LUBAC synthesizes linear ubiquitin chains via a thioester intermediate.

Stieglitz, B; Morris-Davies, AC; Koliopoulos, MG; Christodoulou, E; Rittinger, K

EMBO reports

EMBO open

scientific report

LUBAC synthesizes linear ubiquitin chains via a thioester intermediate

Benjamin Stieglitz, Aylin C. Morris-Davies, Marios G. Koliopoulos, Evangelos Christodoulou & Katrin Rittinger[+]

Division of Molecular Structure, MRC-National Institute for Medical Research, London, UK

The linear ubiquitin chain assembly complex (LUBAC) is a RING E3 ligase that regulates immune and inflammatory signalling pathways. Unlike classical RING E3 ligases, LUBAC determines the type of ubiquitin chain being formed, an activity normally associated with the E2 enzyme. We show that the RING-in-between-RING (RBR)-containing region of HOIP—the catalytic subunit of LUBAC—is sufficient to generate linear ubiquitin chains. However, this activity is inhibited by the N-terminal portion of the molecule, an inhibition that is released upon complex formation with HOIL-1L or SHARPIN. Furthermore, we demonstrate that HOIP transfers ubiquitin to the substrate through a thioester intermediate formed by a conserved cysteine in the RING2 domain, supporting the notion that RBR ligases act as RING/HECT hybrids.

Keywords: E3 ligase; mechanism; thioester; ubiquitination

EMBO *reports* (2012) **13,** 840–846. doi:10.1038/embor.2012.105

INTRODUCTION

The reversible modification of proteins with polyubiquitin chains regulates a multitude of biological functions, and the type of ubiquitin chain attached determines the biological outcome of the modification [1]. Ubiquitin contains seven lysine residues all of which have been shown to form ubiquitin chains *in vivo*. K48-linked and K63-linked chains are at present the most intensely studied type of modification and their roles in proteasomal degradation (K48) [2], DNA repair [3] and cell signalling (K63) [4] are well documented. More recently, a new type of ubiquitin chain was identified, termed linear or M1-linked ubiquitin chains, in which the carboxy-terminal glycine of one molecule forms a peptide bond with the amino-terminal methionine of another. This type of chain has been shown to have an important role in the response to stimulation of the tumour-necrosis factor, interleukin-1β and CD40 receptors and the subsequent activation of the canonical NF-κB pathway, and is catalysed by an E3 ubiquitin ligase complex called LUBAC (linear ubiquitin chain assembly complex) [5]. LUBAC was initially identified to be composed of the two RING-in-between-RING (RBR) domain-containing proteins HOIL-1L (haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1) and HOIP (HOIL-1L-interacting protein) [6]. More recently it was shown to contain a third component, an ubiquitin-binding protein called SHARPIN (Shank-associated RH domain-interacting protein) (Fig 1A). SHARPIN provides the LUBAC complex with a biological activity that is distinct from that of the original HOIP/HOIL-1L complex but does not change the type of ubiquitin chain formed [7–9].

Ubiquitination occurs via an enzymatic cascade involving three enzymes [10]. It is initiated by an E1 activating enzyme that forms a reactive thioester with the C-terminal glycine of ubiquitin in an ATP-dependent fashion. This thioester is passed on to an E2-conjugating enzyme and finally transferred onto the substrate by an E3 ubiquitin ligase. E3 ligases can be divided into two main classes: HECT (homologous to E6AP carboxyl terminus)-type ligases, which form a thioester with ubiquitin before the final transfer onto the substrate, and the much larger family of RING (really interesting new gene) ligases, which act as scaffolds to bring the E2 and the substrate together [11]. Canonical RING domains adopt a cross-brace structure and coordinate two Zn^{2+} ions using conserved cysteine/histidine patterns. The RBR family, which includes HOIP and HOIL-1L, is a subgroup of the RING family of E3 ligases [12] but unlike all other RING E3s contains three Zn^{2+}-binding modules: an N-terminal classical RING (RING1), a central in-between-RING (IBR) domain, which also coordinates two Zn^{2+} ions but adopts a fold different from RING domains [13] and a C-terminal RING domain (RING2), which in the RBR protein HHARI has been shown to bind only a single Zn^{2+} ion with a topology different to that of the classical RING finger (Fig 1B, 1WD2.pdb) [14]. At present, it is not known if coordination of a single Zn^{2+} by RING2 is specific for HHARI or a common feature of other RBR domains. For some members of this RING subfamily it has been shown that the isolated RBR, or even its subdomains, are sufficient for ubiquitin chain synthesis, such as the IBR-RING2 of Parkin [15] or RING2 of HHARI [14]. In contrast, HOIP, the catalytic subunit of LUBAC, is believed to be only functional in complex with HOIL-1L and/or SHARPIN and no active subdomains have been identified [5–9].

