Allosteric activation of the RNF146 ubiquitin ligase by a poly(ADP-ribosylation) signal

Paul A. DaRosa1,2*, Zhizhi Wang2*, Xiaomo Jiang3, Jonathan N. Pruneda1†, Feng Cong3, Rachel E. Klevit1 and Wenqing Xu2

Protein poly(ADP-ribosylation) (PARylation) has a role in diverse cellular processes such as DNA repair, transcription, Wnt signalling, and cell death1–4. Recent studies have shown that PARylation can serve as a signal for the polyubiquitination and degradation of several crucial regulatory proteins, including Axin and 3BP2 (refs 7–9). The RING-type E3 ubiquitin ligase RNF146 (also known as Iduna) is responsible for PARylation-dependent ubiquitination (PARdU)10–12. Here we provide a structural basis for RNF146-catalysed PARdU and how PARdU specificity is achieved. First, we show that iso-ADP-ribose (iso-ADPr), the smallest internal poly(ADP-ribose) (PAR) structural motif, results in robust activation (Fig. 1 and Extended Data Fig. 2), consistent with reports that RNF146 is a PARylation-dependent E3 that is sensitive to subtilisin digestion in the absence of PARdU specificity may be primarily determined by the substrate–TNKS interaction. We propose that the maintenance of unliganded RNF146 in an inactive state may serve to maintain the stability of the RNF146–TNKS complex, which in turn regulates the homeostasis of PARdU activity in the cell.

Two domains can be identified within the 358-residue sequence of RNF146: an amino-terminal RING domain, followed by a WWE domain that binds iso-ADPribose13 (Fig. 1a and Extended Data Fig. 1). RING E3 ligases activate a ubiquitin-conjugating enzyme (E2) to transfer ubiquitin directly from the E2 active site to a lysine residue of a substrate. The intrinsic ability of RING E3s to stimulate ubiquitin (Ub) transfer can be assayed by following Ub transfer from E2 to Ub (activated complex with a thioester linkage) to free amino acid lysine4. Unexpectedly, neither the purified RNF146(RING) domain nor full-length RNF146 significantly enhance the rate of Ub transfer from UbcH5 to lysine (Fig. 1b and Extended Data Fig. 2a). However, addition of the WWE ligands iso-ADPr or PAR to the full-length RNF146 or a fragment containing the RING and WWE domains (termed RNF146(RING-WWE)), but not a RING-only construct, results in robust activation (Fig. 1 and Extended Data Fig. 2), consistent with reports that RNF146 is a PARylation-dependent E3 (refs 10–12). Isothermal titration calorimetry (ITC) analysis shows that iso-ADPr binds to RNF146(RING-WWE) ten times tighter than to the WWE domain alone (dissociation constant (Kd) values of 39 nM and 372 nM, respectively; Extended Data Fig. 3a, b), suggesting that the presence of the RING domain contributes to ligand binding. Furthermore, RNF146(RING-WWE) is sensitive to subtilisin digestion in the absence of iso-ADPr, but is more resistant to proteolysis in the presence of iso-ADPr (Extended Data Fig. 3c), and NMR experiments indicate structural changes within the RING domain of the RING-WWE fragment after iso-ADPr binding (Extended Data Fig. 4). Altogether, the data indicate that both the RING and WWE domains are involved in PARdU binding and that ligand binding affects the conformational and/or stability of the RING domain, leading to increased E3 ligase activity.

The structural basis of RNF146 activation by iso-ADPr or PAR is evident in a 1.9 Å crystal structure of a RNF146(RING-WWE)–UbcH5a–iso-ADPr complex (Fig. 2 and Extended Data Table 1). The RING domain structure is largely similar to other structurally characterized RINGs (see below), and the RNF146(WWE) domain structure is almost identical to an existing crystal structure13 (Extended Data Fig. 5a). The most notable feature is the location of iso-ADPr, which contacts both the RING and WWE domains (Fig. 2b and Extended Data Fig. 5b). Contacts between the WWE domain and iso-ADPr are similar to those previously described13.

Figure 1 | Binding of iso-ADPr or PAR activates RNF146 E3 ubiquitin ligase activity. a, Domain structure of RNF146, with constructs used in this study shown: RNF146(RING), residues 30–89; RNF146(RING-WWE), residues 30–183. Five potential TNKS-binding motifs are shown in orange and numbered I to V. b, Coomassie-stained E2–Ub/lysine reactivity assays of full-length (FL) RNF146 (top) and RNF146(RING-WWE) (bottom) with the E2 UbcH5c. Active RING domains enhance the reactivity of E2–Ub with lysine and therefore speed the disappearance of the E2–Ub species with the corresponding appearance of free E2 and Ub. The WWE domain does not bind ADP-ribose (ADPr)15, M, molecular mass marker.
Although iso in the WWE domain and has van der Waals contacts with the ligand. RING residue Trp 65 forms a hydrogen bond with a main-chain carbocycle that can mediate a hydrogen bond with the adenine ring. In addition, hydroxyl groups on both ribose moieties of iso Lys 61 from the RING domain is within hydrogen-bond distance to hy-

Figure 2 | Crystal structure of the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex. a. Cartoon representation of the RNF146–UbcH5a complex with RING domain coloured blue, WWE domain coloured purple, and UbcH5a coloured green. Zn 2+ ions are shown as grey spheres, and the iso-ADPr ligand is represented as sticks. b. The RNF146–iso-ADPr interface. Left, surface electrostatic view of RNF146(RING-WWE), showing the iso-ADPr/PAR-binding pocket; centre, same view, cartoon representation; right, close-up view of iso-ADPr pocket. Polar contacts between protein and the ligand, iso-ADPr, are indicated by dashed lines; RING residues Lys 61 (magenta) and Trp 65 (orange) are highlighted. Lys 61 from the RING domain is within hydrogen-bond distance to hydroxyl groups on both ribose moieties of iso-ADPr and to a water molecule that can mediate a hydrogen bond with the adenine ring. In addition, RING residue Trp 65 forms a hydrogen bond with a main-chain carbocycle in the WWE domain and has van der Waals contacts with the ligand. Although iso-ADPr is buried in a valley between the RING and WWE domains, the phosphate groups on either end of iso-ADPr are exposed. Thus, the observed ligand orientation is consistent with the notion that RNF146 binds an internal unit of a PAR polymer.

