Mass Spectrometric and Mutational Analyses Reveal Lys-6-linked Polyubiquitin Chains Catalyzed by BRCA1-BARD1 Ubiquitin Ligase*

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The breast and ovarian cancer suppressor BRCA1 acquires significant ubiquitin ligase activity when bound to BARD1 as a RING heterodimer. Although the activity may well be important for the role of BRCA1 as a tumor suppressor, the biochemical consequence of the activity is not yet known. Here we report that BRCA1-BARD1 catalyzes Lys-6-linked polyubiquitin chain formation. K6R mutation of ubiquitin dramatically reduces the polyubiquitin products mediated by BRCA1-BARD1 in vitro. BRCA1-BARD1 preferentially utilizes ubiquitin with a single Lys residue at Lys-6 or Lys-29 to mediate autoubiquitination of BRCA1 in vivo. Furthermore, mass spectrometric analysis identified the Lys-6-linked branched ubiquitin fragment from the polyubiquitin chain produced by BRCA1-BARD1 using wild type ubiquitin. The BRCA1-BARD1-mediated Lys-6-linked polyubiquitin chains are deubiquitinated by 26 S proteasome in vitro, whereas autoubiquitinated CUL1 through Lys-48-linked polyubiquitin chains is degraded. Proteasome inhibitors do not alter the steady state level of the autoubiquitinated BRCA1 in vivo. Hence, the results indicate that BRCA1-BARD1 mediates novel polyubiquitin chains that may be distinctly edited by 26 S proteasomes from conventional Lys-48-linked polyubiquitin chains.

The familial breast and ovarian cancer susceptibility gene product BRCA1 functions in multiple cellular processes that include DNA repair, transcriptional regulation, cell cycle control, and apoptosis (1–4). One possible biochemical function that could contribute to the cellular functions of BRCA1 is the ubiquitin (Ub) ligase activity that arises when BRCA1 forms a RING heterodimer with BARD1 (5–10). Ub-protein isopeptide ligases (E3) catalyze the formation of poly-Ub chains on substrate proteins via isopeptide bonds that link the C-terminal Gly residue of one Ub molecule (activated in an ATP-dependent manner by the enzyme E1) to the εNH2 group of a Lys side chain in another Ub molecule (11). The most common poly-Ub chain is linked through Lys-48 ofUb and serves as a signal for rapid degradation of substrates by the proteasome-dependent proteolysis pathway (12). However, recent studies have revealed roles other than proteolysis for polyubiquitination (13). While Lys-48- and Lys-29-linked chains mediate proteasome-dependent degradation (12, 14), Lys-63-linked chains are a signal for endocytosis, IκB kinase activation, ribosome modification, and DNA repair (15–21). Therefore characterization of the poly-Ub chain linkage is important to predict biological function of Ub ligases.

Several groups have characterized the type of poly-Ub linkages formed by the BRCA1-BARD1 heterodimer. It was reported that the poly-Ub chain built by the BRCA1-BARD1 ligase is linked through Ub residues other than Lys-48, suggesting they may not serve as a degradation signal (6). Another group reported that BARD1 stimulates the formation of both Lys-48- and Lys-63-linked poly-Ub chains and that the BRCA1 autoubiquitylation by BARD1 mostly results in poly-Ub chains linked through Lys-63 (7). However, in studies involving a series of Ub lysine mutants, Baer and co-workers (22) recently reported that BRCA1-BARD1 catalyzes Lys-6-linked poly-Ub chains in vitro. Thus, the type(s) of poly-Ub chains catalyzed by BRCA1-BARD1 has yet to be resolved unambiguously.