Division of Molecular Structure, MRC-National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK
[+]Corresponding author. Tel: +44 20 88162395; Fax: +44 20 88162580;
E-mail: krittin@nimr.mrc.ac.uk

Received 8 April 2012; revised 21 June 2012; accepted 22 June 2012; published online 13 July 2012

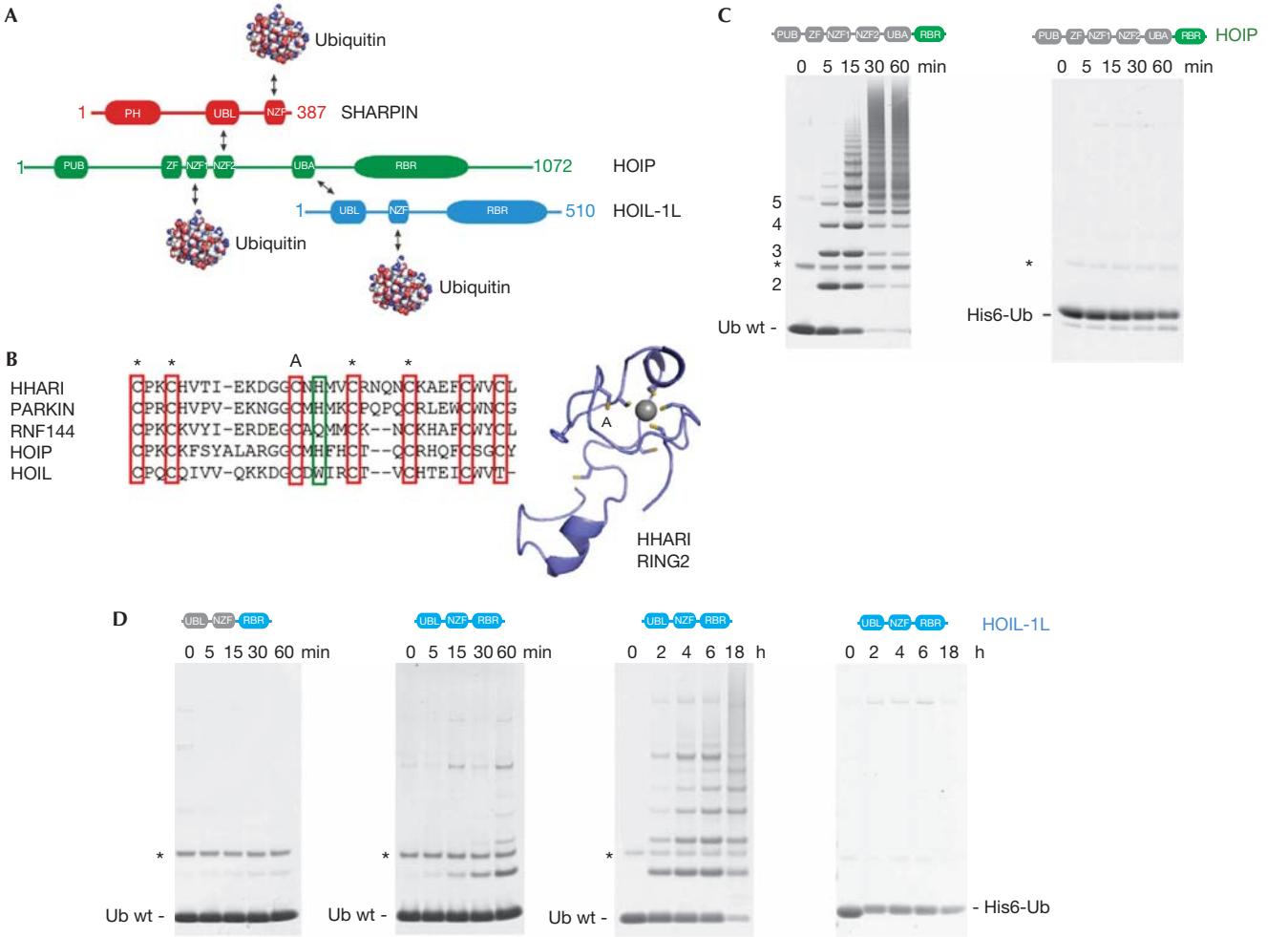

Fig 1 | The composition of LUBAC and the activity of the RBRs. (**A**) Domain structure of the LUBAC subunits, with the domains mediating protein interactions indicated. The colour coding used in this figure (HOIP in green and so on) will be maintained throughout the manuscript. (**B**) Sequence alignment of RING2 domains and structure of HHARI RING2 (1WD2.pdb). Conserved cysteines that are involved in coordination of the Zn^{2+} are indicated by * and the cysteine forming the thioester by **A**. This corresponds to Cys357 in HHARI and Cys885 in HOIP. (**C**) The RBR of HOIP catalyses the synthesis of linear ubiquitin chains. *In vitro* ubiquitination assays with the RBR of HOIP using wt ubiquitin (left) or His_6-ubiquitin (right) as substrate. A star indicates assay components, mostly E2, that have not precipitated during acidification. (**D**) *In vitro* ubiquitination assays covering different time scales with HOIL-1L using wt or His_6-ubiquitin as substrate. The reaction conditions used were identical in **C** and **D**. Gels were stained with Coomassie Blue. The constructs used in **C** and **D** are indicated schematically above the gels with the domains present in the assays highlighted in colour in green for HOIP and blue for HOIL-1L. HOIL-1L, haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1L-interacting protein; LUBAC, linear ubiquitin chain assembly complex; RBR, RING-in-between-RING; RING, really interesting new gene; SHARPIN, Shank-associated RH domain-interacting protein; UBA, ubiquitin-associated; UBL, ubiquitin-like; wt, wild-type.

