using pPARKIN+pUb were too fast to quantify in the discharge assay (Figure S11A, lanes 3

and 5). Similar to the UbFluor MT reaction, pPARKIN shows the highest ubiquitin conjugation efficiency when both pUb and MiroS are present in a native cascade assay (Figure S11D, lane 5).

Together, all of these data suggest that pUb activates pPARKIN by increasing its transthiolation rate in both the E2-independent UbFluor reaction and in the native cascade. However, substrate is required for maximal pPARKIN ubiquitination turnover even in the presence of pUb, because pUb is a poor Ub acceptor for pPARKIN. These results also confirm that UbFluor ST and MT assays can conveniently reveal different aspects of PARKIN ubiquitination mechanisms: ST assays detect transthiolation while MT assays detect both transthiolation and ligation. Furthermore, our results reveal a similar trend between the UbFluor assay and the native reaction under both ST and MT conditions.

Comparison of UbFluor quantitation of PARKIN activity with previous measurements using the native reaction.

Figure 4G compares the maximal rates of UbFluor consumption that we observed in this work to previous AQUA-MS based measurements, which have provided a quantitative measure of PARKIN activity using the native reaction.^(7,64) pPARKIN+pUb gave the highest PARKIN activity in these experiments, therefore, we set the activity of pPARKIN+pUb as a standard value (100%) for comparison.

The apparent activity of non-phosphorylated PARKIN is much higher in the UbFluor assay than in the native reaction experiments. PARKIN activity is ~10% of pPARKIN+pUb activity in the UbFluor assay, while it is only 0.02% of pPARKIN+pUb activity in the native

reaction. This difference may arise because UbFluor detects catalytic site activation apart from E2 binding, unlike the native reaction. This catalytic site activation may reflect (1) physical opening of the catalytic site, exposing the catalytic cysteine (Cys⁴³¹) on RING2 that is otherwise occluded by the UPD, (2) chemical activation of Cys⁴³¹ by neighbouring residues for transthiolation or ligation reactions, or both. In contrast, binding of the E2~Ub complex is strictly required to observe any activation in the native reaction. Therefore, the larger difference between minimal and maximal activity in the native reaction may be due to multiple regulation sites: the E2~Ub interaction site and the catalytic site (Figure S1E).

Aside from higher basal PARKIN activity in the UbFluor assay, the agreement between these two measurements is remarkable. Both measurements find that activation of PARKIN by pUb is less than activation by Ser⁶⁵ phosphorylation, while the greatest activation occurs with both pUb and pPARKIN. This set of findings poses an interesting question: how can UbFluor reproduce the activity trend of PARKIN observed in the native reaction, despite using a distinct transthiolation mechanism? It may be that while E2 binding clearly does not happen in the ByS, the E2-independent UbFluor transthiolation reaction is still relevant to the mechanism of Ub binding during the native transthiolation.

Consistent with this conclusion, Kumar et al. showed that UbCH7-Ub binds to pPARKIN 20-fold tighter than UbCH7 alone, suggesting that ubiquitin binding itself has an important role in native transthiolation.⁽⁶⁶⁾ Furthermore, recent work showed that RING2 and RING1 domains of RBR E3s harbors ubiquitin binding site (48). RING2 ubiquitin binding site is particularly interesting since it was

shown that it recruits E2~Ub thioester for transthiolation reaction. In this respect RING2 domain is functionally similar to the C-lobe of HECT E3 ligases that also has a Ub binding site near the catalytic cysteine. The latter serves to recruit E2~Ub thioester for transthiolation reaction and subsequently retains Ub in HECT E3~Ub thioester for isopeptide ligation step (67-69).

S65E is a poor mimetic of pPARKIN.

Several studies have used overexpressed PARKIN^{S65E} in cells as a phosphomimetic for pPARKIN, but it is unclear whether PARKIN^{S65E} is activated similar to pPARKIN. PARKIN^{S65E} failed to show increased free ubiquitin chain formation compared to PARKIN in a Western blot assay.(7) In apparent contrast, Kumar et al. demonstrated enhanced polyubiquitin chain formation by PARKIN^{S65E} compared to PARKIN.(66) We used the UbFluor-based ByS to quantitatively examine whether pUb activates PARKIN^{S65E} as it does for pPARKIN. We performed titration experiments using PARKIN^{S65E} under the same ST and MT conditions as defined for pPARKIN in Figure 4 (Figure 5A and B). pUb slightly increases the UbFluor consumption rate of PARKIN^{S65E} under both ST and MT conditions. This slight, < 1.5-fold change under ST conditions is similar to what we observed for pUb activation of PARKIN under ST conditions (Figure S8A), but is significantly smaller than the ~7- and 3-fold activation of pPARKIN by pUb under ST and MT conditions, respectively (Figure 5A and B). These results indicate that S65E does not mimic Ser⁶⁵ phosphorylation of pPARKIN in terms of activation by pUb binding.

We further compared the MiroS substrate ubiquitination efficiency of

PARKIN^{S65E} and pPARKIN using both the UbMES-based ByS and the native reaction (Figure 5 C and D). In both reactions, pPARKIN robustly ubiquitinated MiroS while PARKIN^{S65E} had only slight activity (Figure 5 C and D, Lane 2 and 8). As we previously described for the UbFluor assay, unphosphorylated PARKIN showed higher background activity in the UbMES-based ByS reaction than in the native reaction (Figure 5C, lane 6). The PARKIN^{S65E} mutation did not increase Miro ubiquitination significantly above this background in the ByS. This result supports our conclusion that S65E is not a good mimetic of pPARKIN. While this work was in progress, Ordureau et.al. published similar findings (7).

