MUL1-RING recruits the substrate, p53-TAD as a complex with UBE2D2–UB conjugate

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Keywords
MUL1 RING domain; NMR; p53 transactivation domain; UBE2D2; ubiquitin

Introduction
Mitochondrial E3 ubiquitin ligase 1 (MUL1), located in the mitochondrial outer membrane, regulates various biological processes, including mitochondrial dynamics, cell growth, apoptosis, and mitophagy through ubiquitylation and SUMOylation [1]. It is a potential therapeutic target for Parkinson’s disease because its role is similar to that of the PINK1/Parkin pathway [2,3]. MUL1 also has different names, such as mitochondrial-anchored protein ligase (MAPL) [4], mitochondrial ubiquitin (UB) ligase activator of NF-κB (MULAN) [5], growth inhibition, and death E3 ligase (GIDE) [6] and Hades [7]. Sequence-based...
RINGMUL1 alone results in the ubiquitylation of p53 and immunoprecipitation experiments, showed that RINGMUL1 activity is critical for the ubiquitylation of mitofusin, Akt, p53, and ULK1, and responsible for the SUMOylation of dynamin-related protein 1 (Drp1) [1]. MUL1 plays a role in apoptosis via the direct regulation of apoptosis-associated proteins such as NF-κB, Akt, and p53, for which the activity of RINGMUL1 is critical [6–9].

Mitochondrial E3 ubiquitin ligase 1 negatively regulates the exonuclease function of p53 in the mitochondria via ubiquitylation. Moreover, results from cell-based in vivo and in vitro studies, including pull-down and immunoprecipitation experiments, showed that RINGMUL1 alone results in the ubiquitylation of p53 [7]. Although the six Lys residues in the C-terminal region of p53 are ubiquitylated by Mdm2 [10], the K24 residue of p53 is specifically ubiquitylated by the action of RINGMUL1 alone [7]. It has also been reported that RINGMUL1 ubiquitylates the transactivation domain (TADp53, residues 1–73) in the presence of three E2 enzymes (UBE2D1, D2, and D3), but not by UBE2L3 (Ubch7) [11].

Ubiquitylation is generally mediated by the action of the following three enzymes: UB-activating E1, conjugating E2, and ligase E3. E3 plays a key role in determining the target specificity and catalysing UB-transfer from E2 to the Lys side-chain of the target protein, and is mainly classified into two groups according to their E2-binding domains (HECT and RING/U-box); Transfer of the attached donor UB (UBD) directly from E2 to the substrate protein is the key feature of ubiquitylation by RING-E3 [12]. RING-E3 ligase generally contains another domain or region that can facilitate the recruitment of a target protein [13]. Thus, the mechanism underlying the ubiquitylation of p53 by the action of RINGMUL1 alone seems to be unique. Although our recent NMR studies have indicated that non-labelled RINGMUL1 clearly binds 15N-labelled TADp53 (15N-TADp53), its binding affinity (Kd, 1.03 mM) is too weak to support the in vivo ubiquitylation activity of RINGMUL1 alone [14].

To elucidate the mechanism by which RINGMUL1 alone results in the recruitment and ubiquitylation of TADp53, we determined the crystal structure of the RINGMUL1:UBE2D2 complex, and then studied the detailed interactions between TADp53 and the RINGMUL1 complexes with UBE2D2 and UBE2D2–UB mimetics in solution. Additionally, we reported the distinguishing features of RINGMUL1 that markedly enhanced the hydrolysis rate of UBE2D2–UB oxyester (UBE2D2–UBOE), and different dynamic natures of UBE2D2–UBOE depending on the N77 residue of UBE2D2. The higher binding affinity of TADp53 for RINGMUL1:UBE2D2–UBOE than that for RINGMUL1 or UBE2D2 alone depended on the multivalency of their binding, which resulted from the innate characteristics of the intrinsically disordered protein (IDP), TADp53.

## Results

### UBE2D2 exhibits a weak binding affinity for TADp53

We first estimated the binding affinity between 15N-TADp53 and UBE2D2 via chemical shift perturbation (CSP) experiments using two-dimensional (2D) 1H–15N heteronuclear single quantum correlation (HSQC), since the ubiquitylation reaction is also mediated by the action of E2. Certainly, UBE2D2 also bound to 15N-TADp53 (Kd, 735 ± 39 μM; Fig. 1A and Table 1), in which the region containing residues A39 to D55 (AD39) displayed a higher CSP than that exhibited by the region containing residues, S15 to N29 (SN15). The marked decrease in the peak intensities of 15N-TADp53 caused by the binding of RINGMUL1 with a higher Kd value, compared to that caused by the same concentration of UBE2D2, was likely from the heterogeneity of chemical shifts (CSs) induced by the structural flexibility of RINGMUL1 (Fig. 1A,B).

Reciprocal CSP experiments of 15N-UBE2D2 in the presence of TADp53 and AD39 showed that their binding surfaces of UBE2D2 were almost identical, and mainly localized at two regions (Fig. 1E,F) as follows: (a) the main location was in the vicinity of the RING-binding site (x1 and N-terminal x3), while (b) the other location was on the rear side of UBE2D2 (β4-to-loop and x4; Fig. 1J). The fact that the CSP amount of 15N-UBE2D2 induced by the TADp53-binding was considerably higher than that induced by the same concentration of AD39 indicated that the SN15 region also contributed to the binding of UBE2D2. We also ascertained the CSP amounts of 15N-RINGMUL1 induced by TADp53 [14], and compared them to those corresponding to similar concentrations of AD39 and SN15. The CSP patterns of 15N-RINGMUL1 demonstrated by TADp53, AD39, and SN15 were similar, but the CSP amount was higher for TADp53 (Fig. 1G–I). Since each RINGMUL1 and UBE2D2 displayed a weak affinity to TADp53, it was possible that the larger complexes (RINGMUL1:UBE2D2 or RINGMUL1:UBE2D2–UB) display a higher affinity for TADp53.
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Fig. 1. The interactions of TAD_{p53}, UBE2D2, and RINGMUL1 for different counterparts were studied by $^1$H-$^{15}$N HSQC experiments. The CSPs of $^{15}$NTAD$_{p53}$ (A–D), $^{15}$NUBE2D2 (E, F), and $^{15}$NRINGMUL1 (G–I) in the presence of interacting counterparts are shown as bar plots. The residues for which the HSQC crosspeaks were not shown in the free form and the peaks disappeared during the CSP experiments are shown as negative and green bars, respectively. J) The AD39-binding surfaces of $^{15}$NUBE2D2 (panel-F) are shown following the degree of CSP: (a) the front and right side, residues 2–16 (a1) and 94–103 (N-terminal a3), (b) the rear side, residues 70–75 (b3-to-loop) and 119–126 (b4). The reference orientation was defined for UBE2D2 (top, bottom, right, and front, respectively). The length of AD39 in an extended conformation is comparable to that of the long axis of UBE2D2.