In the present study we provide further evidence that BRCA1-BARD1 catalyzes Lys-6-linked poly-Ub chain. In addition to in vitro analyses, in vivo analyses also suggest that the autoubiquitinated form of BRCA1 can be assembled with Ub containing a single Lys residue at Lys-6. More importantly, mass spectrometric analysis identified the Lys-6-linked branched Ub fragment from poly-Ub chains produced by BRCA1-BARD1. These data strongly support the previous data reported by Baer and co-workers (22). When incubated with purified 26 S proteasome in vitro, the Lys-6-linked poly-Ub chains mediated by BRCA1-BARD1 as well as Lys-48-linked chains mediated by ROC1-CUL1 disappear. Disappearance of the Lys-6-linked poly-Ub chains is blocked by Ub aldehyde (Ubal), a potent inhibitor of deubiquitinating enzymes. In contrast, the disappearance of ROC1-CUL1-mediated Lys-48-linked chains is unaffected by the presence of Ubal. Proteasome inhibitors MG132 and LLnL do not stabilize the autoubiquitinated form of BRCA1 in vivo. Hence, the results indicate that the BRCA1-BARD1 Ub ligase catalyzes novel Lys-6-linked poly-Ub chains as a signal recognized, but possibly distinctly processed, by 26 S proteasome relative to traditional Lys-48-linked poly-Ub chain.
MARITAL MATERIALS AND METHODS

**Results**—N-terminally tagged constructs pcDNA3-Myc-BRCA1-1–329, pcDNA3-HA-BARD1, pcDNA3-HA-ROC1, pcDNA3-Myc-CUL1, pET-His6-PK-Ub, pET-His6-UB, pcDNA3-His6-PK-Ub, and pET-His6-UB were purchased commercially. His6-UbcH5c, His6-BRCA1, and BARD1 (Boston Biochem), bovine Ub, and FLAG-Ub (Sigma) were purchased commercially.

His6-PK-Ub was expressed as described previously (5, 23). Ub with a single Lys residue was produced as described above. The Ubs with a single Lys residue were produced as described above. The Mutant Ubs with a single Lys residue (Lys-6, Lys-11, Lys-29, Lys-48, or Lys-63) were substituted with Arg was produced by repeated Lys-Arg site-directed mutagenesis to the six Lys residues. To construct pcDNA3-HA-UB that contained four tandem repeats of HA-Ub for efficient expression in mammalian cells (24). HA and flag were first introduced at the 5’- and 3’-end of HA-UB, respectively, by site-directed mutagenesis. The Ubs with a single Lys residue were produced as described above. After the mutations were verified by DNA sequencing, the XbaI- and SpeI-digested insert fragments were subcloned into the same HindIII and XhoI sites of pET-30a(+) (Novagen) as described previously (5, 23). Ub with a single Lys-Arg mutation (K6R, K11R, K29R, K48R, or K63R) was produced by site-directed mutagenesis. The Ub ligation reaction mixture (30 μl) that contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM NaF, 10 mM potassium acetate, 2 mM ATP, and 0.6 mM DTT as well as 40 ng of E1, 0.3 μg of E2, and 1 μg of purified E3 (His6-tagged proteins). In some experiments E3 immunocomplexes immobilized on protein-A agarose beads as described above were used instead of the purified E3s after washing two times with buffer B containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.01% Nonidet P-40, 1% gelatin, and 1 mM EDTA. The mixture was incubated for 30 min at 37°C with shaking, and the reaction was terminated by boiling in Laemmli SDS loading buffer with a final concentration of 0.1 M DTT. Half of the sample was resolved by SDS-PAGE by autoradiography. The Ub ligation reaction mixture (30 μl) was analyzed by the same procedure without E3. One μg of bovine Ub or FLAG-Ub was used (instead of the 0.75 μg of purified His6-PK-Ub) in the Ub ligation assay for mass spectrometric analysis or in vitro degradation/deubiquitination assay, respectively.

**Immunological Techniques—**Mouse monoclonal antibodies to affinity tags HA (clone 12CA5, Roche Applied Science), Myc (clone 9E10, BabCo), and FLAG (clone M2, Sigma) were purchased commercially. For immunoprecipitation, cells were harvested 36 h after transfection and lysed by incubating at 4°C for 30 min with 0.6 ml/100-mm dish buffer A containing 50 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM dithiothreitol (DTT), 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine. Lysed cells were then centrifuged at 16,000 × g for 10 min. The supernatants (0.3 ml) were mixed with 3 μg of anti-Myc monoclonal antibody, and then the antibody-bound proteins were precipitated with protein-A agarose beads (7.5 μl). The proteins bound to the beads were washed three times with buffer A and used in the Ub ligation assay. To immunoprecipitate in vivo auto ubiquitinated BRCA1, 293T cells transfected with plasmids expressing HA-Ub, Myc-BRCA1, and BARD1 were harvested, lysed in 200 μl of preboiled 1% SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT), and further boiled for an additional 10 min. Lysates were clarified by centrifugation at 16,000 × g for 10 min. Supernatant (100 μl) was diluted 10 times with buffer A, and then the precipitates were washed three times, and half of the sample was resolved on a 7.5% SDS-PAGE, followed by immunoblotting with anti-HA antibody (1 μg/ml). For the immunoblotting, cells were lysed and clarified as described above, and 50 μg of each sample was resolved by SDS-PAGE, followed by immunoblotting.

