Molecular Biology

Structural and biochemical advances on the recruitment of the autophagy-initiating ULK and TBK1 complexes by autophagy receptor NDP52

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The recruitment of Unc-51-like kinase and TANK-binding kinase 1 complexes is essential for selective autophagy and relies on the specific association of NDP52, RB1-inducible coiled-coil protein 1, and Nak-associated protein 1 (5-azacytidine-induced protein 2, AZI2). However, the underlying molecular mechanism remains elusive. Here, we find that except for the NDP52 SKIP carboxyl homology (SKICH)/RB1CC1 coiled-coil interaction, the LC3-interacting region of NDP52 can directly interact with the RB1CC1 Claw domain, as that of NAP1 FIP200-binding region (FIR). The determined crystal structures of NDP52 SKICH/RB1CC1 complex, NAP1 FIR/RB1CC1 complex, and the related NAP1 FIR/Gamma-aminobutyric acid receptor-associated protein complex not only elucidate the molecular bases underpinning the interactions of RB1CC1 with NDP52 and NAP1 but also reveal that RB1CC1 Claw and Autophagy-related protein 8 family proteins are competitive in binding to NAP1 and NDP52. Overall, our findings provide mechanistic insights into the interactions of NDP52, NAP1 with RB1CC1 and ATG8 family proteins.

INTRODUCTION

Autophagy is a highly regulated lysosome-dependent intracellular process that maintains cell homeostasis and/or cope with external stresses via degradation of superfluous or harmful cytosolic constituents, including damaged organelles and cytosol-invading pathogens (1–3). In contrast to the traditional “bulk” autophagy process that nonselectively engulfs cytoplasmic debris for autophagic degradation, a large number of selective autophagy processes that require specific cargo recognition and targeting by the autophagy machinery have been found recently (4–6), such as xenophagy (the selective autophagy of invading pathogens) (7–9), mitophagy (the selective autophagy of dysfunctional mitochondria) (10–12), aggrephagy (the selective autophagy of protein aggregates) (13–15), and ER-phagy (the selective autophagy of endoplasmic reticulum) (16–21). Meanwhile, a set of unique adaptor proteins termed as autophagy receptors have been identified to play essential roles in these selective autophagic processes, such as Sequestosome-1 (SQSTM1/P62), NDP52, Taxi-binding protein 1 (TAX1BP1), Next to BRCA1 gene 1 protein (NBR1), Optineurin, Nix, FUN14 domain-containing protein 1 (FUNDC1), Cell cycle progression protein 1 (CCPG1), Reticulophagy regulator 1 (RETREG1, also known as FAM134B), Testis-expressed protein 264 (TEX264), Reticulon-3 (RTN3), Atlasin-3 (ATL3), and Sec62 in mammals (22–24). As a master factor in selective autophagy, each autophagy receptor contains a cargo-associating domain that can specifically decorate prospective autophagic cargoes, and a unique LC3 (Microtubule-associated proteins 1A/1B light chain 3, MAP1LC3)-interacting region (LIR) that can recognize the key autophagic factors, ATG8 family proteins, including LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2 in mammals (25, 26–29). During selective autophagy, after recognition of relevant autophagic cargo, autophagy receptor can subsequently and effectively induce autophagosome formation in close proximity to the targeting cargo by recruiting relevant autophagy machinery. However, until now, many of the detailed molecular mechanisms underlying the in situ initiation of autophagosome formation by autophagy receptors during selective autophagy are still not well understood.