Table 1. Binding constants ($K_d$, μM) measured by NMR CSP and ITC ($n$, ΔH, kcal mol$^{-1}$) experiments.

| Description                  | $K_d$ (μM) | $n$ | ΔH (kcal mol$^{-1}$) | ΔS (kcal mol$^{-1}$ deg$^{-1}$) |
|------------------------------|------------|-----|----------------------|-------------------------------|
| $^{15}$NTAD$_{p53}$ + UBE2D2 | 736 ± 39   | –   | –                    | –                             |
| $^{15}$NTAD$_{p53}$ + RINGMUL1:UBE2D2 | 171 ± 38 | –   | –                    | –                             |
| UBE2D2 + RINGMUL1            | 28 ± 15$^b$ | 0.71 ± 0.08$^b$ | 1479 ± 906$^b$ | 26.0 ± 4.3$^b$ |
| UBE2D2 + RINGMUL1:UBE2D2     | 13 ± 3     | 0.65 ± 0.13 | 2518 ± 571 | 30.9 |
| RINGMUL1:UBE2D2 + AD39       | 43 ± 18    | 0.80 ± 0.18 | 858 ± 247 | 22.8 |
| RINGMUL1:UBE2D2 + TAD$_{p53}$| 28 ± 10    | 0.69 ± 0.20 | 1061 ± 381 | 24.4 |
| RINGMUL1:UBE2D2                      | 184 ± 13$^b$ | 0.88 ± 0.01$^b$ | –1134 ± 16$^b$ | 13.3 ± 0.1$^b$ |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + AD39 | 175 ± 34 | 0.89 ± 0.25 | –1145 ± 372 | 13.4 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 193 ± 29 | 0.87 ± 0.08 | –1123 ± 130 | 13.2 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 111 ± 31 | 1.01 ± 0.21 | –673 ± 177 | 15.8 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 129 ± 22 | 1.29 ± 0.32$^b$ | –629 ± 125$^b$ | 15.7 ± 0.7$^b$ |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 105 ± 20 | 1.65 ± 0.16 | –596 ± 76 | 16.2 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 151 ± 21 | 1.11 ± 0.16 | –719 ± 122 | 15.1 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 116 ± 16 | 0.95 ± 0.12 | –466 ± 71 | 16.4 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 143 ± 23 | 1.47 ± 0.16 | –735 ± 102 | 15.1 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 34 ± 16$^b$ | 0.67 ± 0.19$^b$ | 485 ± 71$^b$ | 20.6 ± 4.3$^b$ |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 22 ± 4 | 0.54 ± 0.03 | 380 ± 31 | 22.6 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 26 ± 6 | 0.58 ± 0.05 | 538 ± 63 | 22.8 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 57 ± 24 | 0.95 ± 0.33 | 516 ± 211 | 21.2 |
| RINGMUL1:UBE2D2 RAS:UB$_{OE}$ + TAD$_{p53}$ | 33 ± 7 | 0.62 ± 0.05 | 505 ± 59 | 22.2 |

$^a$The HSQC peaks of the AD39 region of TAD$_{p53}$ were traced to obtain the $K_d$ values.; $^b$The average value and standard deviation of the multiple measurements.; The bold values are the average values of the multiple measurements.

Crystal structure of RINGMUL1 in the RINGMUL1:UBE2D2 complex

The crystal structure of the RINGMUL1:UBE2D2 complex was solved at 2.7 Å resolution (Fig. 2A) by molecular replacement (MR) using the previous UBE2D2 coordinate. The structure of RINGMUL1 alone was solved at 1.8 Å resolution by the MR using the RINGMUL1 coordinate in the determined complex structure (Table S1). The crystal of UBE2D2 alone was also obtained during the crystal screens of the RINGMUL1:UBE2D2 complex (Table S1), and the structure (1.8 Å resolution) was almost identical with the previously reported structures (PDB, 2CLW, and 2ESK). Although we also attempted to co-crystallize the RINGMUL1 complexes along with UBE2D2$^{SSR/CSR/CSK-UB^K_{RSR}}$, isopeptide (UBE2D2$^{SSR/CSR/CSK-UB^K_{RSR}}$-$^{IP}$), and UBE2D2$^{SSR/CSR/CSK-UB^K_{RSR}}$, oxester (UBE2D2$^{SSR/CSR/CSK-UB^K_{RSR}}$-$^{OE}$), and RINGMUL1:UBE2D2$^{SSR/CSR/CSK-UB^K_{RSR}}$ complex with the 2,4-dinitrophenyl dye-linked AD39 peptide, in which the colour of the dye was used to determine the specific complex crystal containing the AD39 peptide, the results were unsuccessful.

The failure of molecular replacement (MR) with the NMR ensemble structures of RINGMUL1 (PDB, 6k2k) could be attributed to the structural discrepancy in the region containing residues 329–334 (Fig. 2B). Since the crystal structures of RINGMUL1 are consistent with the $^{15}$N-$^1$H residual dipolar coupling (RDC) data of $^{15}$NRINGMUL1 measured in solution (Fig. 2C), the discrepancy might arise from missing NOE-assignments during the previous automatic CYANA calculation. The greater deviation of the calculated RDC values among the six conformers of the higher resolution crystal structures of the RINGMUL1 alone reflected the existence of innate structural heterogeneity in solution, which substantiated the heterogeneous peak intensity of its HSQC spectrum [14]. The RDC values calculated for the four conformers of RINGMUL1 in the
Fig. 2. Characterizations of the RING\textsubscript{MUL1}:UBE2D2 interaction by X-ray and NMR. (A) Four conformers in the crystal structure of RING\textsubscript{MUL1}:UBE2D2 are superimposed on the basis of UBE2D2. The pairwise RMSD values were calculated for the backbone atoms (CA, C, O, and N) of the well-structured regions (UBE2D2, M1–Y145; RING\textsubscript{MUL1}, A301–P348). The RMSD value of the RING\textsubscript{MUL1} part greatly increases from 0.36 to 1.81 Å, when their coordinates of four conformers were fixed to the superimposed UBE2D2. (B) The previous NMR ensemble structures (PDB, 6K2K; green) were overlaid with the six superimposed conformers of the RING\textsubscript{MUL1} crystal structure. (C) The $^{1}H$–$^{15}N$ RDC values of $^{15}N$RING\textsubscript{MUL1} measured in solution (open circle) were compared to the crystal structures of RING\textsubscript{MUL1} alone (blue) and RING\textsubscript{MUL1} in the complex with UBE2D2 (red). (D) The six conformers of the RING\textsubscript{MUL1} structure (blue) alone are superimposed with the four RING\textsubscript{MUL1} molecules of the RING\textsubscript{MUL1}:UBE2D2 complex (red). The CSPs of $^{15}N$RING\textsubscript{MUL1} (E) and $^{15}N$UBE2D2 (F) in the presence of non-labelled UBE2D2 and RING\textsubscript{MUL1}, respectively are shown as bar plots with the same colours used before. (G) The CSPs of $^{15}N$UBE2D2 induced by RING\textsubscript{MUL1} (panel-F) are mapped to the UBE2D2 structure. RING\textsubscript{MUL1} binding considerably perturbed the specific regions of UBE2D2 (β1-to-β2, and α3 segments) that are used for the binding of UB\textsubscript{BS} and the UB\textsubscript{D}. The residues of UBE2D2 that are close to L306 of RING\textsubscript{MUL1} (< 3.5 Å) are shown as a sphere model (boxed).
RINGMUL1 induced CSP in the wider regions of 15N-UBE2D2