Generally, it is assumed that the linkage specificity of the ubiquitin chain is determined by the last thioester intermediate formed [11,16]. This implies that HECT-type E3 ligases not only select the substrate but also determine the chain linkage [17]. On the other hand, RING-type E3 ligases only mediate substrate selection, while it is the E2 that determines chain linkage specificity. In contrast, LUBAC, despite belonging to the RING family, enforces the generation of linear ubiquitin chains regardless of the cognate E2. A mechanistic explanation for this unique behaviour is at present missing; however, the recent report that HHARI is capable of forming a thioester between a conserved cysteine in RING2 and the C-terminal glycine of ubiquitin provided a first clue to a possible underlying molecular mechanism and has led to the suggestion that RBRs might act as RING/HECT hybrids [18].

Here, we investigate the molecular determinants that allow LUBAC to enforce linear ubiquitin chain synthesis. We demonstrate that the RBR-containing region of HOIP is sufficient to catalyse the formation of linear chains and that the reaction proceeds via a thioester intermediate formed by a conserved cysteine residue in RING2. Importantly, we show transfer of this thioester to a di-ubiquitin substrate forming a tri-ubiquitin product. Furthermore, we establish that the region N-terminal to the RBR of HOIP acts in an autoinhibitory fashion, which can be released

upon complex formation with HOIL-1L or SHARPIN, explaining the earlier observation that HOIP is only active in complex with HOIL-1L and/or SHARPIN.

RESULTS AND DISCUSSION

The RBR region of HOIP synthesizes linear chains

At present, our knowledge about the minimal structural units of LUBAC that are required to promote linear ubiquitin chain synthesis is limited, and it is believed that a complex between HOIP and HOIL-1L or SHARPIN is required for activity [6–9]. We therefore decided to investigate the activity of the RING-containing units, the RBRs, using an *in vitro* ubiquitination assay and sequentially build up to the full-length complex in order to identify the minimum linear chain catalytic machinery. These experiments show that the C-terminal portion of HOIP containing the RBR and a 140 amino acid extension that is required for protein solubility (from now on referred to as RBR-C) is sufficient to efficiently generate ubiquitin chains. In our assay, almost all monoubiquitin is used up after 30 min and polyubiquitin chains with a length of more than 15 ubiquitin moieties are generated (Fig 1C). Importantly, when the same assay was performed using ubiquitin, which contains all seven lysine residues but with an N-terminal His_6-tag, no ubiquitin chains are formed, indicating that the N-terminal Met of ubiquitin participates in the reaction (Fig 1C). These data clearly demonstrate that the RBR-C of HOIP catalyses the formation of linear ubiquitin chains with high specificity and hence represents the activity observed in the native LUBAC complex. Furthermore, we do not observe any autoubiquitination of HOIP (supplementary Fig S1A online) and therefore conclude that all chains formed are unattached.

In contrast to the RBR-C of HOIP, no chain formation was observed with the RBR of HOIL-1L under the same conditions (Fig 1D; supplementary Fig S1B online), in accordance with a previous study indicating that HOIL-1L had no linear chain assembly activity [6]. However, ubiquitination assays using almost full-length HOIL-1L (only lacking a 32-residue C-terminal flexible tail, from now on referred to as HOIL-1L, supplementary Fig S1B online) showed some residual activity after 1 h prompting us to follow the reaction over a longer time period (Fig 1D). Ubiquitination assays run for up to 18 h clearly show that HOIL-1L is, in principle, capable of forming high molecular weight ubiquitin chains, but that the rate of chain formation is very slow. Nevertheless, the chains formed are strictly linear as indicated by the absence of any activity using His_6-ubiquitin as a substrate (Fig 1D). The observation that the full-length protein has a higher activity than the isolated RBR is in clear contrast to Parkin, another member of the RBR family, in which an N-terminal ubiquitin-like (UBL) domain inhibits the activity of the C-terminally located RBR [19] and indicates that intramolecular interactions are not the reason for the low activity of HOIL-1L.

HOIP is autoinhibited in the absence of HOIL-1L

Although activity of the isolated RBR has been shown for some RING E3 ligases [14,15], the observation that the RBR-C of HOIP is sufficient for the generation of linear ubiquitin chains was unexpected as it has been reported that complex formation between HOIL-1L and HOIP, mediated via their respective UBL and ubiquitin-associated (UBA) domains, is required for efficient chain synthesis [6]. To better understand the role of the domains outside the RBRs and that of complex formation on the catalytic activity of HOIP, we examined chain synthesis using increasingly larger constructs of HOIP.