UbFluor can detect PARKIN activation by point mutations.

PARKIN is autoregulated both by mechanisms that block E2~Ub binding and by mechanisms that occlude the catalytic site. Because the UbFluor-based ByS reaction does not include an E2, we may expect it to only detect activation of the catalytic site (Figure S1E). In fact, when used with truncated HECT E3s, the ByS detects mutations that alter activity near the Ub binding site on the C-lobe and near the catalytic cysteine, but not those that alter E2-binding (65). However, for the RBR ligase PARKIN, one report has postulated that the REP:RING1 interface and the RING2:UPD interface are allosterically coupled (11,20). For example it was shown that disrupting REP:RING1 interface with W403A mutation mimics phosphorylated PARKIN. This result suggests that changes in regulation of the E2~Ub binding site may translate to changes in the catalytic site, such that these changes may be observable using the ByS.

To determine the extent to which UbFluor can detect different mechanisms of PARKIN activation, we prepared five constructs: four harbouring point mutations that are known to activate PARKIN, and one harbouring a mutation that abolishes ubiquitination by fully active PARKIN. We assume that the activating mutations represent potential modes of activation by small molecules. Activating mutations disrupt (1) the REP:RING1 interface (A398T and W403A)(46,54), or (2) the UPD:RING2 interface (F463Y and Δ UPD; the Δ UPD construct consists of PARKIN residues 219-465).(46) The inactivating mutation C431A ablates the catalytic cysteine residue(43) (Figure S1E). Importantly A398T is a Parkinson's disease mutation (1). To enable a direct comparison with past data evaluating the effects of these mutations on PARKIN activity, PARKIN point mutations were prepared using a base construct of rat PARKIN 141-465 with an N-terminal GST tag (Δ Ubl, Figure S1E). The Δ Ubl construct lacks the ubiquitin-like domain and linker (1-140), and therefore represents a minimally autoregulated construct that still harbours a low level of ubiquitination activity.(46)

We first confirmed that the E2-independent ByS recapitulates the known properties of Ubl, Δ UPD, and Δ UPD (C431A) using UbMES (Figure 6A). As previously reported from native assays,(46) Ubl exhibited a small amount of activity, UPD had robust activity, and the C431A mutation completely abolished the activity of Δ UPD, demonstrating that the activity of UPD in the ByS assay requires the catalytic cysteine (Lane 1-3, 4-6 and 7-9 respectively, Figure 6A). Next, we examined three activating point mutations in the Δ Ubl background using UbMES. As expected, the F463Y mutation that disrupts the UPD:RING2 interaction showed obvious activation (compare Figure 6A lanes 16-18 to lanes 1-3). We observed modest

activation by the REP:RING1 disrupting mutation A398T and more robust activation by W403A (compare Figure 6A lanes 10-12 and 13-15 to lanes 1-3). While there is no E2~Ub bound structure of PARKIN, a simple explanation for these results is that the W403A mutation may relieve autoinhibition of the Ub binding site in PARKIN which is occupied by UbMES/UbFluor to a larger extent than A398T, or to expose the catalytic cysteine of RING2 domain to a larger extent. Regardless of the unknown structural mechanisms by which these mutations activate PARKIN, our W403A result demonstrates that the E2-independent ByS can detect PARKIN catalytic site activation by significant disruption of the REP:RING1 interface, where E2 enzyme presumably binds, and which is ~50 Å away from the catalytic cysteine of PARKIN.

We next investigated whether the same unnatural activation mechanisms could be detected using the UbFluor assay. To generate a quantitative comparison between mutants, we determined the apparent bimolecular rate of UbFluor consumption (k_{obs}) for each mutant. First, we performed UbFluor assays using four different concentrations of UbFluor under either ST conditions (1 μ M rat PARKIN with 0.25, 0.5, 0.75 and 1 μ M UbFluor) or MT conditions (5 μ M rat PARKIN with 10, 12.5, 15 and 20 μ M UbFluor). Each condition was repeated twice for a total of eight measurements. Next, we obtained the initial UbFluor consumption rate by performing a linear fit of the raw FP decrease at early timepoints (1-5 min for ST; 1-15 min for MT; Figure S12A). We then performed a linear fit of the initial UbFluor consumption rate vs. the UbFluor concentration (Figure S12B). Finally, the slope of this linear fit was divided by the PARKIN concentration to obtain k_{obs} (Figure 6B for MT and Figure S13 for ST).

Using this bimolecular rate analysis, we found that UbFluor detected activation of the Δ UPD construct, as well as activation of the Δ Ubl construct by F463Y and W403A mutations under both ST and MT conditions, similar to the UbMES-based ByS (Figure 6B and Figure S13, respectively). The weak activation by A398T observed in the UbMES assay was not significant enough to be detected in the UbFluor assay, as the activation was smaller than the error in the k_{obs} measurement. We applied the same method to compare k_{obs} of S65E and pPARKIN, and found that in contrast to phosphorylation, the S65E mutation did not significantly increase PARKIN catalytic turnover under either ST or MT conditions (Figure S14).