Based on the complex structures, we examined the interaction between RINGMUL1 and UBE2D2 using CSP experiments. The HSQC spectrum of 15N-RINGMUL1 exhibited high heterogeneity in the presence of only half-molar UBE2D2 and many peaks disappeared (Fig. 2E and Fig. S1A), and many HSQC crosspeaks of 15N-UBE2D2 also disappeared in the presence of RINGMUL1 (Fig. 2F and Fig. S1B), which were likely correlated with the positional plasticity of the crystal structures of RINGMUL1 and UBE2D2 (Fig. 2A). It has been reported that the allosteric effect of the non-covalent binding of UB (UB_{BS}) to the backside of UBE2D2 (α1-to-β2; Fig. 1J, bottom region) increases the binding affinity of RINGRNF38 to UBE2D2{UB_{OE}}, and not to UBE2D2 (K_{d}, 89, 73, 4.6, and 0.36 μM for UBE2D2, UBE2D2:UB_{BS}, UBE2D2{UB_{OE}}, and UBE2D2{UB_{OE}}:UB_{BS}, respectively. Moreover, it also increases the intrinsic lysole reactivity of UBE2D2-UB [16]. Interestingly, the RINGMUL1-binding induced strong CSPs in the regions of 15N-UBE2D2 (α1-to-β2; α2-to-the cleft α3), which were evidently distant from the direct RINGMUL1-interacting regions (Fig. 2G) and were used for conformation with UB_{BS} and UB_{D}.

L306 of RINGMUL1 fits in the cleft formed by α1 (R5, K8, E9, D12) and the N-terminal α3 (T98) of UBE2D2 (Fig. 2G, inset), which may define the molecular basis of the occurrence of higher amounts of CSPs of UBE2D2 induced by the RINGMUL1-binding along with the positional plasticity of RINGMUL1 in the complex. The corresponding residues of other RING domains are mostly conserved as Leu (4auq, RINGBRIC7; 5d1k, RINGRNF25; 5ulh, RINGRNF165; 6fel, RINGTRIM25; 5dm0, RINGARK2C; and 4pql, RINGRNF146), Ile (6w9d, RINGRNF12), and Met (6hpr, RING_CLAPI; 3eb6, RING_CLAP2; 4v3k, RINGRNF4; 4iap4, RINGRNF38), although these residues are changed to Cys (2yho, RING_IDOL), Ala (1fbv, RING_CBTL), Gln (5mnj, RING_MDM2), and Glu (5vzw, RING_TRIM23) at certain instances. The molecular mechanism underlying the activity of UB_Bs remains unclear [17], and the finding that RINGMUL1 induces the exceptionally high levels of CSPs in the regions for inter- and intra-molecular binding of UB_{BS} and UB_{D} supports the hypothesis that the binding of UB_{BS}, UB_{D}, and RINGMUL1 to UBE2D2 is linked allosterically.

The disappeared HSQC crosspeaks in the CSP experiments of UBE2D2 and RINGMUL1 are likely dependent on intermediate binding exchange of the NMR time scale (K_{d}, ~1 μM), resulting in peak broadenings. Both the HSQC spectra of 15N-UBE2D3 and 15N-UBE2D3RS-UB_{OE} could be traceable in the presence of E4BU [15], and the extensive peak disappearance observed in the case of RINGMUL1 was not noted. Through isothermal titration calorimetry (ITC) measurements, we determined the binding affinity to explain the reason for the larger CSP of UBE2D2 in the presence of RINGMUL1. The affinity of RINGMUL1 for UBE2D2 was relatively weaker (~28 ± 15 μM) than the expected one (Table 1 and Fig. S2), since a binding event of this K_{d} value likely causes a CSP change during the CSP experiments. The binding of RINGMUL1 and UBE2D2 was an endothermic reaction (ΔH, 1497 ± 906 cal·mol⁻¹; Table 1), and thus the formation of RINGMUL1:UBE2D2 should be entropy-driven to accomplish the negative Gibbs free energy (ΔG = ΔH – TAS). The K_{d} value of RINGMUL1:UBE2D2 is still lower than those of the other RING/Ub-box:E2 interactions characterized by NMR experiments (E4BU:UBE2D3RS, 97 μM; RING_BRIC7:UBE2D2, too week; RING_BRIC7:UBE2D2{UB_{OE}, 136 μM} [15,18]). The higher binding affinity for UBE2D2 in addition to the positional plasticity of RINGMUL1 causes the apparent CSPs in large areas of UBE2D2. UBE2D2 might have an intrinsic dynamic motion in this area, and the RINGMUL1-binding selects a specific conformation resulting in the CSP on a large area of UBE2D2. However, no clear relaxation

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characterization of the RINGMUL1:UBE2D2–UB mimetics

Prior to investigation on the binding of RINGMUL1:UBE2D2 and RINGMUL1:UBE2D2–UB mimetics to TADp53, we first characterized the stable mimetics of UBE2D2–UB, such as UBE2D2–UBOE or UBE2D2–UB isopeptide (UBE2D2–UBip) by HSQC experiments. Since UBE2G1C90S–UBOE synthesizes K48-linked di-UB even though its activity is lower than that of the thioester [20], UBE2D2C85S (UBE2D2S)–UBOE seems to be an active mimetic of UBE2D2–UB. Therefore, UBK48R, and not wild-type UB, was used to synthesize stable mimetics via E1-mediated conjugation. UBE2D2S22R (UBE2D2R) was used to disrupt the inter-molecular interaction between the attached UB and the backside of different UBE2D2 molecules [21]. To further increase the sensitivity of HSQC experiments, 2H/13C/15N-labelled UBK48R (DCNUBR) instead of 15N-labelled UBK48R (15NUBR) was used for the enzymatic conjugation. The HSQC spectrum of UBE2D2S22R.C85S–DCNUBROE was similar to that of free DCNUBR, except for the C-terminal residues of UB (Figs 3A and 4A). UBE2D2RS–DCNUBR showed that UB and UBOE were stable in buffer solution (pH 6.5), and no apparent hydrolysis was detected after performing the HNCA experiment at 5 °C. However, the addition of RINGMUL1 extensively destabilized UBE2D2RS–DCNUBR, which prevented the acquisition of one clean HSQC spectrum at 25 °C. The half-life of UBE2D2RS–DCNUBR in the presence of RINGMUL1 was estimated to be ~1.7 h, and it increased to ~10 h at 5 °C (Fig. 5). This half-life is considerably less than those previously reported for the complexes of E4B minimal U-Box and UbchH5.C85S–UB oxyester (E4BU:UBE2D3S–UBOE; 10 h at pH 5.75 and 20 °C) [15], RINGBIRC7: UBE2D2S–UBOE (hydrolysis after 1–3 days at pH 7.0 and 4 °C) [18], and UBE2G1C90S–DCNUBR (8.8 h at pH 7.0 and 25 °C), in which the acidic loop of UBE2G1 mimics the effect of the RINGE3 domain [20].