**K6R Mutation of Ub Reduces the Poly-Ub Products Mediated by BRCA1-BARD1—**Ubiquitination acts on numerous biological processes, including the proteasome-dependent proteolysis mediated by Lys-48-linked poly-Ub. The discovery of BRCA1-BARD1 as a Ub ligase therefore prompted us to characterize the poly-Ub chain formation. We have sought to determine which lysine residue(s) of Ub is required for polyubiquitination chain formation by BRCA1-BARD1. Mutant Ubs, in which the Lys residue at position 6, 11, 29, 48, or 63 was individually substituted with Arg (UbK6R, UbK11R, UbK29R, UbK48R, and UbK63R), were labeled with 32P and used to add Ub ligation reactions containing ATP, E1, E2/UbHc5c, His6-BRCA1, and His6-BARD1. The poly-Ub-protein conjugates were resolved by SDS-PAGE by autoradiography. The reaction was fully dependent on enzymes E1, E2 (data not shown) (5), and E3 (Fig. 1A, lanes 1 and 2). The poly-Ub-protein conjugates produced by BRCA1-BARD1 (Fig. 1A, lane 3) were significantly reduced by K6R.
mutation of Ub (lane 4), whereas the reaction did not appear to be significantly affected by other mutations (K11R, K29R, K48R, and K63R) (lanes 5–8). The reduction of polyubiquitinated products was not due to degradation or misfolding of UbK6R in vitro, as it interacts with UbcH5c as well as wild type and the other mutants (Fig. 1B). On the other hand, ROC1-CUL1 with CDC34 failed to catalyze polyubiquitinated product using UbK48R (Fig. 1C, lane 6), consistent with the notion that this E2-E3 mediates Lys-48-linked poly-Ub chain. This demonstrates the specificity of the in vitro Ub ligation reaction using Lys-Arg Ub mutants.

BRCA1-BARD1 Preferentially Utilizes Ub with a Single Lys Residue at Lys-6 or Lys-29—To further analyze the lysine residue of Ub required for polyubiquitination by BRCA1-BARD1, Ub mutants in which all but one Lys residue are substituted with Arg (UbLys-6, UbLys-11, UbLys-29, UbLys-48, and UbLys-63) were produced. Although the single-Lys Ubs were all less effective (Fig. 2A, lanes 3–7) compared with the wild type (lane 2) in the in vitro Ub ligation reaction, there is some preference for UbLys-6 (lane 3) and UbLys-29 (lane 5) to form slower migrating products. The less effective ligation reaction was not due to the failure of interaction of the Ub mutants with UbcH5c (Fig. 2B). Again consistent with previous reports, ROC1-CUL1 with CDC34 catalyzed dimer and trimer formation of UbLys-48 (Fig. 2C, lane 6), demonstrating the specificity of the in vitro Ub ligation reaction using the single-Lys Ubs.

We next tested the single-Lys Ubs in the autoubiquitination of BRCA1 in vivo. To allow detection of autoubiquitinated BRCA1, Ub constructs containing four tandem repeats of HA-tagged Ub (HA-Ub-HA-Ub-HA-Ub-HA-Ub, described as HA-Ub) were constructed for expression in mammalian cells and co-expressed in 293T cells with Myc-BRCA11–772 and BARD1. Cells were boiled in 1% SDS containing buffer to dissociate non-covalently bound proteins. Myc-BRCA11–772 was immunoprecipitated by anti-Myc antibody after the lysate was diluted to 0.1% SDS. Ubiquitinated Myc-BRCA11–772 appears in an anti-HA Western blot as a smear that migrates slower than Myc-BRCA11–772 (Fig. 3A, lane 3). The absence of Myc-BRCA11–772 (Fig. 3A, lane 1), HA-Ub (lane 2), or BARD1 (lane 4) or the use of the breast cancer-derived RING finger mutant BRCA1C61G (lane 5) abolishes the smear, consistent with it being autoubiquitinated BRCA1. To eliminate the possibility that the smear could be contaminated by polyubiquitinated products other than autoubiquitinated BRCA1, we constructed