In the crystal, the E2 enzyme UbcH5a binds to the RING domain at the canonical E2–E3 interface, away from the iso-ADPr-binding site (Fig. 2a). Similar to other RING E3–E2 complex structures, two Zn 2+ -binding loops and the central helix of the RING bend E2 loops 4 and 7. A slight difference in the orientation of the RING and E2 relative to other E2–E3 complex structures is probably due to crystal packing (Extended Data Fig. 6), as the E2–E3 interactions observed in solution by NMR are similar to other well-characterized systems (see below).

Insight into the conformational changes that accompany iso-ADPr binding is provided by comparison to an NMR structure of the unliganded RNF146(RING) domain (Protein Data Bank (PDB) accession 2D8T; RIKEN Structural Genomics/Proteomics Initiative). In the unliganded RING domain the central helix is one turn shorter, with residues 62–66 instead forming a loop that protrudes into the E2–E3 binding interface (Fig. 3a and Extended Data Fig. 7a). Trp 65 makes hydrophobic interactions with Ile 38, Leu 66, Ala 71, and Trp 65 is in a position to block E2 binding. Residues 62–66 adopt the helical structure associated with active RING domains in the iso-ADPr-bound structure. Thus, the RNF146(RING) domain can adopt two different conformations, and binding of iso-ADPr stabilizes an active structure with a functional E2-binding surface.

The proposed model is supported by NMR binding experiments. Addition of unliganded RNF146(RING-WWE) resulted in very minor perturbations to the NMR spectrum of 15N-UbcH5c while extensive perturbations occurred when iso-ADPr was also present (Fig. 3b and Extended Data Fig. 7b). Furthermore, the observed perturbations are highly similar to those observed with other RING-type E3s binding to UbcH5c and to UbcH5a (Fig. 3b and Extended Data Fig. 7c, d).

Mutational analysis was performed to understand the function of key residues in the iso-ADPr-induced conformational switch. Mutation of RING Lys 61, which makes several contacts with iso-ADPr, to Ala or Asp increased the Kd for iso-ADPr to values comparable to that of the WWE domain alone (Kd values of 214 nM (RING-WWE(Lys61Ala))) or 457 nM (RING-WWE(Lys61Asp)) versus 372 nM (WWE); Extended Data Fig. 3a, b). Although the Lys61Asp mutant can still bind ligand, it is not activated by iso-ADPr (Fig. 3c and Extended Data Fig. 8a). Thus, Lys 61 serves to couple ligand binding to the activation of the RING domain. RING Gly 62 may serve to maintain the inactive RING conformation by disrupting the central helix (Extended Data Fig. 8b). Mutation of Gly 62 to Ala in the context of both the RNF146(RING) and RNF146(RING-WWE) constructs was performed. In the absence of ligand, the mutants promote E2–Ub lysine reactivity (Fig. 3c and Extended Data Fig. 8a).
Extended Data Fig. 9a). Co-immunoprecipitation and pull-down assays clusters (ARCs) of TNKS (TNKS(5ARC), residues 173–961) (Fig. 4a) and full-length RNF146 forms a stable complex with the five ankyrin repeat co-migration on size-exclusion chromatography (SEC) showed that S and TNKS. Both glutathione specificity of RNF146 by testing for a direct interaction between RNF146 and the ARCs of TNKS, but a construct that includes motif I (RNF146(1–183)) could not bind to the ARCs of TNKS, with the motif I mutation having a stronger binding to TNKS(5ARC), with the motif I mutation having a stronger effect (Fig. 4a).

Knockdown of RNF146 in cells by short interfering RNA (siRNA) binding to TNKS and subsequent PARylation, RNF146 binds an internal unit of PAR. This causes a conformational change in the RING domain, which activates its ligase activity, enabling the polyubiquitination of substrate. HA, haemagglutinin tag.

Data Fig. 8c). RNF146(RING)(Gly62Ala) also shows increased E2 binding in NMR experiments (Extended Data Fig. 8d). Thus, Gly 62 may have a key role in the conformational transition of the central helix. Likewise, a Trp65Ala mutation in RNF146(RING-WWE) to disrupt Trp 65 interactions in the inactive state also increased basal E3 activity (Fig. 3c). The double mutant Gly62Ala/Trp65Ala of RNF146(RING-WWE) exhibits still greater activity than either of the single mutants (Fig. 3c). The mutational results are consistent with our model in which extension of the RING central helix and repositioning of Trp 65 from the E2–E3-binding site to the RING–iso-ADPr interface constitute the allosteric switch triggered by ligand binding.

Almost all known proteins regulated by PARD, including Axin and 3BP2, are PARylated by TNKS. We sought to understand the specificity of RNF146 by testing for a direct interaction between RNF146 and TNKS. Both glutathione S-transferase (GST) pull-down assays and co-migration on size-exclusion chromatography (SEC) showed that full-length RNF146 forms a stable complex with the five ankyrin repeat clusters (ARCs) of TNKS (TNKS(5ARC), residues 173–961) (Fig. 4a and Extended Data Fig. 4a). Co-immunoprecipitation and pull-down assays using full-length TNKS further support the direct RNF146–TNKS interaction in cells (Extended Data Fig. 4b, c).