Since the isopeptide bond between K85 of UBE2D2 and G76 of UB is believed to be more stable than that of the oxyester, UBE2D2RK–DCNUBRp53 was prepared following the previously reported method [22]. Although UBE2D2RK–DCNUBRp53 was initially thought to be weakly hydrolysed in the presence of RINGMUL1, it was confirmed later to be stable using the SDS/PAGE analysis (Fig. 5). We also prepared UBE2D2RAS–DCNUBOE; the conserved N77 of UBE2D2 that is located near the active C85 residue, stabilizes the oxyanion intermediate during ubiquitylation [23]. The N77A mutation apparently increased the stability of UBE2D2RAS–DCNUBOE, and any HSQC crosspeak of free DCNUBOE was not detected during NMR experiments with RINGMUL1, RINGMUL1:AD39, and RINGMUL1:TADp53. Since UBE2D2RKR–DCNUBRp53 displayed a lower response to the binding of RINGMUL1 and AD39 compared to UBE2D2RS–DCNUBRp53 likely due to the different geometry between the isopeptide and oxyester (Fig. 6A vs Fig. 3B,C), we focused on UBE2D2RS–DCNUBOE and UBE2D2RAS–DCNUBOE as active and stable mimetics, respectively, for further studies to evaluate their detailed binding modes for RINGMUL1 and TADp53.

UBE2D2RS–UBOE was different from UBE2D2RAS–UBOE in terms of closed conformation and interactions with RINGMUL1 and TADp53

We measured each clean HSQC spectrum of UBE2D2RS–DCNUBOE in the presence of RINGMUL1 and RINGMUL1:AD39 at 5 °C. The HSQC spectra of UBE2D2RS–DCNUBOE and free DCNUBR showed that the UB exhibited less intramolecular interactions with UBE2D2RS and favoured an open conformation (Figs 3A and 4A), as previously shown in UBE2D3RS–15N–UBOE [24], RINGE3 activates E2–UB in a closed conformation and renders the nucleophilic attack by a Lys residue of a target protein [25]. The UB of UBE2D2RS–DCNUBRp53 likely assumed a closed conformation in the presence of RINGMUL1 (Figs 3B and 4B). The binding exchange kinetics of UBp53 seemed to shift from a fast (weak binding, CS-moving) to an intermediate (stronger binding, disappearance of peak due to line-broadening) NMR time scale. It has been well known that the residues 6–14 and 41–50 of UBp53 play an important role in switching to a closed conformation via an intramolecular interaction with E2 proteins [15,24,26]. Additionally, the RINGMUL1-binding caused extreme peak-broadening of the C-terminal part of DCNUBOE (residues 70–76; Figs 3B and 4B).

Unexpectedly, the HSQC spectrum of UBE2D2RAS–DCNUBRp53 showed that its UBp53 already adapted to a more closed conformation even in the absence of RINGMUL1 (Fig. 3D). The scale of the dispersion curve supporting the presence of an exchanging motion of μs-ms time scale was identified in the extreme CPMG experiment [19] of 15N–UBE2D2 alone (not shown). Therefore, the large area CSP of UBE2D2 induced by the RINGMUL1-binding does not seem to be dependent on the conformational selection mechanism of UBE2D2 itself.

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CSPs (Fig. 4D) was considerably higher than those of UBE2D2RS-DCNUBR (Fig. 4A) and RING MUL1:UBE2D2RS-DCNUBR (Fig. 4B). The N77 residue of UBE2D2 can form a hydrogen bond with the G76 carbonyl group of the attached UBD, and it was clear that the presence of N77 inhibited the formation of a closed conformation of UBD. Since UBE2D2RS-DCNUBR already adopted a closed conformation, the RING MUL1-binding only caused a marginal CSP of DCNUBRD (Figs 4E and 6B). Moreover, the

Fig. 3. Monitoring the attached UBD of UBE2D2RS-DCNUBR and UBE2D2RS-DCNUBR via 1H-15N HSQC experiments. All NMR experiments were performed at 5 °C, and the HSQC spectra were overlaid to that of free DCNUBR (black). The 1H-15N TROSY-HSQC spectra of 0.1 mM UBE2D2RS-DCNUBR (A), RING MUL1:UBE2D2RS-DCNUBR (1:1) (B), and RING MUL1:UBE2D2RS-DCNUBR:AD39 (1:1:2) (C) were recorded with the reduced 15N-dimension to decrease the acquisition time. (D) The HSQC spectrum of 0.1 mM UBE2D2RS-DCNUBR shows that the N77A mutation of UBE2D2 considerably affects the conformation of UBD in the absence of RING MUL1. The folded peaks are indicated with blue letters. When the peaks of free DCNUBR are only visible, these are indicated with the subscript F.

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RINGMUL1-binding also resulted in the different CSP pattern of UBE2D2 RAS–DCNUBR OE from that of UBE2D2 RS–DCNUBR OE. The CSPs of UBE2D2 RS–DCNUBR OE mainly occurred in residues 41–50 of UB\textsubscript{D} (Fig. 4B,H), whereas those of UBE2D2 RAS–DCNUBR OE mainly occurred in residues 6–14, including T9 located near the bound RINGMUL1 (Fig. 4E,H). The model structures of RINGMUL1:UBE2D2 RS–UB\textsubscript{D} OE...
Recognition of TAD<sub>p53</sub>, IDP by RING<sub>MUL1</sub>:UBE2D2–UB

The estimation of stabilities of (A) UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> and (B) UBE2D2<sup>RS,DCN</sup>UB<sup>R,IP</sup> in the presence of RING<sub>MUL1</sub>. The hydrolyses of UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> and UBE2D2<sup>RS,DCN</sup>UB<sup>R,IP</sup> were assessed by SDS/PAGE analysis. A small amount of E1 fragments (E<sub>1frag</sub> and E<sub>1frag</sub>*) remained after the purification of UBE2D2–UB mimetics. The hydrolysis of UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> was greatly enhanced by the RING<sub>MUL1</sub>-binding, but that of UBE2D2<sup>RS,DCN</sup>UB<sup>R,IP</sup> was not. The half-lives of UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> were 1.67 ± 0.02 and 10.3 ± 1.1 h at 25 and 5°C, respectively.

RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> exhibits an enhanced binding affinity for TAD<sub>p53</sub> compared to UBE2D2:RING<sub>MUL1</sub>

The K<sub>d</sub> values between RING<sub>MUL1</sub>:UBE2D2 and TAD<sub>p53</sub> (171 ± 38 μM) was estimated by the CSP experiments with <sup>15</sup>N-TAD<sub>p53</sub> as increasing the concentration of RING<sub>MUL1</sub>:UBE2D2, in which the HSQC peaks of the AD49 region were traced (Table 1). Similar binding affinities of TAD<sub>p53</sub> and AD39 for RING<sub>MUL1</sub>:UBE2D2 (111 ± 31 and 184 ± 13 μM, respectively) were also confirmed via ITC experiments (Table 1 and Fig. S2). Interestingly, the ΔΗ value of the binding between RING<sub>MUL1</sub>:UBE2D2 and TAD<sub>p53</sub> (~673 kcal·mol<sup>−1</sup>) was higher than that between RING<sub>MUL1</sub>:UBE2D2 and AD39 (~1134 kcal·mol<sup>−1</sup>). Thus, the higher binding affinity of TAD<sub>p53</sub> to RING<sub>MUL1</sub>:UBE2D2 compared to that observed with AD39 was attributed to more entropic contribution (−ΔS) arising from the SN15 region.

Although UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> did not seem to be identical to UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup>, it was used to study the binding thermodynamics of RING<sub>MUL1</sub> and TAD<sub>p53</sub> due to its high stability in solution. The CSPs of UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> were 1.67 ± 0.02 and 10.3 ± 1.1 h at 25 and 5°C, respectively.

The presence of AD39 resulted in the disappearance of the many HSQC crosspeaks of RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> (Figs 3C and 4C). The CSPs of RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> induced by the AD39-binding (Fig. 4F) were much less than those of RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> (Fig. 4C).

The binding of TAD<sub>p53</sub> resulted in higher CSP levels of RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> (Fig. 4G), and the HSQC spectrum (Fig. 6B) showed increased similarity with that of RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup>:AD39 (Fig. 3C).

RING<sub>MUL1</sub>:UBE2D2<sup>RS,DCN</sup>UB<sup>R,OE</sup> exhibits an enhanced binding affinity for TAD<sub>p53</sub> compared to UBE2D2:RING<sub>MUL1</sub>

The AD39 region of TAD<sub>p53</sub> reportedly forms an α-helical structure when binding to Bcl-X<sub>L</sub> [27] and MDM2 [28]. We examined whether the secondary structure of AD39 was changed in the presence of RING<sub>MUL1</sub>:UBE2D2 and TAD<sub>p53</sub> (~673 kcal·mol<sup>−1</sup>) was higher than that between RING<sub>MUL1</sub>:UBE2D2 and AD39 (~1134 kcal·mol<sup>−1</sup>). Thus, the higher binding affinity of TAD<sub>p53</sub> to RING<sub>MUL1</sub>:UBE2D2 compared to that observed with AD39 was attributed to more entropic contribution (−ΔS) arising from the SN15 region.

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of \textit{RINGMUL1:UBE2D2~UB} via circular dichroism (CD) spectroscopy. The $K_d$ value of 129 \textmu M (Table 1) assumed that \(~0.45\) fraction of the AD39 bound the \textit{RINGMUL1:UBE2D2~UB}. However, no increased formation of an $\alpha$-helical structure was identified (Fig. 7). It is likely that TADp53 binds \textit{RINGMUL1:UBE2D2~UB} without the formation of any defined secondary structure.

\textbf{The increased binding affinity of \textit{RINGMUL1:UBE2D2~UB} for TADp53 depends on multivalent interactions}

Tracing of TADp53-binding surfaces via CSP experiments using $^{15N}$\textit{UBE2D2} and $^{15N}$\textit{RINGMUL1} even in the context of stable \textit{RINGMUL1:UBE2D2^{RAS}~UB^{OE}} was impossible, since most HSQC peaks of $^{15N}$\textit{UBE2D2^{RAS}~DCN~UB^{OE}} disappeared in the complexes due to a severe exchange peak-broadening (not shown). Even deuterated \textit{RINGMUL1 (DCN~RINGMUL1)} did not result in an analysable HSQC spectrum in the presence of \textit{UBE2D2^{RAS}~UB^{OE}} (not shown). Therefore, we attempted to map the binding sites for AD39 sequentially using (a) \textit{RINGMUL1:UBE2D2} (Fig. 8A,D, red CSP colour), (b) \textit{RINGMUL1:UBE2D2} (Fig. 8B,D, blue CSP colour), and (c) \textit{RINGMUL1:UBE2D2^{RS}~DCN~UB^{OE}} (Figs 4C and 8E), respectively. The hybrid model structure of a closed conformation (Fig. 4H) was used to show the surfaces of the attached UB$_D$ in \textit{RINGMUL1:UBE2D2^{DCN}~UB^{OE}} that bound to AD39.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{The $^1H$-$^15N$ HSQC spectra of UBE2D2$^{RK}~$DCN$^{UB^{IP}}$ (A) and UBE2D2$^{RAS}~$DCN$^{UB^{OE}}$ (B). The folded peaks are indicated with blue letters. The HSQC spectra of 0.1 mM UBE2D2$^{RK}~$DCN$^{UB^{IP}}$ and 0.1 mM UBE2D2$^{RAS}~$DCN$^{UB^{OE}}$ were recorded at 5 \textdegree C, in the absence and presence of the interacting counterparts. The used concentrations of RINGMUL1, AD39, and TADp53 were 0.1, 0.2, and 0.2 mM, respectively. The HSQC spectrum of UBE2D2$^{RK}~$DCN$^{UB^{IP}}$ was similar to that of UBE2D2$^{RAS}~$DCN$^{UB^{OE}}$ (Fig. 3A); however, changes in its spectra, induced by the binding of RINGMUL1 and RINGMUL1:AD39, were less than those of UBE2D2$^{RAS}~$DCN$^{UB^{OE}}$ (Fig. 3B,C), respectively.}
\end{figure}
UBE2D2 exhibited the presence of two regions that interacted with AD39, wherein the main region was located in the vicinity of the RING-binding site of UBE2D2 (α1 and N-terminal α3; Fig. 1F,J), which was also identified in the CSPs of RINGMUL1:~UBE2D2 (Fig. 8A,D). The AD39-binding to the main region of UBE2D2 could additionally stabilize the closed conformation of the attached UBΔ by shifting RINGMUL1 close to the UBΔ region of UBE2D2~UB. The CSPs of ~RINGMUL1:UBE2D2 by AD39 (Fig. 8B) showed that β1, the unstructured N- and C-terminal regions of ~RINGMUL1 were also perturbed (Fig. 8D, blue CSP colour). The higher amount of CSPs for the same concentration of AD39, compared to ~RINGMUL1 alone (Fig. 8C), indicated that the presence of UBE2D2 increased the binding affinity for AD39. Although the CSPs of ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE induced upon the AD39-binding were strongly coupled to the exchange motions of UBΔ, which also caused the significant CSPs located in the UBE2D2-contacting region of DCN~UBR~OE, the mapping of the CSPs of ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE on the surface model clearly indicated that the interface between ~RINGMUL1 and UBΔ of ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE complex also participated in its interaction with AD39 (Fig. 8E, marked with a cyan circle). The AD39-binding also caused the CSPs in another surface of UBΔ that is distant from the UBE2D2-binding interface (Fig. 8E, marked with yellow circle). Overall, the interaction of AD39 with ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE displayed a multivalency of cumulative weak bindings, which is a typical characteristic of IDP interaction [29].