**Fig. 1. In vitro Ub ligation activity using Ubs with a Lys-Arg mutation.** A. His-tagged RING finger domains of BRCA11–304 and BARD11–189 (1 μg each) were purified from bacteria and incubated with ATP, E1, E2/UbcH5c, and 32P-labeled wild type or mutant Ub as indicated. The reactions were terminated by boiling in SDS- and DTT-containing sample buffer. The samples were then resolved by SDS-PAGE followed by autoradiography. WT, wild type. B, in vitro conjugation of E2/UbcH5c and 32P-Ub was performed as described in A without E3, and the reactions were terminated by the sample buffer without DTT. * indicates nonspecifically dimerized 32P-Ub in the absence of DTT. C, in vitro Ub ligation assays were performed as described in A except that CDC34 and anti-Myc immunocomplexes from Myc-CUL1/HA-ROC1-transfected 293T cells were used as the E2 and E3, respectively.
sequentially truncated Myc-BRCA1 fragments and expressed these in cells. In each case, the smears appear just above the corresponding position of each fragment (Fig. 2A), confirming that they are indeed auto ubiquitinated Myc-BRCA1 products.

Single Lys-HA-Ubs were then co-expressed with Myc-BRCA1 and BRCA1. Autoubiquitinated BRCA1 products were produced when HA-UbLys-6, HA-UbLys-29, or HA-UbLys-63 were co-expressed, consistent with the in vitro Ub ligation assay using the purified Ub mutants (Fig. 2A). In contrast, significantly less autoubiquitinated BRCA1 was detected when other HA-Ub mutants, HA-UbLys-11, HA-UbLys-48, or HA-UbLys-63 were co-expressed (Fig. 2C, top, lanes 4, 6, and 7). A Western blot of total cell lysates indicates that each of the single-Lys Ubs can be similarly incorporated into poly-Ub chains in vivo (Fig. 3C, bottom). This suggests that the contrast among HA-UbLys-6, HA-UbLys-29, and others in autoubiquitination of BRCA1 is not caused by different expression or the inability of the HA-Ub mutants to be incorporated into poly-Ub chains. We note that low levels of auto ubiquitinated BRCA1 were also detected with HA-UbLys-11, HA-UbLys-48, or HA-UbLys-63. These products could represent small populations of BRCA1 containing poly-Ub chains linked via Lys-11, Lys-48, or Lys-63. More likely, the products may be due to poly-Ub chains generated from endogenous Ub that have their chains terminated by the incorporation of a mutant single-Lys Ub.

Identification of Poly-Ub Chain Linkage by LC/MS/MS—We were concerned that the use of mutant Ub molecules could yield unnatural poly-Ub chains if, for example, elimination of the intrinsic Lys residue allows an adjacent Lys residue to be used instead. N-terminal tagging of Ub might also affect the chain formation. We therefore employed a procedure to detect the Lys residue linked in poly-Ub chains produced by BRCA1-BARD1 under more physiological conditions. Wild type untagged bovine Ub, whose sequence is identical to that of human Ub, was added to a Ub ligation reaction containing ATP, E1, UbcH5c, and BRCA1-BARD1 immuno complex. The poly-Ub-protein conjugates were resolved by SDS-PAGE followed by Sypro Ruby staining. The slowest migrating proteins just under the border of the stacking gel (Fig. 4A, indicated with *), were excised, digested with trypsin, and subjected to nanoscale capillary LC/MS/MS analysis. The masses for each possible branched Ub fragment, consisting of the acceptor Ub tryptic fragment linked to amino acids GG or LRGG from the C terminus of the donor Ub, were calculated, and the experimental data were searched for matches. A peptide ion with mz 1517.83 was identified and matched with the Lys-6-linked Ub fragment missing the N-terminal Met residue (QIFVKTLTGK conjugated with LRGG, where K is Lys-6), whereas peptide ions that match the other candidate branched Ub fragments were not observed.