The ARCs of TNKS recognize a consensus motif of RXXGDG, although our recent work suggested that deviations may be tolerated in some circumstances. As RNF146 contains no consensus motifs, we used molecular modelling to identify potential TNKS-binding sequences. Five potential motifs reside in the carboxy-terminal region of RNF146 (motif I to V; Fig. 1a and Extended Data Fig. 1); motif I (residues 193–199) is the most conserved. A C-terminal truncation (RNF146(1–183)) could not bind to the ARCs of TNKS, but a construct that includes motif I (RNF146(1–205)) bind TNKS(5ARC) detectably (Fig. 4a). In the context of full-length RNF146, mutations in either motif I (Gly199Val) or motif IV (Gly337Val/Gly338Val) reduced but did not abrogate the observed binding to TNKS(5ARC), with the motif I mutation having a stronger effect (Fig. 4a).

Figure 4 | RNF146 and TNKS form a tight complex crucial to PARD in vivo. a, GST pull-downs of GST-tagged RNF146 variants with untagged TNKS(5ARC) (residues 173–961), demonstrate a direct interaction between RNF146 and the five ARCs of TNKS. The interaction probably involves several TNKS-binding sites in RNF146 as various RNF146 mutations reduce but do not abolish TNKS binding (inputs are shown in Extended Data Fig. 9d). b, Immunoblots of Axin turnover rescue assay show that both the TNKS–RNF146 interaction and the RNF146 allosteric switch are important for PARD in cells. sipGL2, non-targeted siRNA control; siRNF146, siRNA targeting RNF146. c, Proposed TNKS–RNF146 PARD model. RNF146 is inactive when bound to non-PARylated TNKS in the cell. After substrate binding to TNKS and subsequent PARylation, RNF146 binds an internal unit of PAR. This causes a conformational change in the RING domain, which activates its ligase activity, enabling the polyubiquitination of substrate. HA, haemagglutinin tag.

PARD expression (Fig. 4b). By contrast, expression of the allosteric-switch mutant RNF146(Lys61Asp) resulted in similar Axin levels to the no-RNF146 control. Disruption of the RNF146–TNKS complex via expression of the triple mutant RNF146(Gly199Val/Gly337Val/Gly338Val) also led to increased Axin levels (Fig. 4b). Thus, coupling of PAR binding to the stimulation of E3 activity, and the ability to form an RNF146–TNKS complex are both crucial to cellular PARD regulation and turnover of Axin.

In summary, we propose that RNF146 and TNKS exist as a complex in which the RING domain is predominantly in an inactive state (Fig. 4c). TNKS is responsible for substrate selection and PARylation. Subsequent ubiquitination of the PARylated substrate requires that an internal PAR moiety binds to RNF146 to trigger the allosteric switch to the RING E3-active state. PARylated substrate is probably held in the RNF146–TNKS complex via its interaction with TNKS, as the RNF146–TNKS interaction is required for ubiquitination of substrate in cells. Thus, our studies have revealed specific insights into the regulatory and substrate-recruitment mechanism of PARD and have defined the molecular mechanism by which the RNF146(RING) domain is allosterically switched by non-covalent small molecule binding. These insights may aid in the design of RNF146 inhibitors that may be useful for cancer treatment, as RNF146 overexpression is associated with lung cancer.

Received 10 February; accepted 1 September 2014.
Published online 19 October 2014.

©2015 Macmillan Publishers Limited. All rights reserved
3. Luo, X. & Kraus, W. L. On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1. Genes Dev. 26, 417–432 (2012).

4. Wang, Y., Dawson, V. L. & Dawson, T. M. Poly(ADP-ribose) signals to mitochondrial AIF: a key event in parthanatos. Exp. Neurol. 218, 193–202 (2009).

5. Varga, L. 50 Years of poly(ADP-ribosyl)ation. Mol. Aspects Med. 34, 1043–1045 (2013).

6. Hsiao, S. J. & Smith, S. Tankyrase function at telomeres, spindle poles, and beyond. Biochimie 90, S3–92 (2008).

7. Huang, S. M. et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461, 614–620 (2009).

8. Levaot, N. et al. Loss of Tankyrase-mediated destruction of 3BP2 is the underlying pathogenic mechanism of cherubism. Cell 147, 1324–1339 (2011).

9. Luo, X. & Kraus, W. L. On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1. Nature Struct. Mol. Biol. 18, 1473–1483 (2011).

10. Guettler, S. et al. Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease. Cell 147, 1340–1354 (2011).

11. Wang, Z. et al. Recognition of the iso-ADP-ribose moiety in poly(ADP-ribose) by Tankyrase explains the mechanism of ubiquitin transfer by a RING dimer. Nature Struct. Mol. Biol. 18, 1473–1483 (2011).

12. Kang, H. C. et al. Iduna is a poly(ADP-ribose) (PAR)-dependent E3 ubiquitin ligase that regulates DNA damage. Proc. Natl Acad. Sci. USA 108, 14103–14108 (2011).

13. Zheng, N., Wang, P., Jeffrey, P. D. & Pavletich, N. P. Structure of a c-Cbl–UbcH7 conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. Proc. Natl Acad. Sci. USA 109, 1500–1505 (2012).

14. Wenzel, D. M., Lissounov, A., Brzovic, P. S. & Klevit, R. E. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature 474, 105–108 (2011).