Interestingly, ubiquitination assays using a construct also containing the UBA domain located N-terminal to the RBR show a slower rate of ubiquitin chain synthesis (Fig 2A,B). This suggests that the region N-terminal to the RBR interferes with catalytic activity via an autoinhibitory interaction.

Interaction with HOIL-1L and SHARPIN releases inhibition

The interaction between HOIL-1L and HOIP occurs via their respective UBL and UBA domains [6]. To quantify the binding affinity, we performed isothermal titration calorimetry (ITC) using the isolated domains, which resulted in a K_d value of 3.5 μM (Fig 2C). It is tempting to assume that this interaction might regulate the activity of HOIP. To test this hypothesis, we pre-incubated the HOIP UBA-RBR tandem construct with the UBL of HOIL-1L, and repeated the chain synthesis reaction. Indeed, complex formation restored the activity back to the level of the RBR-C (Fig 2D). This result suggests a model in which the UBL of HOIL-1L competes with an autoinhibitory interaction between the RBR and UBA of HOIP. However, we could not detect any binding between these two domains in isolation by ITC (supplementary Fig S2A online) or see inhibition *in trans* in ubiquitination assays carried out in the presence of increasing concentrations of the UBA (supplementary Fig S2A online), indicating that the mechanism of autoinhibition is not analogous to that identified in Parkin where inhibitory interactions can be detected even *in trans* [19]. In fact, a tight binding between the RBR and UBA might not be favourable for a competitive inhibition mechanism, given that the UBL of HOIL-1L and the UBA of HOIP interact in the low micro-molar range. Alternatively, it is possible that the activation of HOIP involves a more subtle allosteric regulation in a non-competitive fashion.

Remarkably, ubiquitination assays using a complex with HOIL-1L instead of the isolated UBL not only restored the catalytic activity to the level of the HOIP RBR-C but further increased the rate of chain synthesis suggesting that HOIL-1L modulates catalytic efficiency in a second distinctive way. Given the almost negligible activity of HOIL-1L on the time scale of this experiment it is unlikely that the increased activity is due to residual activity of the HOIL-1L RBR (Fig 1D). Nevertheless, to formerly exclude this possibility we repeated the assay with a catalytically inactive HOIL-1L mutant C460A (supplementary Fig S2B online). To our surprise, the activity of this mutant was decreased and comparable to that of the isolated UBL (Fig 2F), suggesting that the RBRs of HOIP and HOIL-1L modulate each other's activity, possibly via a direct interaction. Although we could not detect a direct interaction between the isolated RBR domains (data not shown), it is conceivable that UBA–UBL-mediated complex formation might bring them into close proximity.

The recently identified LUBAC component SHARPIN has also been shown to promote linear chain synthesis when bound to HOIP. To examine if it is equally capable of releasing auto-inhibition, we carried out ubiquitination assays using a much longer construct of HOIP that includes the zinc finger region that contains the binding site for SHARPIN (NZF2, see Fig 1) [7,8] and is only lacking an N-terminal PUB (Peptide:N-glycanase/UBA or