NDP52 (also known as CALCOCO2) is a crucial multifunctional autophagy receptor in mammals and plays important roles in selective autophagic degradations of invading infectious pathogens such as Salmonella enterica Typhimurium and Mycobacterium tuberculosis (30, 31), depolarized mitochondria (32, 33), specific functional proteins such as Endoribonuclease Dicer and Argonaute-2 related to the microRNA pathway (34), and retrotransposon RNA (34). As a multidomain-containing autophagy receptor, NDP52 contains an N-terminal SKIP carboxyl homology (SKICH) domain, an unconventional LIR motif that can specifically bind to LC3C (35), a middle coiled-coil region for the self-dimerization of NDP52 (36), a Galectin8-interacting region (GIR) (37), and two C-terminal zinc fingers that can mediate the interactions of NDP52 with the Myosin6 motor for autophagosome maturation and ubiquitin molecules decorated on relevant cargoes (Fig. 1A) (27, 37–39). Notably, the SKICH domain of NDP52 can directly bind to two closely related adaptor proteins NAP1 and TANK-binding kinase 1-binding protein 1 (TBKBP1, also known as SINTBAD), which, in turn, can recruit the TANK-binding kinase 1 (TBK1) for NDP52 through their respective C-terminal TBK1-binding domains (TBD) (37, 39, 40, 41). The recruitment of TBK1 complex by NDP52 and the kinase activity of TBK1 are both required for the NDP52-mediated selective autophagy processes (7, 42). The active TBK1 can directly phosphorylate NDP52 (32, 43), but the precise downstream effects imposed by this TBK1-mediated NDP52 phosphorylation event are still poorly understood.

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Notably, except for binding to NAP1 and SINTBAD, the SKICH domain of NDP52 are also recently reported to interact with RB1CC1 (also known as FIP200) (44, 45), which is a large subunit of the autophagy initiation ULK complex and mainly contains an N-terminal domain, an LIR motif, and several coiled-coil regions followed by an extreme C-terminal Claw domain (Fig. 1A). The interaction between NDP52 SKICH and RB1CC1 is essential for the recruitment of ULK complex as well as the subsequent initiation of autophagosome formation during NDP52-mediated xenophagy and mitophagy processes (44, 45). However, the detailed molecular mechanisms underpinning the specific interaction of RB1CC1 with NDP52 SKICH as well as the relationship between NAP1 and RB1CC1 in binding to NDP52 SKICH remain elusive.

As a key regulator in the initiation of autophagy, the ULK complex in mammals is composed of four subunits, a serine-threonine ULK1/2 kinase that serves as the catalytic subunit of the ULK complex, ATG101, ATG13, and RB1CC1 (46, 47). In addition to its crucial roles in bulk autophagy, the ULK complex is also recently proved to mediate the initiation of autophagosome formation in several distinct types of selective autophagy processes, including the CCPG1-mediated ER-phagy (20), P62-mediated aggrephagy (48), and NDP52-mediated xenophagy and mitophagy (44, 45). Intriguingly, during those selective autophagy processes, the relevant autophagy receptor can directly interact with RB1CC1, thereby recruiting the ULK complex to the targeting cargo for the subsequent autophagosome biogenesis (20, 44, 45, 48). Specifically, the autophagy receptors CCPG1 and P62 can directly recognize the C-terminal Claw domain of RB1CC1 mediated by their respective FIP200-binding regions (FIR) (20, 48), and, in contrast, autophagy receptor NDP52 is reported to interact with the second C-terminal coiled-coil region of RB1CC1 (44, 45). Other autophagic proteins including ATG16L1, the NDP52/TBK1-binding NAP1 and SINTBAD, are also reported to interact with RB1CC1 Claw through their FIR regions (45, 49, 50). Very recently, our work revealed that the interactions of RB1CC1 Claw with autophagy receptors CCPG1 and Optineurin can be tuned by the