15. Zheng, N., Wang, P., Jeffrey, P. D. & Pavletich, N. P. Structure of a c-Cbl–Ubch7 complex: RING domain function in ubiquitin-protein ligases. Cell 102, 533–539 (2000).

16. Das, R. et al. Allosteric regulation of E2:E3 interactions promote a processive ubiquitination machine. EMBO J. 32, 2504–2516 (2013).

17. Dong, H. et al. Structural basis for autoinhibition and phosphorylation-dependent activation of c-Cbl. Nature Struct. Mol. Biol. 19, 184–192 (2012).

18. Bentley, M. L. et al. Recognition of UbcH5c and the nucleosome by the Bmi1/Ring1b ubiquitin ligase complex. EMBO J. 30, 3285–3297 (2011).

19. Campbell, S. J. et al. Molecular insights into the function of RING finger (RF)-containing proteins HRNF8 and HRNF68 in Ubc13/Mms2-dependent ubiquitylation. J. Biol. Chem. 287, 23900–23910 (2012).

20. Yin, Q. et al. E2 interaction and dimerization in the crystal structure of TRAF6. Nature Struct. Mol. Biol. 16, 656–666 (2009).

21. Pruneda, J. N. et al. Structure of an E3:E2–Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. Mol. Cell 47, 933–942 (2012).

22. Plechanovová, A., Jaffray, E. G., Tatnam, M. H., Naismith, J. H. & Hay, R. T. Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. Nature 489, 115–120 (2012).

23. Hodson, C., Purkiss, A., Miles, J. A. & Walden, H. Structure of the human FANCL RING-Ube2T complex reveals determinants of cognate E3–E2 selection. Structure 22, 337–344 (2014).

24. Lee, H., Buettow, L., Sibbett, G. J., Cameron, K. & Huang, D. T. BiRC7–E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. Nature Struct. Mol. Biol. 19, 876–883 (2012).

25. Brzovic, P. S. et al. Binding and recognition in the assembly of an active BRCAl/BARD1 ubiquitin-ligase complex. Proc. Natl Acad. Sci. USA 100, 5646–5651 (2003).

26. Morrone, S., Cheng, Z., Moon, R. T., Cong, F. & Xu, W. Crystal structure of a Tankyrase-Axin complex and its implications for Axin turnover and Tankyrase substrate recruitment. Proc. Natl Acad. Sci. USA 109, 1500–1505 (2012).

27. Gao, Y. et al. Overexpression of RNF146 in non-small cell lung cancer enhances proliferation and invasion of tumors through the Wnt/β-catenin signaling pathway. PLoS ONE 9, e85377 (2014).

28. Andrabi, S. A. et al. Iduna protects the brain from glutamate excitotoxicity and stroke by interfering with poly(ADP-ribose) polymer-induced cell death. Nature Med. 17, 692–699 (2011).

Acknowledgements We thank P. Brzovic and N. Zheng for discussions and editorial comments. We are grateful to the staff at Advanced Light Source (ALS) beamlines BL 8.2.1 and 8.2.2 for assistance with synchrotron data collection. This work was supported by National Institutes of Health (NIH) grant RO1 GM099766 to W.X. and R.E.K. and NIH T32 GM07270 to P.A.D.

Author Contributions P.A.D and Z.W. performed experiments. X.J. performed cell-based assays. F.C. and J.N.P. provided critical insight. P.A.D, Z.W., R.E.K. and W.X. wrote the paper. All authors provided editorial comments.

Author Information The atomic coordinates and structure factors of the RNF146–UbcH5a–iso-ADPr complex are deposited in the Protein Data Bank (PDB) with the accession code 4QPL. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.X. (wxu@uw.edu) and R.E.K. (klevit@uw.edu).
METHODS

Plasmids, protein expression and purification. Human RNF146 was subcloned into a pET-28a vector with an N-terminal His, and T7 tags and a tobacco etch virus (TEV) cleavage site, and a pGEX-6P-2 vector with an N-terminal GST and C-terminal His, tag. Mouse RNF146(RING-WWE) (residues 30–183) has an identical protein sequence to human RNF146 in this region, was subcloned into a pGEX-4T-1 vector with an N-terminal GST tag and TEV cleavage site. GST-tagged human tankyrase 1 fragment containing all five ankyrin repeat clusters (TNKS(SARC); residues 173–961) was expressed from a pGEX-4T-1 vector with an N-terminal GST tag and TEV cleavage site. Full-length mouse TNKS was cloned into a pET-15b plasmid. Mutants and truncations of RNF146 were generated using site-directed mutagenesis (Stratagene) and confirmed by sequencing. BRC1–BAR1 (residues 1–112 and 1–115 respectively), UbcH5a, UbcH5b, UbcH5c, Ub, UbcH5c(Ser22Arg/Cys85Ser), and wheat E1 were purified as previously described 30.

The oxyesterase mutants and truncations of RNF146 were generated using site-directed mutagenesis and TEV cleavage overnight at 4°C. The untagged TNKS(5ARC) was subsequently purified by ion exchange on a HiTrap Q column (GE Healthcare) and SEC using a Superdex 200 10/300 column (GE Healthcare). Protein concentrations were determined by their ultraviolet absorbance at 280 nm, and confirmed with Coomassie-stained SDS–PAGE.