Together, these results demonstrate that UbFluor can recapitulate the majority of the effects of PARKIN mutations observed using UbMES. In addition, these results confirmed that UbFluor assays are robust to changes in the UbFluor concentration. We determined k_{obs} values using initial rates obtained from four different UbFluor concentrations under both ST and MT conditions, and found that the rate of each mutant relative to the wild type Δ Ubl construct is conserved at all UbFluor concentrations used (Figure S15). (70) The robustness of the UbFluor assay to different concentrations of probe validates its use for single-concentration and end-point high-throughput screening assays.

Finally, after determining that the UbFluor-based ByS can quantitatively detect both natural and unnatural activation mechanisms of the RBR E3 ligase PARKIN, we performed an initial test of the dynamic range of the UbFluor assay for high-throughput screening to identify small molecules that affect RBR E3 ligase activity. We monitored UbFluor

consumption by pPARKIN and GST-tagged HHARI RBR E3 ligases in a 384 well plate using a microplate reader (Synergy 4, Figure S16). Each E3 ligase (1.0 μ M) was incubated with either DMSO (0.2%; negative control) or iodoacetamide (1 mM; positive control) for 1 hour. UbFluor (5 μ M) was then added and endpoint FP readings were recorded after 1 and 2 hours of reaction. The 2-3-fold difference between the endpoint values of these positive and negative controls further demonstrates the feasibility of using UbFluor for high-throughput screening of bioactive molecules for RBR E3 ligases under MT conditions.

Discussion

We began this work by applying our previously developed UbMES-based ByS assay to study the RBR E3 ligase PARKIN (65). Despite the fact that PARKIN, like other RBRs, is very cysteine-rich (45), we found that the reaction with UbMES was highly specific for the catalytic cysteine and that the E2-independent bypassing system recapitulates many aspects biochemical mechanisms of PARKIN. Our results demonstrate that in the absence of any E2 enzyme, pPARKIN can build polyUb chains with specific linkages and select target lysine residues within substrates *in vitro*.

After confirming that the E2-independent ByS reaction recapitulated the native activity of PARKIN, we used the novel probe UbFluor to quantitatively evaluate PARKIN activation by both natural mechanisms (pUb and substrate) and unnatural mechanisms (PARKIN mutations that disrupt REP:RING1 and UPD:RING2 interface). Simple titration experiments using UbFluor enabled us to quantitatively compare and rank the native activation mechanisms acting on PARKIN: Ser⁶⁵-phosphorylation and pUb. Our titration

experiments also provided new insight on pPARKIN+pUb activity. We found that although pUb strongly activates transthiolation step of PARKIN and E2~Ub/UbFluor, maximal ubiquitination turnover by pPARKIN is only observed when a substrate or free lysine is present, suggesting that pUb itself is not a good ubiquitin acceptor for pPARKIN. Importantly, most efforts on studying the mechanisms of PARKIN activation have been focused on the activating role of PINK1 and pUb. Here we show that substrates that provide acceptor lysines can also enhance PARKIN catalytic turnover. Thus the local concentration of the substrate can affect Parkin activity as evident from ByS and native ubiquitination systems (Figure 4G, S11).

We would like to highlight the remarkable simplicity of the assay: only two reagents are needed, and by changing the ratio of UbFluor and enzyme we can quantify transthiolation and isopeptide ligation steps. Furthermore, we can easily test the effect of other protein cofactors such as pUb and substrates such as Miro on PARKIN activity, and investigate their roles on the enzyme mechanism (i.e. activation of transthiolation step vs isopeptide ligation step). We envision that our findings will expand the use of UbFluor probe for a robust quantifying of the enzymatic activity of HECT, RBR, and bacterial HECT-like and NEL E3 ligases, upon structural mutations or in the presence of protein partners such as pUb or substrates.

Importantly, our data demonstrated that UbFluor can detect changes in the ligation efficiency of PARKIN under MT conditions, despite the fact that the signal change from FP occurs during transthiolation (Figure 4 and Figure S11). This is an essential property of the UbFluor

probe for high-throughput screening, as our goal is to identify molecules that increase ligation efficiency as well as the transthiolation step.

Lastly, we demonstrated several properties of the UbFluor assay that indicate its suitability for high-throughput screening to identify small molecules affecting RBR E3 ligase activity. We found that the E2-independent UbFluor reaction can, at least in some cases, detect significant activation at the REP:RING1 site of PARKIN. In addition, the UbFluor-based end-point assay detected activity changes of both PARKIN and HHARI caused by iodoacetamide with a strong signal change in a 384 well-plate format. Because UbFluor is stable at 100 mM lysine, direct consumption of UbFluor by small molecules containing amines is not expected. However, because high concentrations of free thiols (such as BME and cysteamine) release Fluor-SH, small molecules with free thiols should be avoided. Overall, these results provide a very encouraging demonstration that UbFluor can be used in a highly simplified high-throughput screening assay to detect allosteric activators or inhibitors of PARKIN and perhaps other RBR E3 ligases. Further use of UbFluor in application to HECT/RBR/HECT-like bacterial/NEL E3s will be reported in the future.

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Author Contributions

S.P., S.E.R, S.A.V. designed the study. S.P., P.F., and D.T.K. conducted experiments, and collected the data. S.P., P.F., D.T.K. S.E.R., and S.A.V. analyzed the data. The manuscript was written by S.E.R., S.P., and S.A.V. All authors participated in discussions about the manuscript and approved the final version.

To whom correspondence should be addressed: Tel. 713-743-9616; E-mail: avstatsy@central.uh.edu

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Figure/Scheme captions:

Figure 1. Chemical activation of the C-terminus of ubiquitin as a thioester (e.g. UbMES or UbFluor) can bypass the need for E1, E2 and ATP, downsizing the 5-component native cascade reaction (E1, E2, ATP, PARKIN, and Ub) to 2 components (PARKIN and ByS probes). Transthioylation with UbMES releases a mercaptoethanesulfonate group (MES), while transthioylation with UbFluor releases fluorescein thiol.