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**Fig. 1. Biochemical and structural characterizations of the interaction between NDP52 SKICH domain and RB1CC1 coiled-coil region.** (A) A schematic diagram showing the domain organizations of RB1CC1, NDP52, NAP1, and TBK1. In this drawing, the RB1CC1/NAP1, RB1CC1/NAP1, NDP52/NAP1, and NAP1/TBK1 interactions are further highlighted and indicated by two-way arrows. (B) Overlay plot of the analytical gel filtration chromatography (AGFC) of the interaction between RB1CC1(1286-1395) and NDP52(10-126), A$_{280}$ absorbance at 280 nm. (C) The isothermal titration calorimetry (ITC)–based measurement of the binding affinity of NDP52(10-126) with RB1CC1(1286-1395). The dissociation constant (K$_d$) error is the fitted error obtained from the data analysis software when using the one-site binding model to fit the ITC data. DP, the differential power measured by the ITC machine; $\Delta H$, the heat change measured by the ITC machine. (D) Ribbon diagram showing the overall structure of NDP52(10-126) in complex with RB1CC1(1286-1395). In this drawing, NDP52(10-126) is shown in forest green and RB1CC1(1286-1395) is shown in purple. (E) Combined ribbon and surface representation showing the overall architecture of NDP52(10-126)/RB1CC1(1286-1594) complex with the same color scheme and orientation as in (D).
phosphorylation in CCPG1 FIR2 and Optineurin LIR, respectively (S1). We solved the crystal structures of RB1CC1 Claw in complex with the phosphorylated CCPG1 FIR2 and Optineurin LIR and elucidated the detailed molecular mechanism underpinning the interactions of RB1CC1 Claw with CCPG1 and Optineurin as well as their potential regulations by kinase-mediated phosphorylation (S1). Unfortunately, because of lacking relevant complex structures, the underlying molecular mechanisms governing the interactions of RB1CC1 with other currently known binding partners, especially NDP52 and NAP1, are still largely unknown.

Here, we biochemically and structurally characterize the interactions of NDP52 and NAP1 with RB1CC1 and found that, in addition to the NDP52 SKICH/RB1CC1 coiled-coil interaction, the NDP52 LIR region can also directly bind to the Claw domain of RB1CC1, similar to that of NAP1 FIR. We determine the high-resolution crystal structures of the NDP52 SKICH/RB1CC1 coiled-coil complex and the NAP1 FIR/RB1CC1 Claw complex and uncover the molecular mechanism by which NDP52 and NAP1 recognize RB1CC1. In addition, we elucidate that NAP1 and RB1CC1 are competitive in binding to the SKICH domain of NDP52, but the presence of the NAP1 FIR motif can stabilize the NDP52/RB1CC1/NAP1 ternary complex formation. Moreover, we demonstrate that similar to that of NDP52 LIR, the FIR motif of NAP1 can selectively bind to mammalian ATG8 family proteins, and the TBK1-mediated phosphorylation of NDP52 LIR can specifically enhance the interactions of NDP52 LIR with ATG8 family proteins. The determined crystal structure of GABARAP in complex with NAP1 FIR elucidates the detailed molecular mechanism governing the selective interactions of NAP1 FIR with ATG8 family members and suggests that RB1CC1 and ATG8 family proteins are mutually exclusive in interacting with NAP1 FIR and NDP52 LIR.

RESULTS

Biochemical characterizations of the interaction between NDP52 SKICH domain and RB1CC1 coiled-coil region

To elucidate the molecular mechanism governing the specific interaction between NDP52 SKICH and RB1CC1, we first conducted detailed sequence alignment analyses of the NDP52 SKICH regions and the RB1CC1 C-terminal coiled-coil region (residues 1286 to 1395) from different species (figs. S1 and S2), which were reported to mediate the NDP52 and RB1CC1 interaction in previous studies (44, 45). In keeping with their critical functional roles, the sequence alignment results revealed that these regions of NDP52 and RB1CC1 are highly conserved during evolution (figs. S1 and S2). Then, we purified the RB1CC1(1286-1395) fragment and the NDP52(10-126) fragment that includes the entire NDP52 SKICH domain and used the analytical gel filtration chromatography (AGFC)–based assay to test their interaction. The result showed that RB1CC1(1286-1395) can directly bind to NDP52(10-126) (Fig. 1B). However, the small peak shift of RB1CC1(1286-1395) in the presence of NDP52(10-126) on the AGFC profile indicated that the interaction between RB1CC1(1286-1395) and NDP52(10-126) is relatively weak (Fig. 1B). Consistently, quantitative iso-thermal titration calorimetry (ITC) analysis revealed that NDP52(10-126) can directly interact with RB1CC1(1286-1395) with a dissociation constant ($K_D$) value of about 12.5 μM (Fig. 1C). Further truncation analyses of RB1CC1(1286-1395) revealed that the C-terminal portion of RB1CC1(1286-1395) (residues 1343 to 1395) is responsible for the interaction with NDP52(10-126) (Fig. 1B and fig. S3).