Lysine reactivity assay. For Coomassie or Oriole (Biolad)–stained gels, UbcH5–Ub conjugates were generated in a solution containing 100 μM E2, 1.5 μM wheat E1, 200 μM Ubs (lys-deficient (K0) mutant: Lys6Arg, Lys11Arg, Lys29Arg, Lys33Arg, Lys48Arg, Lys63Arg and Lys27Met), 2.5 mM MgCl2 and 2 mM ATP (Sigma–Aldrich) in PBS at 37°C for 30 min. The conjugate was purified by SEC before being dialysed overnight at 4°C. The untagged TNKS(SARC) was subsequently purified by anion exchange column using a Q column (GE Healthcare). Full-length mouse TNKS1 was partially purified by Ni2+ NTA resin (GE Healthcare). Full-length His–T7-RNF146 was purified with a Ni2+ NTA resin (GE Healthcare), followed by ion exchange on a HiTrap Q (GE Healthcare) and SEC using a Superdex 200 10/300 column (GE Healthcare). Protein concentrations were determined by their ultraviolet absorbance at 280 nm, and confirmed with Coomassie-stained SDS–PAGE.

iso-ADPr for 1 h at room temperature. The reaction was quenched with 5% SDS loading buffer and the resulting digest was resolved by SDS–PAGE and stained with Coomassie.

SEC–MALSS. SEC–MALSS experiments were performed at room temperature with a Superdex 200 10/300 column (GE Healthcare) and a miniDANW TREOS MALSS detector (Wyatt Technology). About 200 μg of full-length RNF146, TNKS(SARC), and TNKS(5ARC)–RNF146 complex was injected in each run. The column was run with buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM DTT. The light scattered by a protein is directly proportional to its weight-average molecular mass and concentration.

Cysteine and data collection and structure determination. Cysteine and data collection were performed at the ALS, beamline 8.2.1. All diffraction data were processed by the HKL2000 package in the space group P212121. The structure was determined, at 1.9 Å resolution, by single-wavelength anomalous dispersion (SAD) using one data set collected at a wavelength of 1.28295 Å, which was also used for refinement (Extended Data Table 1). The zinc sites and the initial phases were determined by PHENIX 35. Four zinc sites were found in one asymmetric unit, and the experimental electron density map clearly showed the presence of two RNF146 (RING-WWE)–UbcH5a complexes with two ligands in one asymmetric unit. The complex model was improved using iterative cycles of manual rebuilding with the program COOT 36 and refinement with Refmac5 37. There are no Ramachandran outliers (96.0% most favoured, 4.0% allowed).

NMR spectroscopy. Two-dimensional 1H–15N HSQC–TROSY experiments were performed on a Bruker 500 MHz AVANCE II NMR spectrometer. All data were obtained with 200 μM 15N-labelled protein. Data were processed with NMRPipe 38, and peak intensities (I) and chemical shift perturbations (CSPs) were measured in NMRView 39 (One Moon Scientific). Peak intensity changes of 1N-labelled E3 (UbcH5c(Ser22Arg/Cys85Ser)) were measured relative to free E2 (K0 bound to free), and peaks affected by binding were identified by one standard deviation away from Iref. CSPs were buffer exchanged into 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM DTT using SEC on a Superdex 75 10/300 column (GE Healthcare), eluting at a final concentration of ~15–20 μM. The ligand, iso-ADPr, was diluted in the HEPES buffer (100 μM). Both ligand and protein were degassed before use. Ligand was injected in 5 μl quantities every 5 min for a total of 25–40 injections into a 1.4218–ml protein chamber. Data were analysed using Origin 7.0, fitting curves to a one-site model.
Full-length TNKS pull-down assays were performed using GST–RNF146 (Arg163Ala), a mutant that is deficient in binding poly(ADP-ribose)11. Proteins were resolved via SDS–PAGE and the full-length TNKS, GST and GST–RNF146 (Arg163Ala) were visualized by western blot analysis using anti-TNKS (rabbit polyclonal antibody, Abcam ab86279) and anti-GST (mouse monoclonal antibody, GenScript A00865) antibodies.

**Immunoprecipitation.** HEK293T cells (ATTC, CRL-11268) were transfected with indicated plasmids with Fugene HD (Promega). Thirty-six hours after transfection, cells were lysed with immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, supplemented with protease inhibitors and phosphatase inhibitors) and cleared by centrifugation. Flag-tagged proteins were immunoprecipitated from lysates with Flag-agarose beads (Sigma-Aldrich), and washed in immunoprecipitation buffer. Proteins bound to the beads were resolved by SDS–PAGE and analysed by immunoblotting. Cells were authenticated by single nucleotide polymorphism testing and confirmed as mycoplasma negative by a PCR-based assay.

**RNF146 knockdown and rescue assay.** siRNA-mediated knockdown and cDNA overexpression of RNF146 were described previously11. siRNA-resistant HA-tagged RNF146 (wild type) and indicated mutants were subcloned into pcDNA4-TO. T-REx-293 cells (Life Technologies) were transfected with these plasmids and selected with Blasticidin to establish stable lines that express RNF146 in a doxycycline-inducible manner. siRNAs against RNF146 and luciferase (as negative control) were transfected with Lipofectamine RNAiMax (Life Technologies). Sequences of siRNAs used are: RNF146 sense, 5'-GCACGUUUCUGCUAUCUAdTdT-3', antisense, 5'-UGAUAGCAGAAAACGUGCdTdT-3' (Qiagen); pGL2 (luciferase), sense, 5'-CGUACGCGGAAUACUUCGAdTdT-3', antisense, 5'-UCGAAGUUCGCUACGdTdT-3' (Dharmacon). Seventy-two hours after siRNA transfection and doxycycline induction, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were resolved by SDS–PAGE and analysed by immunoblotting with rabbit anti-Axin1 (monoclonal antibody, Cell Signaling Technology 2075), rat anti-HA (monoclonal antibody, Roche 12158167001), and mouse anti-tubulin (monoclonal antibody, Sigma TS168). T-REx-293 cells were authenticated by single nucleotide polymorphism testing and confirmed as mycoplasma negative by a PCR-based assay.