Scheme 1. Synthesis of UbFluor.

Figure 2. The PARKIN/ByS recapitulates several aspects of the native cascade. (A) ByS reaction of pPARKIN, UbMES, and the MiroS substrate. Lane 4 shows pPARKIN autoubiquitination and chain formation, while Lane 6 shows pPARKIN autoubiquitination, chain formation, and MiroS substrate ubiquitination. Notably, UbMES has no ubiquitination activity in the absence of pPARKIN, or in the presence of catalytically inactive C431A pPARKIN (lanes 5 and 7). Hyd. indicates hydrolyzed UbMES before the reaction. (B) pPARKIN undergoes autoubiquitination and MiroS ubiquitination using UbMES. Mutation of the primary phosphorylation site (S65A) or the catalytic cysteine (C431A) blocks this activity. P indicates PARKIN, M indicates MiroS and * indicates impurities. PARKIN (S65A) was pretreated with TcPINK1 under the same reaction conditions as PARKIN, prior to its use in assay. (C) ByS ubiquitination depends on the concentration of UbMES and requires pPARKIN. (D) pPARKIN/ByS forms K48- and K63-linked chains, similar to the native cascade. The reaction with pPARKIN, MiroS and the corresponding UbMES were analyzed by western blot. A K48R

mutation in UbMES blocks formation of K48-, but not K63-linked chains (lane 3), while a K63R mutation in UbMES blocks formation of K63-, but not K48-linked chains (lane 4). (E) MALDI-TOF analysis of a slice from the stacking gel of a native cascade reaction and the ByS reaction from (D), lane 2, confirming K48 and K63 chain formation. (F) MiroS ubiquitination by pPARKIN using the ByS (1 μ M pPARKIN and 30 μ M UbMES) proceeds on a similar timescale to common native reaction conditions (100 nM UBE1, 1 μ M Ubch7, 1 μ M pPARKIN, 30 μ M Ub, and 4 mM ATP). P indicates PARKIN and M indicates MiroS.

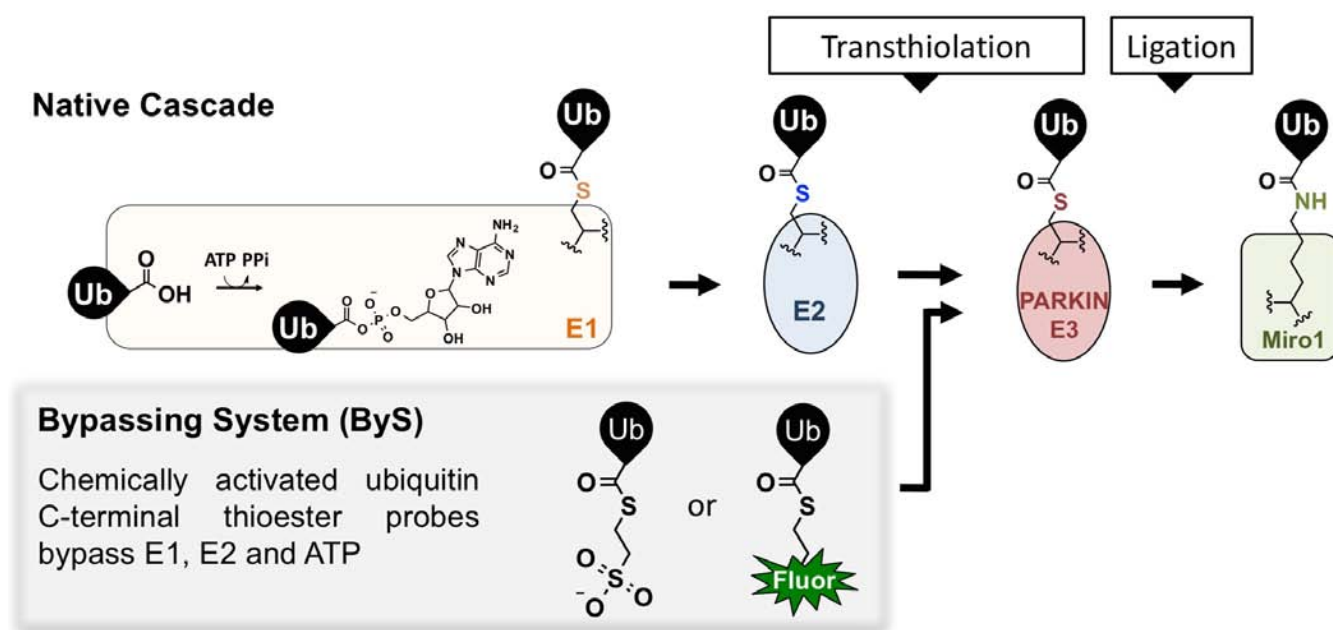
Figure 3. UbFluor reports pPARKIN activity in real-time by fluorescence polarization. (A) Design of the ByS reaction using UbFluor and PARKIN. (B), BME (lane 2) or pPARKIN (lane 4) can release free fluorescein (SFluor) from UbFluor but not pPARKIN^{C431A} (lane 6). (C) Gel-based analysis of UbFluor/pPARKIN reaction. At the indicated time points, the reaction was stopped by addition of non-reducing Laemmli buffer and analyzed by SDS-PAGE. A time-dependent decrease in the intensity of the UbFluor band and increase in the free fluorescein band were observed. (D) FP-based analysis of UbFluor (0.5 μ M) consumption by the indicated amount of pPARKIN (0, 1, 2, 3, 4, and 5 μ M). Mean $\pm$ SEM of 3 measurements for each concentration of pPARKIN are shown. (E) Plot of the initial rates (0-5 min) from (D) showing that the rate of UbFluor consumption increases linearly with pPARKIN concentration ($R^2=0.996$).