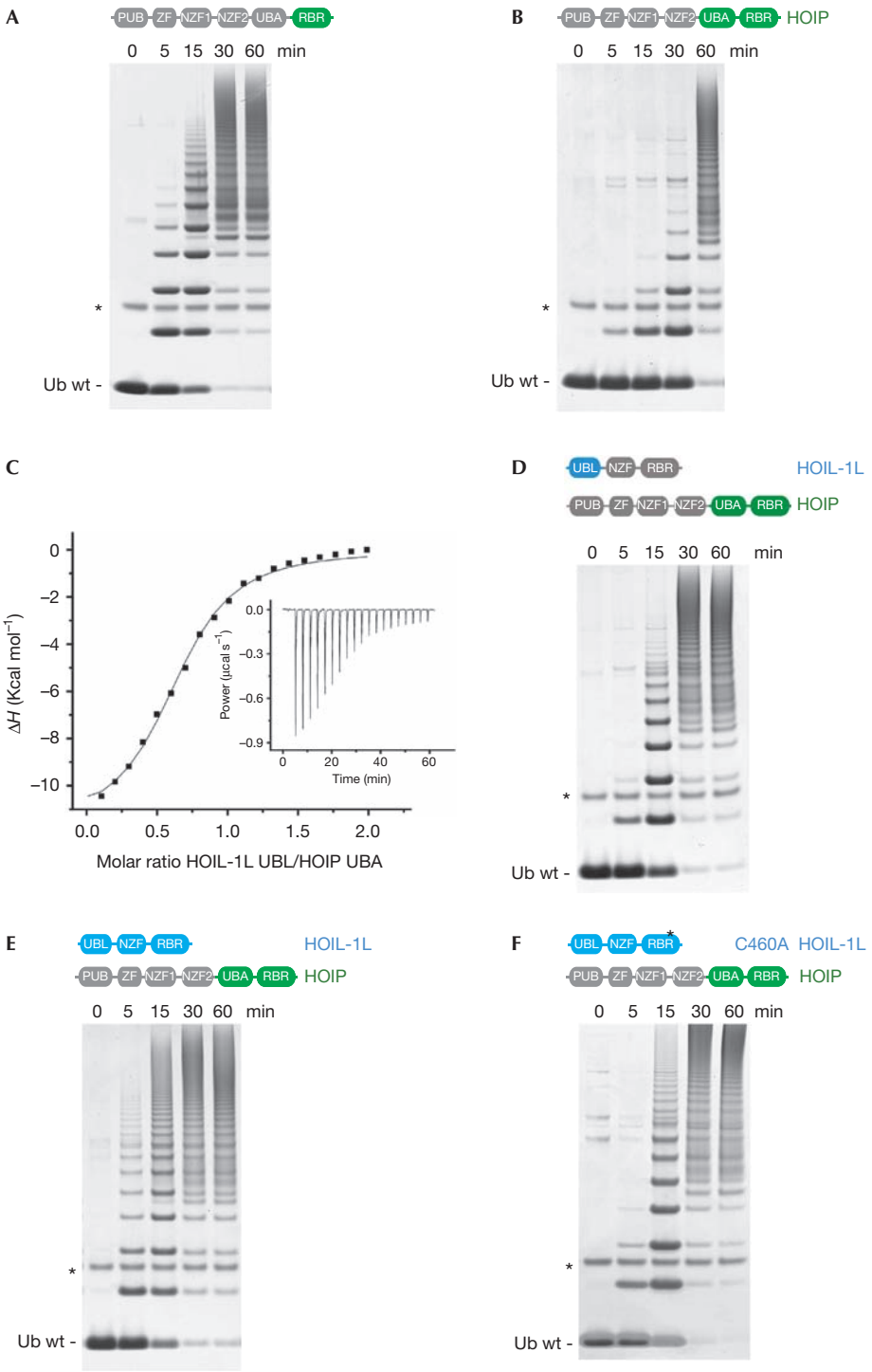

Fig 2 | Inhibition of the catalytic activity of HOIP by adjacent domains and release through complex formation with HOIL-1L. (**A**) Comparison of ubiquitin chain synthesis by HOIP constructs containing the RBR (as Fig 1C) and (**B**) UBA-RBR. (**C**) ITC titration between the UBA of HOIP and UBL of HOIL-1L. (**D**) Ubiquitination assay with a HOIP UBA-RBR/HOIL-1L UBL complex. (**E**) Comparison of the activity of a complex between HOIP UBA-RBR and wt HOIL-1L versus (**F**) a complex with a catalytically dead HOIL-1L mutant (C460A). Stars indicate assay components that have not precipitated during acidification. The colour coding and reaction conditions used are as in Fig 1C,D. HOIL-1L, haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1L-interacting protein; RBR, RING-in-between-RING; UBA, ubiquitin-associated; UBL, ubiquitin-like; wt, wild-type.

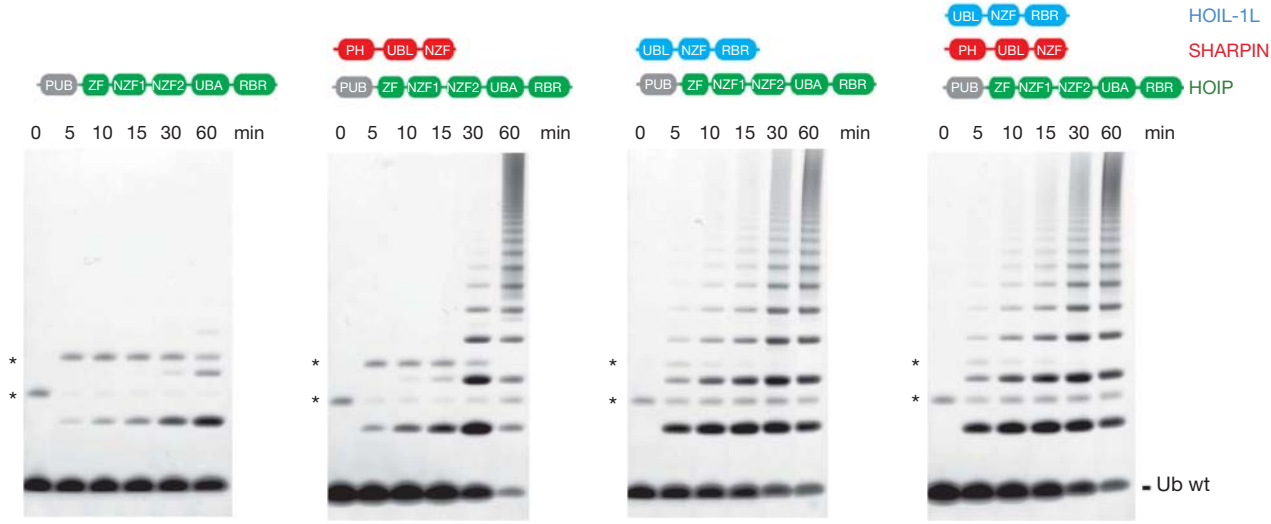

Fig 3 | Comparison of linear ubiquitin chain synthesis by different LUBAC complexes. The gels show the increase in the rate of chain formation upon addition of SHARPIN or HOIL-1L to a HOIP ZFs-UBA-RBR construct. There is no further increase in the rate of chain formation in the trimeric complex. Stars indicate assay components that have not precipitated during acidification. HOIL-1L, haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL-1L-interacting protein; LUBAC, linear ubiquitin chain assembly complex; RBR, RING-in-between-RING; SHARPIN, Shank-associated RH domain-interacting protein; UBA, ubiquitin-associated.