29. Brzovic, P. S., Lissounov, A., Christensen, D. E., Hoyt, D. W. & Klevit, R. E. A. UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. Mol. Cell 21, 873–880 (2006).
30. Pruneda, J. N., Stoll, K. E., Bolton, L. J., Brzovic, P. S. & Klevit, R. E. Ubiquitin in motion: structural studies of the ubiquitin-conjugating enzyme–ubiquitin conjugate. Biochemistry 50, 1624–1633 (2011).
31. Otwinowski, Z. & Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode Vol. 276 (Academic, 1997).
32. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D 66, 213–221 (2010).
33. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. D 66, 486–501 (2010).
34. The CCP4 suite. Programs for protein crystallography. Acta Crystallogr. D 50, 760–763 (1994).
35. Delano, W. L. & Brünger, A. T. Helix packing in proteins: prediction and energetic analysis of dimeric, trimeric, and tetrameric GCN4 coiled coil structures. Proteins 20, 105–123 (1994).
36. Delaglio, F. et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293 (1995).
37. Johnson, B. A. & Blevins, R. A. NMR View: A computer program for the visualization and analysis of NMR data. J. Biomol. NMR 4, 603–614 (1994).
38. Yeh, T. Y. et al. Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell-cell contact and protein poly(ADP-ribosyl)ation. Biochem. J. 399, 415–425 (2006).
Extended Data Figure 1 | Multiple sequence alignment of RNF146 orthologues. The coloured bars above the sequence alignment indicate regions of interest in human RNF146: RING domain (blue), WWE domain (purple), and potential TNKS-binding motifs, numbered I to V (orange). Although there are no apparent RXXGDG TNKS-binding motifs, the five potential binding motifs indicated here are based on the TNKS–substrate interface plasticity demonstrated by a recent crystal structure of the Axin–TNKS complex.26
Extended Data Figure 2 | Both PAR and iso-ADPr can activate RNF146 E3 ligase activity. a, Coomassie-stained E2—Ub/lysine reactivity of the RNF146(RING) domain with and without iso-ADPr. The RING domain does not enhance E2—Ub conjugate reactivity in the absence or presence of ligand. b, Intrinsic lysine reactivity of the UbcH5c—Ub conjugate with and without iso-ADPr. iso-ADPr does not enhance the reactivity of the conjugate in the absence of RNF146. c, Oriole-stained E2—Ub/lysine reactivity with increasing iso-ADPr (3 min after lysine addition). The rate of E2—Ub/lysine reactivity is increased as a function of [iso-ADPr] up to 5 μM ligand addition (1.2 equiv.), consistent with the affinity of RNF146 for iso-ADPr (see Extended Data Fig. 3). d, Auto-ubiquitination of full-length RNF146 in the absence or presence of iso-ADPr or PAR polymer. Image shows western blot for T7-tagged RNF146. Because full-length RNF146 and the RING-WWE fragment have similar abilities to enhance E2—Ub reactivity (see Fig. 1), the additional auto-ubiquitination seen with PAR is probably due to increased local concentration of RNF146 near PAR polymers, allowing auto-ubiquitination in trans. e, E2/lysine reactivity of UbcH5a, UbcH5b, and UbcH5c ubiquitin conjugates with RNF146(RING-WWE) in the absence or presence of iso-ADPr (Coomassie-stained). All three isoforms function with ligand-activated RNF146. f, Technical triplicates of RNF146(RING-WWE) E2—Ub/lysine reactivity assays (Oriole-stained; left) and a plot of relative densitometry values of the E2—Ub conjugate (right). Error bars indicate the mean ± s.d. from three separate experiments. All times are given in minutes. 'No E3' samples do not contain RNF146.
Extended Data Figure 3 | Both RNF146(RING) and RNF146(WWE) domains contribute to iso-ADPr binding. **a**, Summary of iso-ADPr binding ($K_d$ values) for RNF146(RING-WWE) obtained from the ITC titrations in the current work, and for the WWE-only fragment (previously published; indicated by asterisk)\(^1\). These data indicate that the RING domain contributes to iso-ADPr binding. **b**, Raw ITC titrations of RNF146(RING-WWE) fragments: (left to right) wild type, Lys61Ala, and Lys61Asp. **c**, Limited proteolysis of RNF146(RING-WWE) and of a construct of RNF146 including the linker between the RING and WWE domains, and the WWE domain (RNF146(linker-WWE); residues 83–183). Both seem to result in the same product when treated with subtilisin. The RING-WWE construct is more resistant to subtilisin in the presence of ligand.
Extended Data Figure 4 | $^1$H–$^{15}$N HSQC-TROSY spectra of RNF146 reveal a conformational change in the RING domain after iso-ADPr binding.

a, RNF146(RING-WWE) spectra in the absence (black) and presence (red) of saturating iso-ADPr concentrations show a marked change in most amide chemical environments. 
b, Overlay of the RNF146(RING-WWE) spectrum (black) with the spectrum of the RNF146(RING)-only domain (green) in the absence of iso-ADPr. Nearly all RING-only peaks overlay with a peak in the RING-WWE fragment spectrum, confirming that the RING-only domain adopts the same conformation as the RING domain in the larger fragment. 
c, Overlay of the liganded RING-WWE spectrum (red) with the isolated RING domain (green) shows very few corresponding peaks between the two spectra, indicating environment changes of most RING domain peaks in the presence of iso-ADPr consistent with a conformational change. Notably, there are no changes in the spectrum of the RING-only construct when iso-ADPr is added under these conditions (data not shown). 
d, Close-up of c to illustrate that the RING domain samples a minor conformational state in liganded RNF146(RING-WWE). The minor peaks all correspond to RING peaks of unliganded RNF146 (black arrows). Therefore, the RING domain can still sample the non-activated conformation when saturated with ligand. Spectra were obtained with 200 μM protein and 300 μM iso-ADPr (saturating conditions).
Extended Data Figure 5 | Comparison of ligand binding in the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex and in the WWE-only structure. 