We confirmed that the recombinant bovine Ub used in the study is missing its N-terminal Met residue by LC/MS/MS analysis. The parental peptide ion was further analyzed by collision-induced dissociation spectrum (MS/MS) to confirm the peptide sequence. The fragment possesses two N termini. Therefore we calculated the theoretical mz of the MS/MS fragment from the two straight fragments, QIFVKTLTGK and LRGGKTLTGK, each possessing one N and one C terminus, and we compared this with the experimental data (Fig. 4B). Other spikes shown in Fig. 4B included more complicated fragments as represented by QIFVGGKTLTGK conjugated with LRGGKTLTGK, each possessing one N and one C terminus, and we compared this with the experimental data (Fig. 4B).
characterized previously we next necessarily tested whether or not it signals degradation. We first employed an in vitro degradation assay using purified 26 S proteasome and the polyubiquitination products catalyzed by BRCA1-BARD1. Poly-Ub chains were produced by reactions containing ATP, FLAG-Ub, E1, UbcH5c, and anti-Myc immunocomplexes precipitated from lysates of 293T cells expressing Myc-BRCA11–772 and BARD1. Immunoblotting with anti-FLAG antibody nicely detected the polyubiquitination products after incubation for 60 min (Fig. 5A, top, lane 3). The protein complex containing polyubiquitinated products immobilized on the beads was then incubated with 26 S proteasome supplemented with MgCl2 and ATP. The polyubiquitinated products disappeared after incubation with 30 or 60 nM purified 26 S proteasome (Fig. 5A, top, lane 5 or 6, respectively), whereas incubation with buffer alone did not alter the level of polyubiquitinated products (lane 4). We blotted the same reaction with anti-Myc antibody to detect the autoubiquitinated substrate, Myc-BRCA1. Mono- and poly-

![Image](http://www.jbc.org/Downloaded_from)
ubiquitinated forms of Myc-BRCA1 were detected 5 and 60 min after the Ub ligation reaction, respectively (Fig. 5A, bottom, lanes 2 and 3). The majority of the non-ubiquitinated Myc-BRCA1 correspondingly disappeared as the polyubiquitinated form appeared (Fig. 5A, bottom, lane 3). Consistent with the anti-FLAG immunoblotting the autoubiquitinated Myc-BRCA1 disappeared after incubation with 26 S proteasome (Fig. 5A, bottom, lanes 5 and 6).

Purified 26 S proteasomes have an intrinsic deubiquitinating activity that rapidly releases poly-Ub chains from substrates before their degradation by the 20 S proteasomes. To determine whether the observed disappearance of polyubiquitinated products is due to degradation or to the deubiquitinating activity in the 26 S proteasome fraction, a deubiquitination inhibitor, Ubal, was added to the reaction. The decrease in autoubiquitinated Myc-BRCA1 observed upon incubation with 30 nM 26 S proteasome (Fig. 5B, top, lane 2) was slightly inhibited or completely blocked by the presence of 1 or 10 μM Ubal, respec-
Fig. 5. In vitro deubiquitination of autoubiquitinated BRCA1 and polyubiquitinated protein products produced by BRCA1-BARD1. A, in vitro Ub ligation assays were performed with ATP, E1, E2/Ubch5c, FLAG-Ub, and anti-Myc immunocomplexes from Myc-BRCA1–BARD1-transfected 293T cells for the indicated lengths of time. The reactions were terminated by washing with cold washing buffer, and the polyubiquitinated protein complexes immobilized on the beads were either boiled in SDS- and DTT-containing sample buffer (lanes 1–3) or subjected to the reaction buffer containing the indicated amounts of 26 S proteasome (lanes 4–6). The reactions were terminated by boiling in SDS- and DTT-containing sample buffer. Half of the sample was then resolved by SDS-PAGE followed by immunoblotting (WB) with anti-FLAG (top) or anti-Myc (bottom). IP, immunoprecipitation. B, in vitro Ub ligation assay was performed as described in A except that CDC34 and anti-Myc immunocomplex from Myc-CUL1/HA-ROC1-transfected 293T cells were used as E2 and E3, respectively, for the bottom panel. The autoubiquitinated Myc-BRCA1 (top) or Myc-CUL1 (bottom) was incubated with 26 S proteasome (lanes 2–4) in the presence or absence of the indicated amount of Ubal. * indicates IgG.