ubiquitin regulatory X (UBX)-containing proteins) domain of yet unidentified function. This experiment shows that, similar to HOIL-1L, SHARPIN is capable of releasing autoinhibition of HOIP, although to a lesser extent than HOIL-1L (Fig 3), and supports the notion that the release of autoinhibition of HOIP does not rely solely on competition between its RBR and the UBL of HOIL-1L for binding to the UBA of HOIP. Interestingly, the activities of HOIL-1L and SHARPIN are not additive as no increase in the rate of chain formation is observed with the trimeric HOIP/HOIL-1L/SHARPIN complex (Fig 3). This observation indicates that the primary role of SHARPIN in the LUBAC complex might not be to support the catalytic machinery but rather to provide further functionality such as substrate selection or possibly localization.

Ubiquitin transfer proceeds via a thioester intermediate

In contrast to RING E3 ligases, HECT ligases determine the linkage specificity of the ubiquitin chain formed in a reaction that proceeds via a thioester intermediate. It has recently been shown that the RBR ligase HHARI also forms a thioester intermediate leading to the suggestion that RBRs might be a new class of E3s that act as RING/HECT hybrids [18]. At present, it is not clear if this behaviour is a specific property of HHARI or if this hybrid mechanism might apply more generally to RBR family proteins. To investigate this possibility, we developed a fluorescence-based ubiquitination assay aimed at trapping the thioester intermediate. This assay is designed such that individual steps in the ubiquitination cascade are visualized by following the activity of a fluorescent, Cy5-labelled ubiquitin. This molecule can act as a ubiquitin donor and form a thioester but is N-terminally blocked and hence cannot accept an ubiquitin thereby preventing chain formation. As shown in Fig 4A, incubation of Cy5-ubiquitin with E1 and ATP results in the formation of a high molecular weight adduct that is sensitive to treatment with a reducing agent indicating that the E1-thioester has been formed (lanes 3 and 4). Addition of E2 induces transthiolation of the ubiquitin onto the E2, forming another dithiothreitol (DTT)-sensitive product (lanes 5 and 6). Once the RBR-C of HOIP is added to this mixture the ubiquitin is transferred onto the RBR, forming an adduct that is sensitive to treatment with reducing agents, clearly demonstrating that the reaction product formed is a thioester and not autoubiquitination of the RBR-C (lanes 7 and 8). Importantly, addition of a C-terminally blocked di-ubiquitin that can only act as a ubiquitin acceptor to this mixture results in the quantitative transfer of the activated ubiquitin from HOIP onto the substrate forming a covalent product (lane 9) as indicated by its insensitivity to reducing agents (lane 10). A gel stained for total protein content of this reaction is shown in supplementary Fig S4 online.

The cysteine responsible for thioester formation in HHARI has been identified as Cys357 [18], which is completely conserved across RBRs but not involved in coordination of the Zn^{2+} ion in HHARI (Fig 1B) [14]. At present it is not known if coordination of a single Zn^{2+} ion by RING2 is specific for HHARI or a general feature of RBRs as no structures are available for other RING2 domains. To test if the equivalent cysteine in HOIP, Cys885, might also be forming the thioester, we mutated it to alanine and tested its catalytic activity. This mutant was catalytically inactive as shown in the Cy5-ubiquitin-based assay (Fig 4B) as well as in the conventional *in vitro* ubiquitination assay (supplementary Fig S3 online). To ensure that this loss of activity is not due to unfolding of the protein, we made the corresponding serine mutation (C885S) to test for formation of an oxyester. As shown in Fig 4C, this mutant was active and formed an adduct with HOIP clearly indicating that removal of Cys885 does not trivially remove a residue required for Zn^{2+} coordination resulting in unfolded protein. The adduct formed was insensitive to treatment with DTT but undergoes acid-catalysed ester hydrolysis (Fig 4C), demonstrating that it does not contain an isopeptide linkage.