The overall structure of the NDP52 SKICH/RB1CC1(1286-1395) complex

To uncover the molecular mechanism underlying the specific interaction between NDP52(10-126) and RB1CC1(1286-1395), we sought to determine their complex structure. However, initial attempts using conventional methods to purify the NDP52(10-126)/RB1CC1(1286-1395) complex failed, likely due to the weak interaction between NDP52(10-126) and RB1CC1(1286-1395). Therefore, we only mixed NDP52(10-126) with RB1CC1(1286-1395) in a 1:1 molar ratio and performed a crystal screening using this protein mixture. Fortunately, using this approach, we obtained good crystals that diffracted to 2.64 Å. Using molecular replacement method with our previously determined apo-form structure of NDP52 SKICH domain [Protein Data Bank (PDB) ID: 5Z7A] (36), we successfully solved the NDP52(10-126)/RB1CC1(1286-1395) complex structure (table S1). In the final complex structure model, each asymmetric unit contains one NDP52(10-126)/RB1CC1(1286-1395) complex, which is composed of one RB1CC1(1286-1395) dimer and two NDP52 SKICH molecules, forming an elongated 2:2 stoichiometric heterotetramer (Fig. 1, D and E). In the complex structure, the two RB1CC1(1286-1395) molecules mainly adopt two continuous $\alpha$ helices and head-to-head pack against each other to form a parallel coiled-coil dimer with an overall length of ~157 Å (Fig. 1, D and E), although one RB1CC1(1286-1395) helix has an obvious kink configuration in its middle region (Fig. 1D), presumably due to crystal packing (fig. S4A). Meanwhile, two NDP52 SKICH molecules, each of which adopts a $\beta$-sandwich immunoglobulin-like architecture consisting of seven antiparallel $\beta$ strands, symmetrically bind to the C-terminal region of the RB1CC1 coiled-coil dimer through their solvent-exposed $\beta4$ and $\beta5$ regions, consistent with our aforementioned biochemical results (fig. S3). Notably, there is no direct contact between two NDP52 SKICH molecules in the complex structure (Fig. 1, D and E), and the overall architecture of NDP52(10-126)/RB1CC1(1286-1395) complex is distinct from any currently known structures revealed by the protein structure comparison server Dali (52). In addition to RB1CC1, the SKICH domain of NDP52 was also demonstrated to interact with NAP1 (7), and, previously, we had determined the structures of the isolated NDP52 SKICH domain and the NDP52 SKICH/NAP1(33-75) complex (36). Further structural comparison analyses revealed that the overall structure of NDP52 SKICH in the NDP52(10-126)/RB1CC1(1286-1395) complex is highly similar to that of the apo-form SKICH domain and the SKICH domain in the NDP52(10-126)/NAP1(33-75) complex, except for the $\beta4$/$\beta5$-connecting loop that directly engages in the binding with RB1CC1 (fig. S4, B and C). Notably, when binding to the SKICH domain of NDP52, the coiled-coil domains of RB1CC1 and NAP1 pack against two completely different surfaces of NDP52 that locate at the opposite sides of the NDP52 SKICH domain (fig. S4D), revealing that the SKICH domain of NDP52 adopts distinct approaches to associate with RB1CC1 and NAP1.

The molecular interfaces in the NDP52 SKICH/RB1CC1(1286-1395) complex

In the determined NDP52 SKICH/RB1CC1(1286-1395) complex structure, there are two different types of interaction interfaces, the RB1CC1 coiled-coil dimerization interface and the NDP52 SKICH/RB1CC1 binding interface (Fig. 1, D and E). In particular, the dimerization of RB1CC1 coiled-coil is mediated by extensive interactions of RB1CC1 FIR with CCPG1 and Optineurin as well as their underlying molecular mechanisms governing the interactions of RB1CC1 with other currently known binding partners, especially NDP52 and NAP1.
hydrophobic and polar interactions between two RB1CC1 helices (residues 1287 to 1394) and can be further divided into three segments of interactions spanning from the N terminus to the C terminus of RB1CC1(1286-1395) (Fig. 2A).