TADp53 resulted in stronger CSPs of ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE compared to AD39 (Fig. 4F,G). The occurrence of synergistic interactions by TADp53, from AD39 to SN15, enhanced its binding affinity for ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE. The lengths of the extended AD39 region and the long horizontal axis of UBE2D2 were ~65 and ~50 Å (Fig. 1J), respectively, and the binding stoichiometry (N) of ~RINGMUL1:UBE2D2~RS~DCN~UBR~OE for TADp53 determined by the ITC measurements was close to 1 (0.67 ± 0.19; Table 1). Therefore, TADp53 seemed to encompass whole interaction regions involving ~RINGMUL1, UBE2D2, and UBΔ.

### Discussion

**Differential characteristics of various UBE2D2–UB mimetics upon ~RINGMUL1 binding**

Closed conformation of E2–UB reportedly plays an important role in the RING-E3-mediated ubiquitylation [15,17,30]. The UBΔ molecules of RING:UBE2D1~RS~UBE~OE (PDB, 4auq) and various other RING:E2–UBIP complexes (PDB, 4ap4, 4v3k, 5fer, 5mnj, 5vgw, and 6hpr) have the common converged position via the linchpin interactions (Fig. 9A). However, the closed conformation of UBΔ in the recent crystal structure of RINGRNF12:UBE2D2~RK~–UBIP [31] is different from those of the linchpin structures (Fig. 9B). Middleton et al. reported that direct contact between the UBΔ and RINGRNF12 was absent, indicating that although UBΔ in RINGRNF12:E2–UB might possess a range of conformational spaces, locking of UBΔ in the prime conformation via additional intramolecular RING-to-UBIP linchpin contact could be critical for ubiquitylation [31]. Interestingly, the CSP pattern of UBE2D2~RS~DCN~UBR~OE induced by ~RINGMUL1 (Fig. 4E) is well matched with the linchpin structure (Fig. 9C), in which the T9 of UBΔ has a close contact with the RINGE3 domains. However, that of UBE2D2~RS~DCN~UBR~OE by ~RINGMUL1 (Fig. 4B) is correlated with the structure of RINGRNF12:UBE2D2~RK~–UBIP (Fig. 9C). Although the molecular basis for the activation of UBE2D2–UB mediated by the ~RINGMUL1-binding remains unknown, a special UBE2D2-binding mechanism...
seems to be mediated by RINGMUL1. It may be postulated that subtle structural changes result in the occurrence of allosterically linked inter- and intra-molecular interactions of RINGMUL1:UBE2D2–UBE2D2RS–UBE2D2RS in which one instance among the positional plasticity of the bound RINGMUL1 favoured the transient structure of UBE2D2, thereby stabilizing a closed conformation conducive for the hydrolysis of the attached UB

The activation of UBE2D2–UB via a closed conformation of UBD induced by the RINGMUL1–binding

Fig. 8. The binding surfaces of RINGMUL1:UBE2D2 and RINGMUL1:UBE2D2RS–UBE2D2RS for AD39. The CSPs of 0.2 mM RINGMUL1:15NUBE2D2 (A), 15NRINGMUL1:UBE2D2 (B), and 15NRINGMUL1 (C) in the presence of 0.5 mM AD39 are shown as bar plots, respectively. The amount of CSPs induced by the AD39-binding is higher in 15NRINGMUL1:UBE2D2 than that in 15NRINGMUL1 alone. (D) The CSPs of RINGMUL1:15NUBE2D2 (panel-A) and 15NRINGMUL1:UBE2D2 (panel-B) induced by the AD39-binding are indicated on the surface model via red and blue tones, respectively. (E) The CSPs of 0.1 mM RINGMUL1:UBE2D2RS–UBE2D2RS by 0.2 mM AD39, as shown in Fig. 4C, are indicated on the surface model of RINGMUL1:UBE2D2–UBE2D2RS. The AD39-binding also caused the CSPs in the other surfaces of UBD that are distant from the UBE2D-binding interface; (a) the cyan circled area, residues 13–14 and 28–34, (b) the yellow circled area, residues 58–61.
depends critically on the presence of N77. Although detailed characterization of a closed conformation of RINGMUL1:UBE2D2RS–UBR OE remains further studies, our NMR data showed that the intra- and intermolecular interactions of UBE2D2RS–UBR OE were different from those of UBE2D2RAS–UBR OE in terms of the RINGMUL1- and TADp53-bindings. To explain the different closed conformations of UBE2D2RS–DCNUBR OE and UBE2D2RAS–DCNUBR OE conceptually, the Gibbs free energy of the RINGMUL1-induced closed conformation of UB D (ΔG system) can be divided into the overall contact energy between UBE2D2 and UB D (ΔG UB contacts) and the local energy at the junction of thioester (ΔG UB junction). The high energy state of ΔG UB junction that activates UBE2D2–UB and is critically dependent on the presence of N77 could be supported by decreasing ΔG UB contacts relying on the RINGMUL1 binding (Fig. 9D).