Figure 4. UbFluor confirms multiple activation states of PARKIN. Titrations in the left panels (A, C, and E) were performed under ST conditions (5 μ M PARKIN, 0.5 μ M UbFluor), while the right panels (B, D, and F) were performed under MT conditions (2 μ M PARKIN, 20 μ M UbFluor). Each titration was repeated twice and all data are shown. Solid lines join the rates determined from one titration experiment, while dashed lines join rates determined from a second titration. A-B, Ub or pUb titration into a UbFluor reaction with PARKIN or pPARKIN. C-D, MiroS titration into pPARKIN+25 μ M pUb. E-F, Lysine titration into pPARKIN+25 μ M pUb. G, Comparison of PARKIN activation measured using UbFluor with previous data. UbFluor measurements under ST and MT conditions and SDS-PAGE based quantification are reported in this work. AQUA-MS data were reported in by Ordureau et al(7,64). For comparison of UbFluor to AQUA-MS, the maximal rate corresponding to each state is determined as a percentage of pPARKIN+pUb activity. Cartoons depict the relative activity of each PARKIN species, with unmodified PARKIN having the lowest activity and pPARKIN+pUb+MiroS having the highest. * rate was too fast to accurately resolve.

Figure 5. S65E is a poor mimetic of pPARKIN. (A-B) ST (A) and MT (B) experiments titrating Ub or pUb into a UbFluor reaction with PARKIN^{S65E} (green triangles) were compared to PARKIN (orange circles) and pPARKIN (blue diamonds) titrations from Figure 4. Conditions were as described in Figure 4. Each titration was repeated twice and all data are shown. Solid lines join the rates determined from one titration experiment, while dashed lines join rates determined from a second titration. (C-D) Ubiquitination of MiroS by pPARKIN, pPARKIN^{C431A}, PARKIN, or PARKIN^{S65E} were performed by (C) the UbMES-based ByS reaction or (D) the native reaction. Conditions for these experiments were identical to those used in Figure 2F. * indicates ubiquitinated MiroS.

Figure 6. UbFluor can quantify PARKIN activation or inactivation by known point mutations. (A) UbMES assay on Δ Ubl, Δ UPD, Δ UPD (C431A), and activating PARKIN mutations in the Δ Ubl background (A398T, W403A, and F463Y). Experiments with each mutant were performed using 30 μ M UbMES and 1.0 μ M PARKIN for the time indicated. W403A and F463Y were run on a separate gel. (B) Bar graph showing bimolecular reaction rates of UbFluor consumption under MT conditions by PARKIN mutants as described above using the UbFluor assay. Errors are based on linear fits of data from two repeats of UbFluor consumption data at 4 different concentrations of UbFluor (8 total measurements).