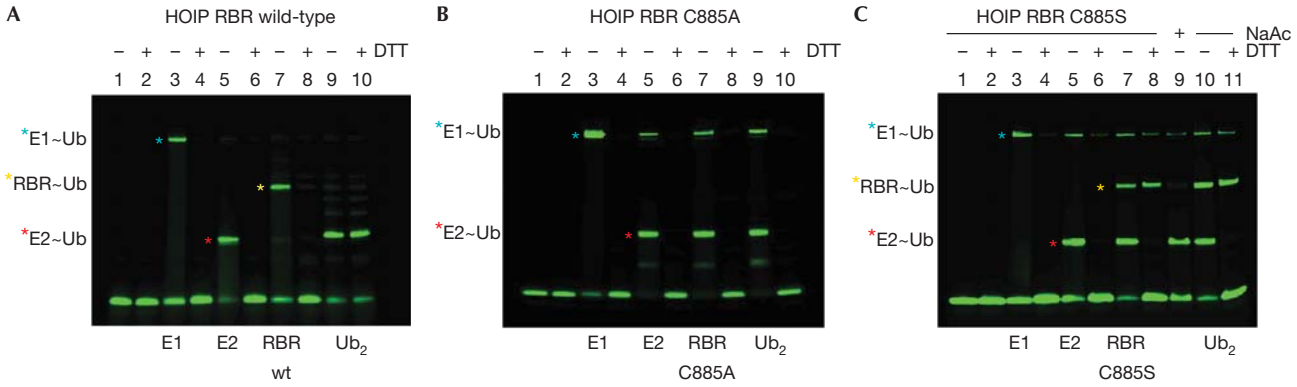

Fig 4 | Ubiquitination assays using Cy5-labelled ubiquitin show the formation of a HOIP-thioester. The gels shown only monitor the Cy5-labelled ubiquitin; gels stained for total protein content are shown in supplementary Fig S4 online. (**A**) Assay using wt HOIP-RBR-C: lanes 1/2 ubiquitin + E1 before addition of ATP, lanes 3/4 + ATP, lanes 5/6 + E2, lanes 7/8 + E3 and lanes 9/10 + diUb substrate. Uneven numbered lanes are − DTT, even numbered + DTT. (**B**) Assay using HOIP-RBR C885A, same conditions as in (**A**), no ubiquitin is transferred to E3 in lane 7, indicating that no thioester is formed. (**C**) Assay using HOIP-RBR C885S. An adduct is formed upon addition of RBR C885S that is not sensitive to treatment with reducing agent and cannot be transferred to a substrate indicating formation of an oxyester. Lanes 1–8 are equivalent to reactions in panels A and B. Incubation with DTT does not reduce the adduct formed with HOIP-RBR-C but does reduce the E2-thioester (lane 8). Lane 9: the reaction mixture is treated with NaAc, which hydrolyses the RBR-adduct. Lanes 10 + 11: addition of diUb substrate to the reaction mixture. Blue, yellow and red stars indicate the bands representing E1~Ub, RBR ~ Ub and E2 ~ Ub, respectively. DTT, dithiothreitol; HOIP, haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1-interacting protein; RBR, RING-in-between-RING; wt, wild-type.

CONCLUSIONS

We show here that the RBR-C of HOIP is sufficient to catalyse the synthesis of linear polyubiquitin chains. However, this activity is inhibited by the remainder of the protein but can be released upon addition of HOIL-1L or SHARPIN, nicely explaining why earlier studies could only detect activity in the context of the complex. Furthermore, our data indicate that HOIL-1L not only releases autoinhibition but can also synthesize linear ubiquitin chains. Although the catalytic activity of HOIL-1L in isolation is very slow, our data suggest that it becomes relevant in complex with HOIP and contributes to the linear chain synthesis activity of the heterocomplex via an as yet unidentified mechanism. It is tempting to speculate that this might include a direct interaction between the two RBRs of HOIP and HOIL-1L, brought into close proximity by their respective UBA and UBL domains, similar to those RING ligases that act as homo and heterodimers [20].

Our experiments have now shown that the RBR of HOIP forms a thioester via a conserved cysteine in RING2, and we demonstrate for the first time that this thioester intermediate is subsequently transferred to a substrate. These data provide a first molecular insight into the mechanism underlying the ability of LUBAC to enforce the synthesis of linear ubiquitin chains. LUBAC shows reactivity with a range of E2s, including UbcH7 that is normally not active with RING E3 ligases. Taken together, our data provide new evidence that the RING/HECT hybrid mechanism suggested by Klevit and colleagues for the action of HHARI [18] might be a more general mechanism for RBRs and might require this RING subfamily to be reclassified within the ligase superfamily.

METHODS

Protein expression, labelling and purification. All constructs used in this study were expressed in pET-47b, pET-49b (Merck Millipore) or pGEX-6P1 (GE Healthcare). Domain boundaries of the constructs used are listed in supplementary information online. All proteins used were purified by affinity chromatography on glutathione sepharose (GST-tagged) or Ni-NTA Superflow resin (His-tagged), followed by ion-exchange chromatography if necessary (after removal of the GST-tag by Precission Protease) and size-exclusion chromatography (SEC). Protein concentrations were determined by ultraviolet spectrometry at 280 nm using calculated extinction coefficients. Bovine mono-ubiquitin was purchased from Sigma and further purified by SEC. His6-Cys-ubiquitin was labelled with Cy5 maleimide mono-Reactive Dye (GE Healthcare) according to the manufacturer's instructions and further purified by SEC. Further details are provided in supplementary information online.