In vivo interaction of p53 and RINGMUL1:E2–UB

The multivalency of TADp53 originating from its innate IDP characteristics contributes to its enhanced binding affinity for RINGMUL1:UBE2D2–UB, which is supported by the estimated binding thermodynamic parameters (Table 1). The formation of the triple complex (RINGMUL1:UBE2D2–UB) provides additional surfaces for adopting TADp53. TADp53 caused the
appreciable CSPs in the various regions of RINGMUL1:UBE2D2 and RINGMUL1:UBE2D2RAS–UBOE (Fig. 8D,E), which is a typical characteristic of the IDP interaction, multivalency [29], and thus the TAD$_{p53}$-binding for RINGMUL1:UBE2D2RAS–UBOE is hardly converged to one binding mode. It is likely that the IDP nature of TAD$_{p53}$ and its multivalency occurring during binding to RINGMUL1:UBE2D2–UB may synergistically increase the binding affinity and affect the dynamic nature of UB$_D$. The binding stoichiometry (N) between RINGMUL1:UBE2D2RAS–UBOE and TAD$_{p53}$ is close to 1, and the AD39 region of TAD$_{p53}$ is primarily recognized as increasing the size of complexes from RINGMUL1 or UBE2D2 alone to RINGMUL1:UBE2D2RAS–UBOE. Therefore, the AD39 region is preferentially located in two interfaces (a) between the xl of UBE2D2 and RINGMUL1, and (b) between UB$_D$ and RINGMUL1, and then the SN15 region including K24 faces the accessible junction between UB$_D$ and UBE2D2, which can render the thioester bond susceptible to the nucleophilic attack by K24.

The measured $K_4$ values between RINGMUL1:UBE2D2RAS–UBOE and TAD$_{p53}$ (34 $\mu$m) may not be sufficient for achieving in vivo ubiquitylation of p53 by MUL1. However, native RINGMUL1:UBE2D2–UB could exhibit a higher affinity for TAD$_{p53}$, since RINGMUL1:UBE2D2RAS–UBOE displayed a stronger dynamic nature of UB$_D$ in response to the AD39-binding than that observed with RINGMUL1:UBE2D2RAS–UBOE. It is also well documented that the transcription-independent pro-apoptotic function of p53 is associated with stress-induced translocation of p53 to the mitochondria [32–34]. In vitro physical interaction between the C-terminal domain of p53 and negatively charged phospholipids has also been reported [35]. Therefore, the reduction in the dimensionality of the interaction from 3D to 2D in the mitochondrial outer membrane may enhance the interaction between MUL1 and p53, thereby facilitating rapid ubiquitylation of the translocated p53 for subsequent processes.

The complex formed by RINGMUL1 with UBE2D2–UB evidently aid the recruitment of TAD$_{p53}$ and this ubiquitylation mechanism has not been reported thus far. The in vivo regulation and proteostasis of IDPs that are dominantly observed in humans are important for understanding the mechanisms underlying the development of human diseases [29,36]. The ubiquitylation of IDPs could be one of the most commonly observed in vivo regulation mechanisms, in which direct recognition of IDPs by other RING$_{E3}$:E2–UB complexes may also be useful.

Materials and methods

Protein expression and purification

The human RINGMUL1 domain (residues 298–352), human TAD$_{p53}$ (residues 1–73), and UBE2D2 conjugating enzymes were prepared following the previously reported methods [11,14]. Non-tagged UB$_{K48R}$ proteins were prepared as per the previously reported method [37]. For the preparation of NMR samples, size exclusion chromatography (SEC) was performed using the NMR-buffer (pH 6.5, 50 mM MES, 50 mM NaCl, 5 $\mu$m ZnSO$_4$, and 1 mM DTT). In the other cases, SEC was performed with a buffer (pH 7.5, 25 mM Tris-HCl, 100 mM NaCl, and 1 mM DTT). The concentrations of all proteins were estimated using their extinction coefficient at 280 nm [38].

For the NMR experiments, the proteins were expressed in M9 minimal media after incubation for 6 h at 30 °C following isopropyl β-D-1-thiogalactopyranoside (IPTG) induction at 0.7–0.8 OD at 600 nm. Isotope-labelled proteins were expressed by growing Escherichia coli cells in M9 minimal media (1 L) supplemented with isotope-labelled ammonium chloride (1 g) and glucose (2 g). CELTONE base powder (1 g), vitamins, and trace metals were added to enhance cell growth. All isotope-labelled materials were purchased from Cambridge Isotope Laboratories Inc. The detailed composition of media and the culture method used for protein deuteration are described in previous reports [39].

The mouse E1 gene was cloned into the pRSET-A vector, and then the plasmid was transformed into E. coli Rosetta (DE3). The E1 protein was expressed in LB medium for 6 h at 25 °C after IPTG induction at 0.7–0.8 OD at 600 nm. The cultured cells were resuspended in buffer (pH 8.0, 25 mM Tris-HCl, 0.5 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM PMSF). Non-specific protease activity was reduced by adding EDTA-free protease inhibitor cocktail tablets (Sigma-Aldrich, Seoul, Korea). After cell disruption by sonication, the supernatant was applied to a Histrap HP column (GE Healthcare, Seoul, Korea) as soon as possible. The E1 protein was eluted using 150 ml imidazole gradient with buffer (pH 8.0, 25 mM Tris-HCl, 0.5 mM NaCl, 0.5 mM imidazole, and 10 mM 2-mercaptoethanol). The protein fractions were dialysed into buffer (pH 8.0, 20 mM Tris-HCl and 1 mM DTT), and then was applied to a Hitrap-Q HP column (GE Healthcare). Elution was performed using a 150 mM NaCl gradient with buffer (pH 8.0, 20 mM Tris-HCl, 1 mM NaCl, and 1 mM DTT). The purified E1 protein was concentrated to ~2.0 mg·mL$^{-1}$, roughly estimated via SDS/PAGE, and then stored in a ~80 °C refrigerator.

Preparation of the UBE2D2–UB mimetics and estimation of their stabilities in solution

All mimetics, namely, UBE2D2–UBOE [20] and UBE2D2–UB$_{IP}$ [22], were enzymatically synthesized as previously
reported methods. The mimetics were synthesized via E1-mediated enzymatic reactions of E2 proteins (0.1 mM) in the presence of twice the amount of non-tagged DCN\textsubscript{UB}\textsuperscript{R} protein. Briefly, the reactions of UBE2D2\textsuperscript{RS} and UBE2D2\textsuperscript{RS}\textsubscript{AS} were performed in buffer (pH 9.0, 50 mM CHES, 150 mM NaCl, 1 mM DTT, 4 mM ATP, and 5 mM MgCl\textsubscript{2}) with ~4 μM E1 for 6 h at 35 °C. UBE2D2-UB\textsubscript{IP} was synthesized with ~6 μM E1 in buffer (pH 10.0, 50 mM CAPS, 150 mM NaCl, 1 mM DTT, 4 mM ATP, and 5 mM MgCl\textsubscript{2}) for 24 h at 35 °C. The synthesized UBE2D2-UB mimetics were purified by SEC using the Superdex-75 column in buffer (pH 6.5, 50 mM MES, and 50 mM NaCl).