a, Left, superposition of the WWE domain of the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex (purple) and the previous iso-ADPr/WWE structure (cyan, PDB code 3V3L). Middle and right, WWE residues involved in binding iso-ADPr in the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex (purple) (middle), and in the previous iso-ADPr/WWE structure (cyan) (right). Waters are shown as non-bonded spheres; hydrogen bonds are shown as dashed lines. Side-chain contacts between ligand and protein are maintained in both structures.

b, Stereoview of the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex ligand-binding site showing the $2F_o - F_c$ map (grey mesh) contoured at 1.5σ. The ligand and waters are well defined within the binding site. Waters are shown as red non-bonded spheres, iso-ADPr is shown in cyan, and the RING and WWE domains are coloured as in Fig. 2a.

c, Stereoview of the iso-ADPr-binding site indicating residues within 4.5 Å of the ligand. Protein and ligand are represented as sticks, waters as red non-bonding spheres, and hydrogen bonds as dashed yellow lines. The RING and WWE domains and ligand are coloured as in Fig. 2a.
Extended Data Figure 6 | Rotation and crystal packing at the E2–E3 binding interface of the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex.

a, Superposition of the E2 in the RNF146(RING-WWE)–UbcH5a–iso-ADPr (coloured as in Fig. 2a) with a representative RING E3–E2 structure, the Bmi1–Ring1b–UbcH5c complex (grey) (PDB code 3RPG)18. The WWE domain is excluded for clarity. Boxes show close-up views of the RING domains revealing a rotation of the RING domain relative to the E2. Bottom right, RING domains rotated 90° to show the E2 binding surface of the E3s. The RING of the RNF146(RING-WWE)–UbcH5a–iso-ADPr structure is rotated relative to Ring1b–UbcH5c and other E3–E2 complexes15–20,23 (indicated by red arrow) when the E2s are aligned.

b, Close-up view of the E2–E3 interface of the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex shows that RING residue Arg 74 (yellow) is too far (7.7 Å) from the E2 Gln 92 (magenta) carbonyl to make the hydrogen bond observed in activated E3–E2–Ub structures17,21,22,24. The side chain of Arg 74 in the RING domain packs against Phe 128 (orange), a WWE domain residue of a symmetry-related molecule in this crystal form. It is likely that crystal packing interferes with the formation of the ‘allosteric’ hydrogen bond.

c, E2–Ub/lysine reactivity with RNF146(RING-WWE)(Arg74Ala) shows a dependence of RNF146 activity on the allosteric arginine17,21,22,24 with or without ligand. Because RNF146 activation requires Arg 74, which does not make contacts with the E2 in our structure, and because RNF146 shows canonical E2 binding in solution (see Extended Data Fig. 7), we conclude that the orientation of RNF146 in the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex is probably an unproductive E2–E3 association. The observed rotation is probably a crystallographic artefact.
Extended Data Figure 7  |  RNF146–iso-ADPr binding allows the RING domain to bind and activate a ubiquitin conjugating enzyme (E2).  

**a.** Left, superposition of the RING domain of unliganded RNF146 (PDB code 2D8T; grey, Trp 65 is shown as orange spheres) with the RNF146(RING-WWE)–UbcH5a–iso-ADPr complex (coloured as in Fig. 2a), shows a clash of Trp 65 with UbcH5a at the E2–E3 binding interface. This clash is observed when the RNF146(RING) structure (2D8T) is aligned with all other E2–E3 structures. 

**b.** Peak broadening (top; intensity relative to free E2) and CSPs (bottom) of 15N-UbcH5c(Ser22Arg/Cys85Ser) resonances (data are from the spectra shown in Fig. 3b). Histograms shown in blue compare the spectral properties of free E2 to E2 plus RNF146(RING-WWE); histograms shown in red compare free E2 to E2 plus RNF146(RING-WWE) and iso-ADPr. Dashed lines indicate one standard deviation from the mean value of the liganded (red) plots. Values below and above the dashed lines for the relative intensities and CSPs respectively are plotted on the E2 surface shown in c and Fig. 3b. 

**c.** Left, the RNF146(RING-WWE) binding surface inferred from data in b (light blue, on green E2), is compared with (right) the BRCA1–BARD1 binding surface on E2 (yellow, on green E2; residues 1–112 and residues 26–115, respectively) previously inferred by an analogous experiment. When the NMR perturbations are mapped to the surface of UbcH5c, the revealed binding sites are very similar, and are consistent with previously reported binding surfaces for RING E3s on free ubiquitin conjugating enzymes. 