Figure 1



Scheme 1

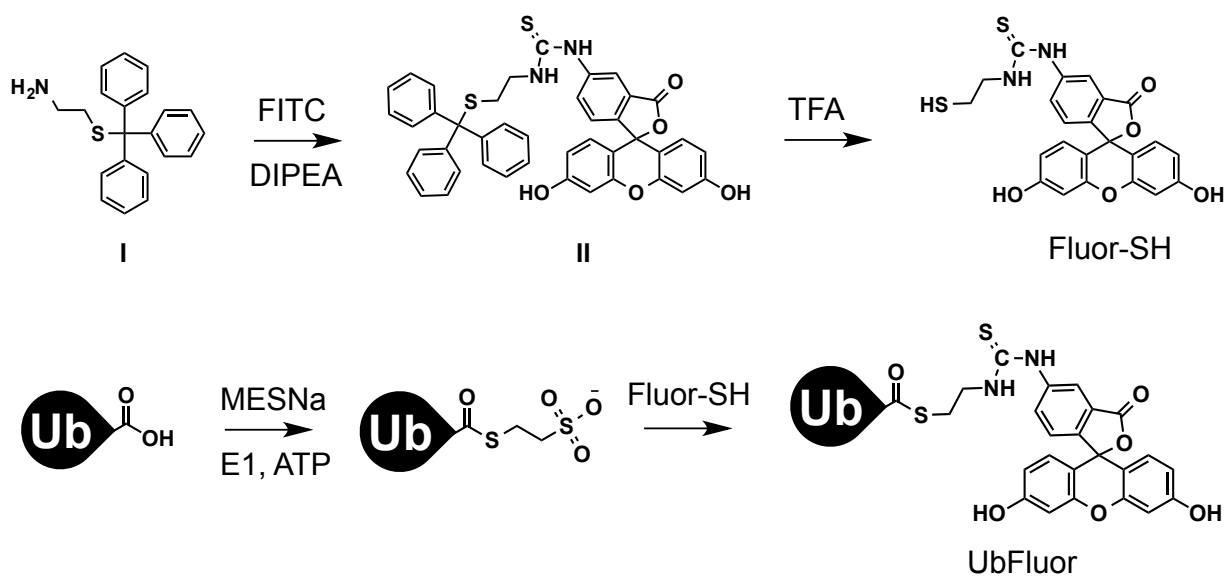


Figure 2

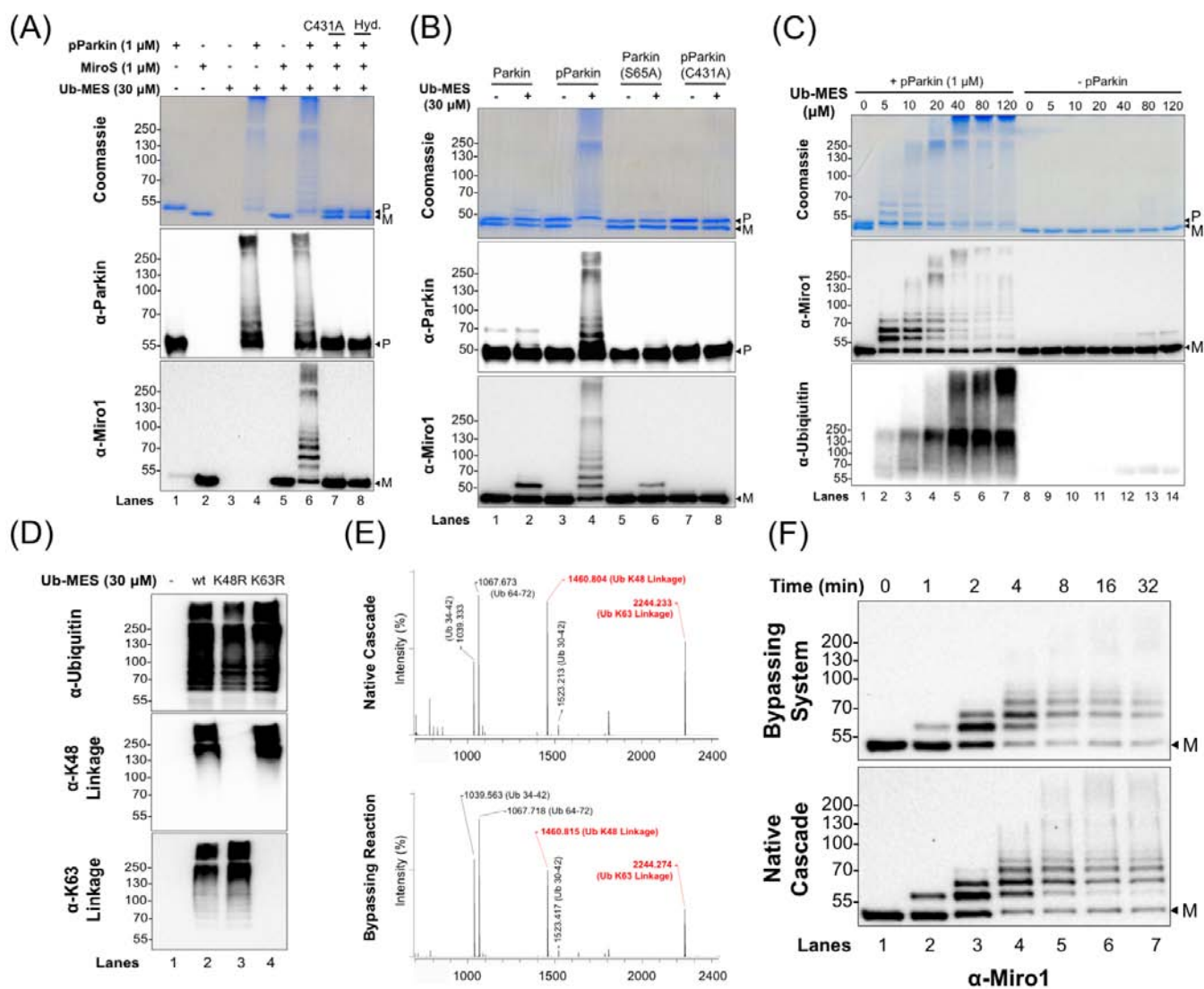


Figure 3

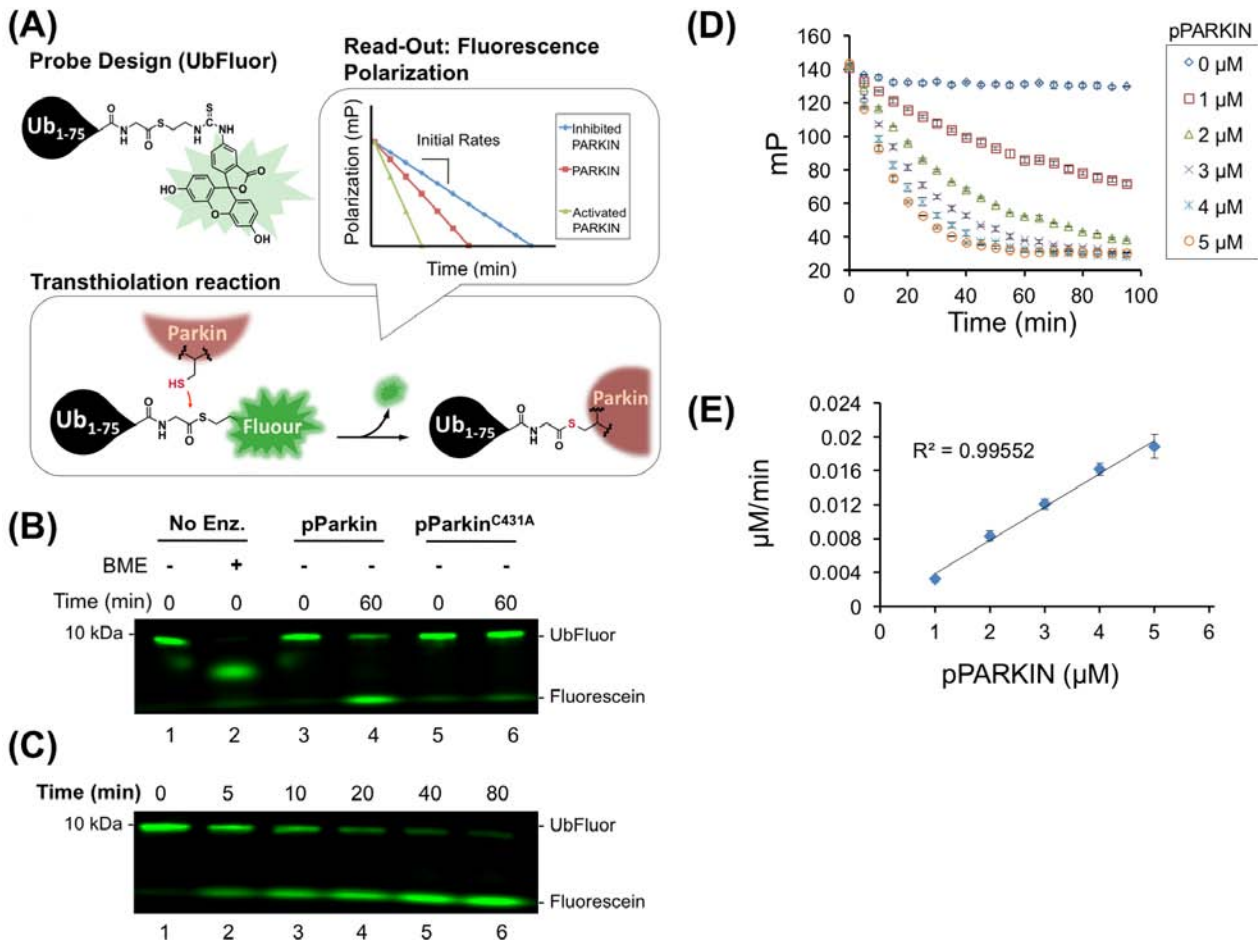


Figure 4