In the complex structure, the two NDP52 SKICH molecules symmetrically bind to two homodimeric interfaces located at the opposite sides of the C-terminal part of the RB1CC1 paired helices, each burying a total surface area of ~785 Å² (Fig. 1, D and E). Detailed structural analysis uncovered that the interacting interface between NDP52 SKICH and RB1CC1 is mainly formed by residues from the β3, β4, and β5 strands as well as the β3/β4, β4/β5, and β5/β6 connecting loops and accommodates RB1CC1 residues located in the C-terminal region of the dimeric RB1CC1 coiled-coil through both hydrophobic and polar interactions (Fig. 2, B and C). In particular, the hydrophobic side chain groups of Y69, T71, F72, and Y97 of NDP52 SKICH pack against a hydrophobic patch formed by the hydrophobic side chains of L1367 and L1371 from one helical RB1CC1 chain and the side chain of I1368 from the other chain of the RB1CC1 dimer (Fig. 2C). In addition, the backbone carboxyl groups of R67, Y69, and W74 together with the polar side chain groups of Y69, Y70, W74, T76, Y96, and Y97 from NDP52 interact with the S1370, D1366, D1364, R1363, and Q1360 residues from one chain of the RB1CC1 dimer and the R1375, S1372, and Q1361 residues from the other chain of the RB1CC1 dimer to form 11 highly specific hydrogen bonds (Fig. 2C). Furthermore, two Arg-Glu pairs (Arg⁶⁰NDP52-Glu¹³⁸⁰RB1CC1 and Arg⁶⁷NDP52-Glu¹³⁸¹RB1CC1), two Arg-Asp pairs (Arg⁶⁰NDP52-Asp¹³⁷⁴RB1CC1 and Arg⁶⁷NDP52-Asp¹³⁷⁵RB1CC1) of salt bridges further strengthen the specific interaction between NDP52 SKICH and RB1CC1 interaction (Fig. 2C). In accordance with their important structural roles, all of these key binding interface residues of RB1CC1 and NDP52 are highly conserved across different eukaryotic species (figs. S1 and S2). Using ITC analyses, we further verified the specific interactions between NDP52 SKICH and RB1CC1 coiled-coil observed in the complex structure (fig. S5). In agreement with the aforementioned structural data, the ITC results showed that point mutations of key interface residues either from NDP52 SKICH or RB1CC1 coiled-coil—such as the F72E, R67E mutations of NDP52 or the L1367A, R1375A, and E1381A mutations of RB1CC1—essentially abolished or largely reduced the specific interaction between NDP52 SKICH and RB1CC1(1286-1395) in our ITC-based analyses (Fig. 2D and fig. S5).