Each purified 0.1 mM UBE2D2\textsuperscript{RS}-UB\textsuperscript{R,OE} and UBE2D2\textsuperscript{RS}-UB\textsuperscript{R,IP} was incubated with 0.15 mM RINGMUL1 in the NMR-buffer. The reaction mixtures were aliquoted and then were incubated at 25 and 5 °C, respectively. The reaction was stopped at an appropriate time by adding the SDS sample buffer, and then was stored at ~20 °C before the SDS/PAGE analysis. The protein bands of UBE2D2-UB mimetics were quantified with the ImageJ program (https://imagej.nih.gov/ij/). The half-lives of UBE2D2\textsuperscript{RS, DCN}\textsubscript{UB}\textsuperscript{R,OE} in the presence of RINGMUL1 were estimated by fitting to the equation of single exponential decay.

Preparation of peptide samples

SN15 (residues 15–30) and AD39 (residues 39–57) peptides, as well as the colour-dye derivative form of AD39, were purchased from PEPTRON Inc. (Daejeon, Korea). In the dye attached AD39 peptide, the Lys residue coupled to 2,4-dinitrophenyl dye (K-DNP) was attached to the C-terminal of the native form (AMDDLMSPDDEIQWFTED/K-DNP). All peptides were dissolved in an appropriate experimental buffer, and then an equimolar amount of NaOH as that of the Asp/Glu residues was added to maintain the solution pH value.

NMR experiments

To assign the backbone CSs of UBE2D2, the HNCACB, HN(CO)CA(CB), HNCO, and HN(CA)CO spectra were recorded using the Bruker 800 and 900 MHz spectrometers equipped with TCI-cryogenic probe. The HSQC crosspeaks of the mutated proteins (UBE2D2\textsuperscript{RS}, UBE2D2\textsuperscript{RS, AS}, UBE2D2\textsuperscript{R, K}, and UB\textsuperscript{R}) and their UB-conjugated forms were assigned by additional HNCA experiments. In the absence of additional comments, NMR experiments were conducted in the NMR buffer (pH 6.5, 50 mM MES, 50 mM NaCl, 5 μM ZnSO\textsubscript{4}, and 5% D\textsubscript{2}O) at 25 °C. The CSP data of HSQC peaks were processed using the equation, $\Delta N = \frac{H_0 - H}{H_0 + H}$, where $\Delta H$ and $\Delta N$ represent the CS differences of \textsuperscript{1}H and \textsuperscript{15}N, respectively. Analysis of the protein amide CSs deposited in the Biological Magnetic Resonance Bank (No. 36251) showed that the average distribution of \textsuperscript{15}N-CSs was 6-fold greater than that of \textsuperscript{1}H-CSs [40]. All NMR data were processed using the NMRFame program [41] while spectral analyses were conducted using the NMRFAM-SPARKY program [42].

Determination of crystal structures of the RINGMUL1 and UBE2D2:RINGMUL1 complex

Detailed crystallization conditions of the RINGMUL1 protein (15 mg·mL\textsuperscript{-1}) and the UBE2D2:RINGMUL1 complex (10 mg·mL\textsuperscript{-1}) have already been reported [11]. The crystal of UBE2D2 alone was obtained under the conditions of the reservoir buffer containing 0.1 mM sodium citrate (pH 5.6), 0.5 M ammonium sulfate, and 1.0 M lithium sulfate during the screening of the UBE2D2:RINGMUL1 crystal. The diffraction data were collected at beamline 7A at the Pohang Accelerator Laboratory, and data were indexed, scaled, and merged using the HKL-2000 software [43].

To solve the structure of RINGMUL1, we performed molecular replacement (MR) using the Phaser program [44]. Neither the recent NMR ensemble structures of RINGMUL1 (PDB code, 6K2K) nor the homology model calculated using the Phyre2 (Protein Homology/Analogous Recognition Engine) web portal [45] yielded the correct MR results. Therefore, the crystal structure of the UBE2D2:RINGMUL1 complex was solved by MR using the coordinate of UBE2D2 (PDB code, 2ESK), and then the crystal structure of RINGMUL1 alone was solved by MR using the coordinate of RINGMUL1 in the complex. Interactive model building and structure refinement were performed using the Coot program [46] and the PHENIX software suite [47]. The calculated crystal structures were validated using the MolProbity web portal [48].

Biophysical analyses

All protein samples, except the peptide samples, were prepared using a buffer (pH 6.5, 50 mM MES, 50 mM NaCl, 5 μM ZnSO\textsubscript{4}, and 1 mM TCEP) through dialysis or SEC. ITC experiments were performed using the Auto-iTC200 micro-calorimeter (Malvern Panalytical, Malvern, UK) at 10 °C. The low concentrated protein was loaded in the sample cell, and 15–30 higher concentrated titrant protein was placed in the syringe. ITC data were analysed using the MICROCAL ORIGIN™ software.

Circular dichroism spectra were recorded using the J-715 CD instrument (JASCO, Tokyo, Japan) with 0.05 cm path-length circular CD cell to measure high-concentration protein samples (0.1 mM, > 3.0 mg·mL\textsuperscript{-1}). All protein and peptide samples were prepared using a buffer (pH 6.5, 50 mM MES, 50 mM NaCl, and 5 μM ZnSO\textsubscript{4}).

Structure presentations and analyses

Visualizations of all structures were performed using the Chimera program [49]. The CSP data were presented via a
The PDB coordinates of UBE2D2 (PDB ID: 7BOL), RING\textsubscript{MUL1} (PDB ID: 6M2D), and RING\textsubscript{MUL1}:UBE2D2 (PDB ID: 6M2C) were deposited to protein data bank (PDB).

Acknowledgements
This work was supported by the KBSI grants (T39632 and C130000) and by the National Research Foundation grants funded by the Korean government (NRF-2017R1E1A1A01074403, NRF-2019M3E5D4069903, and NRF-2019M3A9C40476156). This work was also supported by the KRIBB Research Initiative Program (KGM9952112).

Conflict of interest
The authors declare no conflict of interest.

Author contributions
SWC, CKL, and KSR planned this research project. MSL and SOL performed the NMR and X-ray experiments, respectively. JC performed most of the additional experiments. MR, MKL, JHK, and EH were involved in the design and data interpretation of additional experiments including ITC. Overall data analyses were performed by JC and KSR. The manuscript was written by JC, SWC and KSR.

Peer review
The peer review history for this article is available at https://publons.com/publon/10.1111/febs.16360.

Data availability statement
The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** The interactions between RING_MUL1 and UBE2D2 were monitored by $^1$H–$^{15}$N HSQC experiments.

**Fig. S2.** The representative ITC data of the RING_MUL1-mediated interactions.

**Table S1.** Statistics of data collections and structure refinements.