***In vitro* ubiquitination assays.** Assays were performed with 1 μM Ube1, 5–30 μM UbcH7 or UbcH5, 5–30 μM of the relevant HOIP, HOIL-1L and SHARPIN components and 200 μM ubiquitin in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$ and 10 mM ATP. Reactions were incubated at 30 °C for 1 h and samples taken at set time intervals. Further details of the assay are in supplementary information online.

***In vitro* ubiquitination assays using Cy5-labelled ubiquitin.** One micromolar Cy5-ubiquitin was mixed with 2 μM Ube1 (lanes 1, 2), and 1 mM ATP was added to start the reaction (at 25 °C). After 10 min, samples were taken (lanes 3, 4) and 10 μM UbcH5 was added. After 10 min, samples were taken (lanes 5, 6) and 20 μM HOIP was added. Samples were taken after 15 s (lanes 7, 8) to detect the thioester, and 15 μM diUb-His_6 was added and further samples (lanes 9, 10) taken after 10 min. To monitor formation of the oxyester, the reaction was allowed to proceed for 15 min and carried out at 37 °C instead of 25 °C as oxyester formation is very slow at the lower temperature. To hydrolyse the oxyester, the reaction mixture was incubated for 1 min in 4 M urea followed by incubation in 0.5 M NaAc at pH 4 for 3 min at 25 °C. Gels were scanned with a Storm 860 Scanner (GE Healthcare).

Isothermal titration calorimetry. ITC measurements were performed using an ITC200 MicroCalorimeter (GE Healthcare). All samples were dialysed into buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl and 1 mM TCEP. Titrations were performed at 20 °C with 50 μM of HOIP RBR or HOIL-1L UBL loaded into the cell and 500 μM of HOIP UBA into the syringe. ITC data were analysed with the Origin7 software (MicroCal) supplied by the manufacturer.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We are grateful to K. Iwai for the E1 virus, and I. Dikic, F. Ikeda, D. Komander and F. Randow for cDNAs and plasmids. We thank S. Howell for mass spectrometry, and S. Smerdon, R. Rana and N. Brown for helpful discussions. This work was supported by the Medical Research Council, UK, grant U117565398 and Wellcome Grant 094112/Z/10/Z.

Author contributions: B.S., A.C.M.-D., M.G.K., E.C. and K.R. prepared the samples; B.S., A.C.M.-D. and M.G.K. carried out experiments; and K.R. wrote the manuscript. All authors designed experiments, discussed results and commented on the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Pickart CM, Fushman D (2004) Polyubiquitin chains: polymeric protein signals. *Curr Opin Chem Biol* **8:** 610–616
2. Hershko A, Ciechanover A (1992) The ubiquitin system for protein degradation. *Annu Rev Biochem* **61:** 761–807
3. Huang TT, D'Andrea AD (2006) Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol* **7:** 323–334
4. Chen ZJ, Sun LJ (2009) Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* **33:** 275–286
5. Walczak H, Iwai K, Dikic I (2012) Generation and physiological roles of linear ubiquitin chains. *BMC Biol* **10:** 23
6. Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K, Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J* **25:** 4877–4887
7. Gerlach B *et al* (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* **471:** 591–596
8. Ikeda F *et al* (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. *Nature* **471:** 637–641
9. Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata S, Tanaka K, Nakano H, Iwai K (2011) SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex. *Nature* **471:** 633–636
10. Pickart CM (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70:** 503–533
11. Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78:** 399–434
12. Eisenhaber B, Chumak N, Eisenhaber F, Hauser MT (2007) The ring between ring fingers (RBR) protein family. *Genome Biol* **8:** 209
13. Beasley SA, Hristova VA, Shaw GS (2007) Structure of the Parkin in-between-ring domain provides insights for E3-ligase dysfunction in autosomal recessive Parkinson's disease. *Proc Natl Acad Sci USA* **104:** 3095–3100
14. Capili AD, Edghill EL, Wu K, Borden KL (2004) Structure of the C-terminal RING finger from a RING-IBR-RING/TRIAD motif reveals a novel zinc-binding domain distinct from a RING. *J Mol Biol* **340:** 1117–1129
15. Matsuda N, Kitami T, Suzuki T, Mizuno Y, Hattori N, Tanaka K (2006) Diverse effects of pathogenic mutations of Parkin that catalyze multiple monoubiquitylation in vitro. *J Biol Chem* **281:** 3204–3209
16. Ye Y, Rape M (2009) Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* **10:** 755–764
17. Kim HC, Huibregtse JM (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity. *Mol Cell Biol* **29:** 3307–3318
18. Wenzel DM, Lissounov A, Brzovic PS, Klevit RE (2011) UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature* **474:** 105–108
19. Chaugule VK, Burchell L, Barber KR, Sidhu A, Leslie SJ, Shaw GS, Walden H (2011) Autoregulation of Parkin activity through its ubiquitin-like domain. *EMBO J* **30:** 2853–2867
20. Budhidarmo R, Nakatani Y, Day CL (2012) RINGs hold the key to ubiquitin transfer. *Trends Biochem Sci* **37:** 58–65