The Steady State Level of Autoubiquitinated BRCA1 Is Not Affected by Proteasome Inhibitors in Vivo—We next investigated the in vivo effect of the proteasome on Lys-6-linked poly-Ub chains by testing whether the steady state level of autoubiquitinated BRCA1 can be stabilized by proteasome inhibitors. Myc-BRCA1–BARD1, and HA-Ub (wild type or Lys-6) were co-expressed in 293T cells. Thirty h after transfection cells were treated with proteasome inhibitor MG132, LLnL, or solvent Me2SO for 6 h. Autoubiquitinated Myc-BRCA1 was then immunoprecipitated and blotted as described above. None of these reagents affected the steady state level of Myc-BRCA1 autoubiquitinated with wild type HA-Ub (Fig. 6, lanes 1–4) or HA-UbLys6 (lanes 5–8). This suggests that the Lys-6-linked poly-Ub chain is not a proteasomal degradation signal in vivo.

DISCUSSION

The fact that deleterious missense mutations in the RING finger domain of BRCA1 found in familial breast cancer all abolish the Ub ligase activity of BRCA1-BARD1 suggests a strong connection between this activity and the tumor suppressor function of BRCA1 (5, 7, 25, 26). However, the manner in which the Ub ligase activity contributes to the biological functions of BRCA1 remains to be determined. There are two key types of information required to elucidate the role of the BRCA1-BARD1 Ub ligase. One is, of course, the identity of its specific substrate(s). The other is the type of poly-Ub chain built by BRCA1-BARD1 and what biological consequence it signals. The studies reported herein identify the Lys linkage of poly-Ub catalyzed by BRCA1-BARD1. Three lines of evidence demonstrate that BRCA1-BARD1 catalyzes poly-Ub chain through the Lys-6 residue of Ub. First, K6R mutation of Ub dramatically reduces the poly-Ub products mediated by BRCA1-BARD1 in vivo (Fig. 1). We note that this result is inconsistent with a previous report that Lys-48 and Lys-63 of Ub are important for the polymer formation by BRCA1-BARD1 (7). There are two clear differences between our experimental protocol and that in the earlier report that may account for the different results. (a) To preserve the native properties of Ub as much as possible, we used the conservative Lys-Arg mutation rather than the Lys-Ala substitution. Also, (b) we used a Hi56 affinity tag on our mutant Ubs, whereas the earlier study employed glutathione S-transferase-tagged Ubs, which will exist as dimers in solution. Second, BRCA1-BARD1 preferentially utilizes UbLys6 or UbLys20 to mediate autoubiquitination of BRCA1 in vivo. Third, mass spectrometry analysis identified the Lys-6-linked branched Ub fragment from poly-Ub chain produced by BRCA1-BARD1 using wild type Ub. The results clearly indicate that BRCA1-BARD1 catalyzes Lys-6-linked poly-Ub chain formation. The significance of poly-Ub chain formation catalyzed by BRCA1-BARD1 in vivo using UbLys20 is not clear at present. BRCA1-BARD1 may catalyze heterogeneous poly-Ub chains. However, despite extensive effort, we failed
to detect any branched Ub fragments other than that at Lys-6. Lys-29 may be used by the BRCA1-BARD1-E2 complex when the preferred Lys-6 side chain is not available.

Whereas the roles of poly-Ub chains linked through Lys-29, Lys-48, and Lys-63 have been studied extensively, there is only limited information available on Lys-6-linked poly-Ub chains. In vitro, the yeast E2, RAD6, catalyzes the addition of up to six poly-Ub chains have not been widely studied to date, other Ub ligases that mediate Lys-6-linked poly-Ub chain formation, besides BRCA1-BARD1, may exist in nature. Mass spectrometry analysis combined with mutational analysis, as shown in this report, may contribute to the discovery of such ligases.

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