The LIR region of NDP52 can directly interact with RB1CC1 Claw domain

Intriguingly, further AGFC-based biochemical assays showed that, in contrast to that of the NDP52(10-126)/RB1CC1(1286-1395) interaction (Fig. 1B), a longer fragment of NDP52, NDP52(1-316) that includes the SKICH domain, the unconventional LIR motif, and the coiled-coil region of NDP52 (fig. S2) can effectively interact with the RB1CC1(1286-1394) fragment that contains the Claw domain in addition to the C-terminal coiled-coil region of RB1CC1 (Fig. 3A and fig. S1). Moreover, using analytical ultracentrifugation analyses, we further elucidated that the NDP52(1-316) and RB1CC1(1286-1394) fragments both form dimers in solution, and they can effectively interact with each other to form a large and inhomogeneous binary complex (Fig. 3B and fig. S6). On the basis of this observation, we inferred that there might be additional interaction sites between NDP52 and RB1CC1 except for the NDP52 SKICH/RB1CC1 coiled-coil binding site. Our AGFC-based assays demonstrated that the NDP52(128-316) fragment, which contains the LIR motif and the coiled-coil domain of NDP52 but lacking the SKICH domain (fig. S2), can directly interact with the Claw-containing RB1CC1(1450-1594) and RB1CC1(1490-1594) fragments but not RB1CC1(1396-1489) that lacks the Claw domain (Fig. 3C and fig. S7, A and B). In contrast, the SKICH-containing NDP52(10-126) fragment is unable to interact with RB1CC1(1450-1594) (fig. S7C). Further fragment truncation analyses together with AGFC-based assays elucidated that the LIR region of NDP52 (residues 129 to 141) can specifically interact with RB1CC1 Claw domain (Fig. 3D and fig. S7D). Consistently, the NDP52(10-141) fragment that includes the SKICH domain and the following LIR region of NDP52 can also directly interact with RB1CC1 Claw domain (Fig. 3E). Notably, given that RB1CC1 Claw domain forms a symmetrical dimer (48), the dimerization of NDP52 LIR induced either by the coiled-coil domain of NDP52 [the NDP52(128-316) fragment] or by the artificial fusion of NDP52 LIR with the dimeric NAP1(33-75) (the NDP52-NAP1 chimera) can remarkably promote the interaction between NDP52 and RB1CC1, when comparing with that of the monomeric NDP52 LIR fragment in the AGFC-based assays (Fig. 3, D and F, and fig. S7, D and E). Unfortunately, likely because of potential allosteric changes induced by the NDP52 LIR/RB1CC1 Claw interaction, we could not use ITC-based assays to quantitatively measure the binding affinity $K_d$ values of RB1CC1 Claw with different NDP52 fragments that contain the NDP52 LIR motif. Therefore, we used the powerful nuclear magnetic resonance (NMR) spectroscopy to further validate the two interaction sites between NDP52 and RB1CC1. We chose the NDP52(10-141) fragment, as it contains both the RB1CC1-binding SKICH domain and the LIR region of NDP52. Titrations of $^{15}$N-labeled NDP52(10-141) with unlabeled RB1CC1(1490-1594) or RB1CC1(1286-1395) proteins showed that many peaks in the $^1$H-$^15$N heteronuclear single-quantum coherence (HSQC) spectrum of NDP52(10-141) undergo obvious peak broadenings or chemical shift changes (Fig. 3, G and H), confirming that NDP52(10-141) can directly bind to the RB1CC1 Claw domain and the RB1CC1(1286-1395) fragment. Since the NDP52 SKICH domain had been well assigned in the $^1$H-$^15$N HSQC spectrum of NDP52(10-126) in our previous study (36), therefore, we could easily differentiate the NMR peaks corresponding to the LIR region from that of the SKICH domain in the $^1$H-$^15$N HSQC spectrum of NDP52(10-141) by comparing the two $^1$H-$^15$N HSQC spectra of NDP52(10-141) and NDP52(10-126) (fig. S8). Further detailed analyses revealed that the NMR peaks corresponding to the LIR region of NDP52 in the $^1$H-$^15$N HSQC spectrum of NDP52(10-141) show obvious changes when titrated with the Claw domain of RB1CC1 (Fig. 3G) but display little changes in the presence of RB1CC1(1286-1395) (Fig. 3H), further confirming that the LIR region of NDP52 is directly involved in the interaction with RB1CC1 Claw but not RB1CC1(1286-1395). Collectively, all these data clearly demonstrated that the LIR region of NDP52 contains another RB1CC1-binding site, which can specifically interact with the Claw domain of RB1CC1.