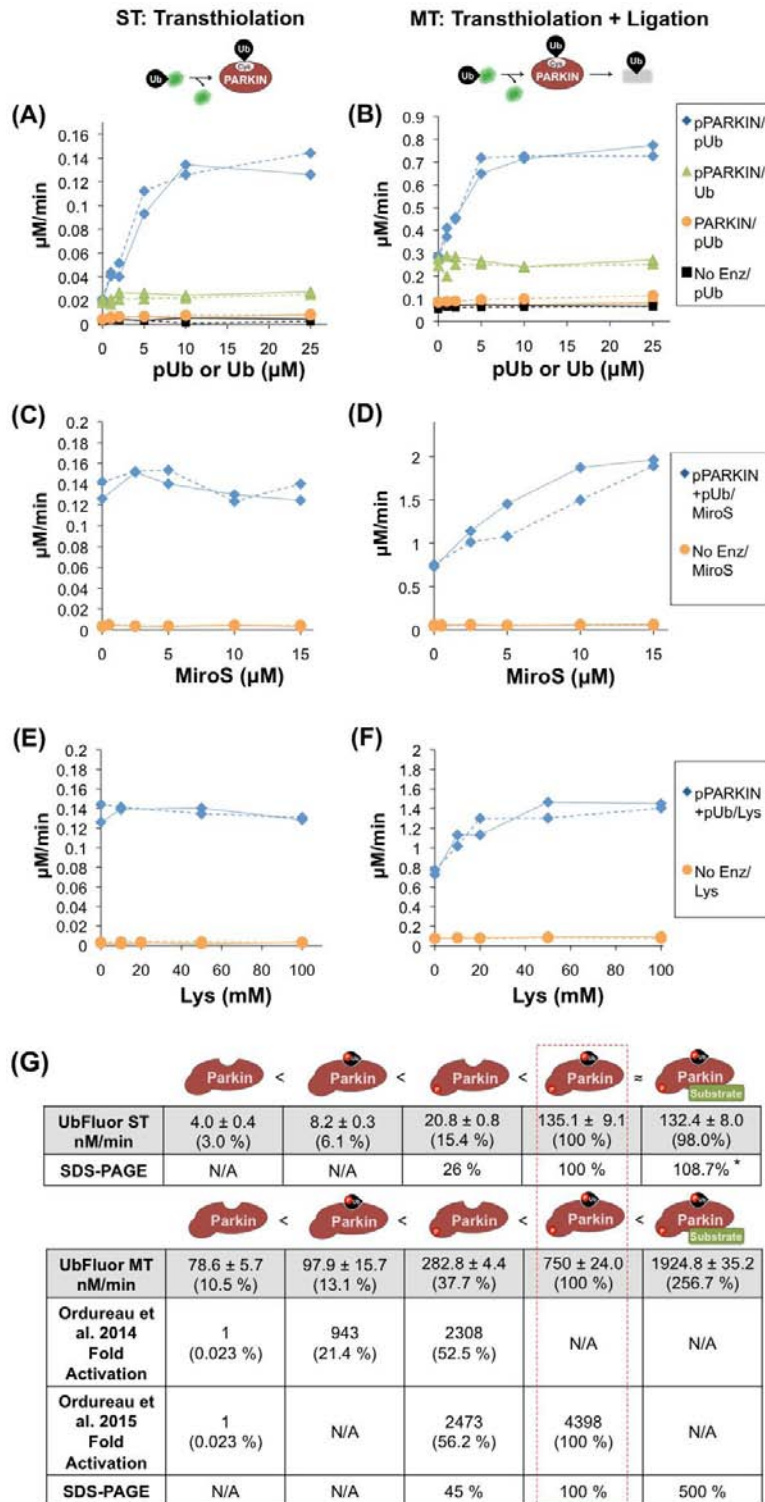


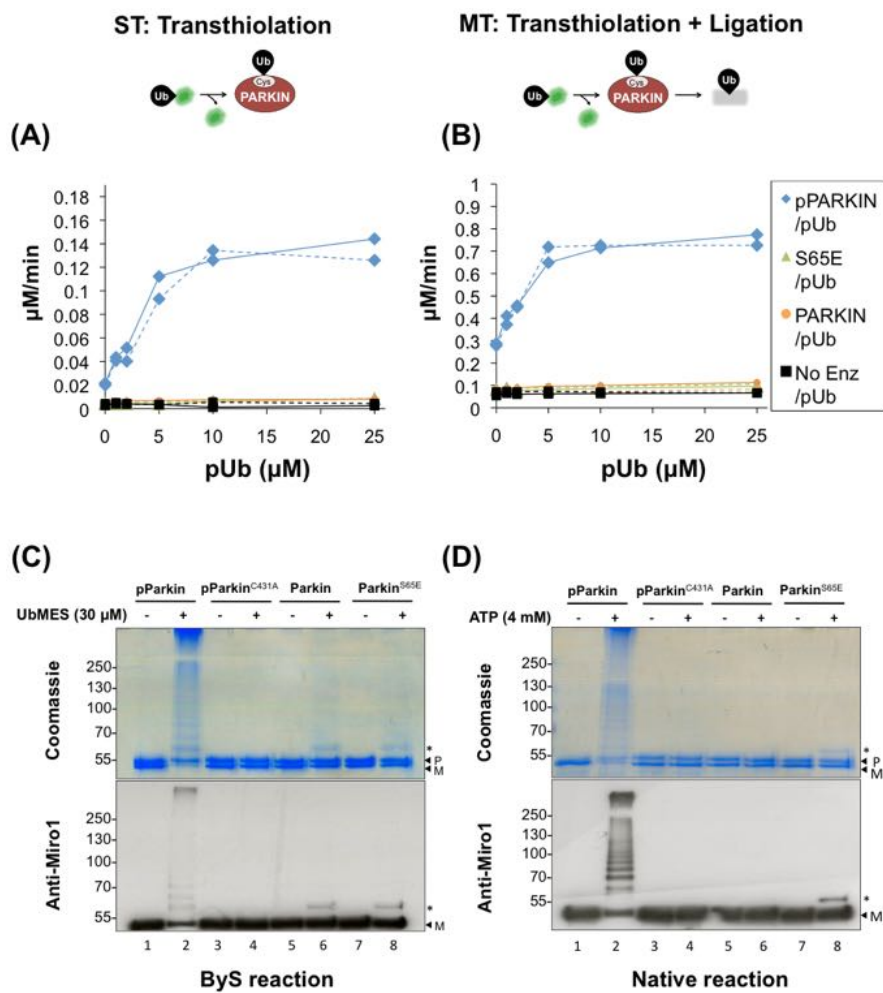
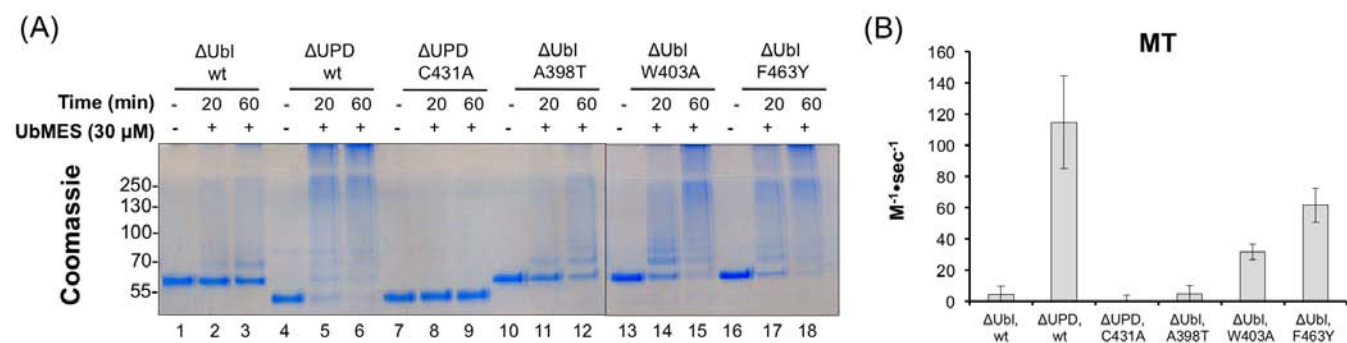
Figure 5

Figure 6



UbMES and UbFluor: Novel Probes For RBR E3 Ubiquitin Ligase PARKIN

Sungjin Park, Peter K Foote, David T Krist, Sarah E Rice and Alexander V Statsyuk

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