**d.** Chemical shift perturbations and broadening of resonances from 15N-E2–O–Ub conjugate (UbcH5c(Ser22Arg/Cys85Ser)–O–Ub) after RNF146(RING-WWE)–iso-ADPr binding (determined by the same method as shown in b, but with only 0.125 mol. equiv. E3 added to minimize hydrolysis of the E2–O–Ub oxyester during NMR data collection). Left, perturbed residues are mapped onto UbcH5b (magenta on green E2) and ubiquitin (yellow on red ubiquitin). Centre and right, perturbed residues mapped onto the structure of E2–O–Ub as it appears in the E3/E2–O–Ub complex of BIRC7–UbcH5b–Ub (PDB code 4AUQ; BIRC7 not shown for clarity) show that the surfaces highlighted in the left panel are buried in the ‘closed’ state. The data show that RNF146 activates the E2–Ub conjugate by inducing the closed conformation. Because only the most perturbed residues are mapped to the E2–Ub surface, the E3 binding surface is not highlighted on the E2 in d.
Extended Data Figure 8 | Stabilizing helix 1 of RNF146 activates the RING domain.  

a, Complete images of gels shown in Fig. 3c (Oriole-stained) for Gly62Ala, Trp65Ala, Gly62Ala/Trp65Ala (GAWA), Lys61Ala, and Lys61Asp mutants of RNF146(RING-WWE) with or without iso-ADPr. Gly62Ala and GAWA mutants show reduced enhancement with iso-ADPr relative to wild type, probably owing to a clash of the Ala side chain with a turn in the WWE domain at position 62 (data not shown).  

b, Alignment of RNF146(RING) solution structure (PDB code 2D8T; white) and the crystal structure determined in this study (blue) shown in stereoview. Side chains are excluded for clarity; the backbone is represented by sticks. Comparison of the conformation of Gly 62 in the two structures suggests a need for a small side chain at position 62 to allow the structural transition from the inactive to active form of RNF146.  

c, Anti-HA western blot of the E2—Ub/lysine reactivity assay of RNF146(RING-WWE) compared with RNF146(RING) and RNF146(RING)(Gly62Ala) showing enhanced reactivity for the Gly to Ala mutation.  

d, Left, $^1$H–$^{15}$N HSQC-TROSY of $^{15}$N-UbcH5c(Ser22Arg/Cys85Ser) in the presence of 0.0 (black), 0.25 (red), 0.5 (green) and 1.0 (magenta) mol. equiv. of RNF146(RING)(Gly62Ala). Right, the same experiment performed with wild-type RNF146(RING). The most perturbed residues, indicated by letter and position (S100, etc.), show increased chemical shift perturbations for the RNF146(RING)(Gly62Ala) mutant.
Extended Data Figure 9 | RNF146 directly interacts with TNKS. a, Left, SEC profiles of untagged TNKS(5ARC) (blue), His₆T₇-RNF146 (red), and a 1:1 mixture of these proteins (green). Numbers above the peaks indicate the average mass obtained by multi-angle static light scattering (MALS) for each peak. His₆T₇-RNF146 and TNKS(5ARC) co-migrate as a single peak with an apparent mass of 128 kDa. Right, Coomassie-stained SDS–PAGE analysis of the SEC peaks in left panel show the presence of both proteins within the peak of the TNKS(5ARC)–His₆T₇-RNF146 complex (bottom right).

b, GST pull-down of partially purified full-length mouse tankyrase-1 (FL-mTNKS1) with GST-tagged RNF146(Arg163Ala) (PAR-binding deficient RNF146 mutant). Full-length mTNKS1 can be pulled down by GST–RNF146, but not GST. c, Co-immunoprecipitation of HA–RNF146 variants with transiently transfected flag-tagged TNKS(Met1207Val) (catalytically inactive mutant). The Met1207Val mutation prevents auto-PARylation of TNKS and therefore PAR-mediated interactions between RNF146 and TNKS. Under the experimental conditions, both the motif I mutant, Gly199Val, and the motifs I + IV mutant, Gly199Val/Gly337Val/Gly338Val, markedly reduce the RNF146–TNKS interaction. d, Coomassie-stained SDS–PAGE of proteins used in the GST pull-down assay shown in Fig. 4a (inputs). Samples were used in a 1:2 ratio (3 μM GST–RNF146 to 6.7 μM TNKS(5ARC)) for these GST pull-down experiments.
**Extended Data Table 1 | Data collection, phasing and refinement statistics**

| Data collection                        | RNF146(RING-WWE)/Ube3H5α/Uso-ADP·Zn\(^{2+}\) SAD |
|----------------------------------------|---------------------------------------------------|
| Space group                            | P2\(_1\)2\(_1\)2                                         |
| Cell dimensions                        |                                                   |
| \(a, b, c\) (Å)                        | 133.67, 61.69, 94.35                                 |
| \(\alpha, \beta, \gamma\) (°)         | 90, 90, 90                                         |
| Resolution (Å)                         | 50.0 - 1.90 (1.96 - 1.90)*                         |
| \(R_{	ext{merge}}\)%                  | 9.2 (45.9)*                                        |
| \(I/\sigma I\)                         | 34.4 (2.5)*                                        |
| Completeness (%)                       | 95.6 (71.41)*                                      |
| Redundancy                             | 7.8 (4.6)*                                         |

| Refinement                              |                                                   |
| Resolution (Å)                         | 50.0 - 1.90                                       |
| No. reflections (test set)              | 55779 (2994)                                      |
| \(R_{	ext{work}}/R_{	ext{free}}\)    | 18.6 / 22.2                                       |
| No. atoms                               |                                                   |
| Protein                                 | 4886                                              |
| Ligand                                  | 72                                                |
| \(Zn^{2+}\)                             | 4                                                 |
| Water                                   | 371                                               |
| B-factors                               |                                                   |
| Protein                                 | 16.3                                              |
| Ligand                                  | 31.0                                              |
| \(Zn^{2+}\)                             | 31.5                                              |
| Water                                   | 42.4                                              |
| R.m.s deviations                       |                                                   |
| Bond lengths (Å)                        | 0.009                                             |
| Bond angles (°)                         | 1.4                                               |

This diffraction data set was collected from a single crystal.
5% randomly selected reflections were used as a test set.
*Highest resolution shell is shown in parentheses.