The overall structure of the NAP1 FIR/RB1CC1 Claw complex

Next, we intended to determine their complex structure to elucidate how RB1CC1 Claw domain recognizes the LIR motif of NDP52. Unfortunately, despite many attempts, we failed to solve the complex structure either using x-ray crystallography or NMR spectroscopy,
likely due to the weak and dynamic nature of this RB1CC1 Claw and NDP52 LIR interaction. In addition to the LIR region of NDP52, the FIR regions of NAP1, SINTBAD, ATG16L1, and CCPG1 were also reported to interact with the Claw domain of RB1CC1 (20, 45, 49, 50). Further detailed sequence alignment analysis of those currently known RB1CC1 Claw-binding regions revealed a consensus core sequence \( \Psi \Theta XX \Theta \) (where \( \Psi \) represents an acidic Asp residue; \( \Theta \) represents a bulk hydrophobic Ile, Leu, or Val residue; and \( X \) represents any residues) shared by those RB1CC1-binding proteins (Fig. 4A), indicating that they might adopt a similar binding
mode to interact with RB1CC1 Claw domain. Then, we focused on the interaction of RB1CC1 Claw domain with the FIR motif of NAP1 (residues 6 to 16), which is strictly conserved across different vertebrate species (fig. S9). Using the AGFC-based assay, we confirmed that NAP1 FIR (residues 6 to 16) can directly bind to the Claw domain of RB1CC1 (Fig. 4B). Further quantitative fluorescent polarization (FP)-based analyses showed that the synthetic NDP52 LIR and NAP1 FIR peptides can both directly interact with RB1CC1 Claw, and, notably, NAP1 FIR binds to RB1CC1 Claw with a 10-fold stronger binding affinity than that of NDP52 LIR (Fig. 4C). Considering the relatively stronger binding between NAP1 FIR and the Claw domain of RB1CC1, we sought to determine the NAP1 FIR/RB1CC1 Claw complex structure. After numerous trials, we finally managed to solve the high-resolution crystal structure of the
The interactions of RB1CC1 (1490-1594) with NAP1 (6-16) (green) and NDP52 (129-141) (purple). The KFP-based assays measure the AGFC analysis of the interaction between NAP1 (6-16) and RB1CC1 (1490-1594). (C) Combined surface representation and ribbon-stick model showing the charge-charge interactions between RB1CC1 Claw and NAP1 FIR in the complex structure.

**The binding interface between NAP1 FIR and RB1CC1 Claw**

In the NAP1 FIR/RB1CC1 Claw complex structure, each NAP1 FIR packs extensively with a solvent-exposed groove formed by the β4 and β5 strands as well as the β4/β5-connecting loop of the monomeric RB1CC1 Claw domain (Fig. 4, D to F). Further structural analyses of the binding interface of the NAP1 FIR/RB1CC1 Claw complex elucidated that the hydrophobic side chain of I9 from NAP1 FIR occupies a hydrophobic pocket on the Claw domain formed by the side chains of RB1CC1 C1565, A1567, F1574, V1576, and F1582, and, meanwhile, the hydrophobic side chain of NAP1 L12 residue packs against a small hydrophobic groove formed by the aromatic side chain of Y1564 and the aliphatic side chain of K1581 from RB1CC1 (Figs. 4E and 5A). In addition, the negatively charged E6, D7, D8, and E15 residues of NAP1 FIR form specific charge-charge and hydrogen bonding interactions with the positively charged R1584, R1573, K1569, K1568, and K1562 residues of RB1CC1 Claw (Figs. 4F and 5A). Furthermore, the polar side chain of NAP1 H14 has a specific hydrogen bonding interaction with the negatively charged side chain of RB1CC1 E1561 residue (Figs. 4F and 5A), and the backbone groups of C10, L12, N13, and E15 residues located at the β strand region of NAP1 FIR interact with the backbone groups of RB1CC1 Q1566, Y1564, and K1562 residues to form seven strong backbone hydrogen bonds (Figs. 4F and 5A). In accordance with
their important structural roles, all of these key binding interface residues of RB1CC1 and NAP1 are highly conserved across different species (figs. S1 and S9). Using AGFC and FP assays, we further verified the interactions between NAP1 FIR and RB1CC1 Claw observed in the complex structure. In agreement with our structure data, the obtained results showed that point mutations of key interface residues—such as the D8A, I9Q, H14A, and E15A mutations of NAP1 FIR or the Y1564S, K1569A, R1573E, and F1574Q mutations of RB1CC1 Claw—all significantly decrease or essentially abolish the specific interaction between NAP1 FIR and RB1CC1 Claw (Fig. 5B and figs. S12 and S15, A to D).

**NDP52 LIR and NAP1 FIR share a similar binding mode to interact with RB1CC1 Claw**

On the basis of the determined NAP1 FIR/RB1CC1 Claw complex structure together with our detailed sequence alignment analysis (Figs. 4A and 5A), we inferred that NDP52 LIR should adopt a similar binding mode as that of NAP1 FIR for interacting with RB1CC1 Claw. Further structural modeling analysis showed that the LIR region of NDP52 may interact with RB1CC1 Claw domain in a similar approach as that of NAP1 FIR (Fig. S13A), and the lack of corresponding NAP1 H14 and E15 residues in NDP52 LIR likely rationalizes the fact that NDP52 LIR has a weaker binding ability to the Claw domain of RB1CC1 compared with NAP1 FIR (Fig. 4, A, C, and E, and fig. S13A). To further confirm our hypothesis, we designed a series of NDP52 LIR mutations including the E130A, E131A, D132A, I133S, and V136R mutations of NDP52 LIR, which are corresponding to the critical Claw-binding E6, D7, D8, I9, and L12 residues of NAP1 FIR, respectively (Fig. 4A). As expected, using AGFC- and FP-based assays, we demonstrated that those mutations of NDP52 LIR all significantly disrupt the interaction between NDP52 LIR and RB1CC1 Claw (Fig. 3C and fig. S13, B to F). Conversely, the Y1564S, K1569A, R1573E, and F1574Q mutations of RB1CC1 Claw, which were proved to affect the NAP1 FIR/RB1CC1 Claw interaction (Fig. 5B and figs. S12, E to H, and S15, A to D), also largely attenuate or essentially abolish the specific interaction of NDP52 LIR and RB1CC1 Claw (Fig. 5B and figs. S14 and S15, E to F). Overall, on the basis of these structural and biochemical results, we concluded that NDP52 LIR and NAP1 FIR share a general binding mode to interact with the Claw domain of RB1CC1. Therefore, our biochemical and structural characterizations elucidated that there are two different binding sites between NDP52 and RB1CC1, the NDP52 SKICH/RB1CC1 coiled-coil interaction site and the NDP52 LIR/RB1CC1 Claw interaction site. Using AGFC and coimmunoprecipitation (co-IP) assays, we further validated the dual binding sites of NDP52 and RB1CC1 observed in this study. In line with our structural data, double mutations of the key interface residues located in the two different binding sites of NDP52—such as R67E/D132A, R67E/I133S, and F72E/D132A of NDP52—or, conversely, the key binding interface residues of RB1CC1—such as R1375A/R1573E, R1375A/F1574Q, and L1367A/R1573E of RB1CC1—all largely diminished or essentially abolished...
the specific interaction between NDP52 and RB1CC1 in vitro and in cells (Fig. 5, C to E, and fig. S16).

**The FIR motif of NAP1 is essential for the stable RB1CC1/NAP1/NDP52 ternary complex formation**

Our aforementioned structural analyses revealed that NDP52 SKICH domain uses two distinct and nonoverlapping binding interfaces to interact with the coiled-coil domains of RB1CC1 and NAP1 (fig. S4D), suggesting that RB1CC1 and NAP1 might simultaneously bind to the SKICH domain of NDP52. However, unexpectedly, further AGFC coupled with SDS–polyacrylamide gel electrophoresis (PAGE) assays together with analytical ultracentrifugation analyses revealed that NAP1(33–75) can directly compete with RB1CC1(1286–1594) for binding to NDP52(1–316), thereby disrupting the formation of the large RB1CC1(1286–1594)/NDP52(1–316) binary complex [the backward shift rather than the forward shift of the major complex peak in the AGFC profile of RB1CC1(1286–1594)/NDP52(1–316) complex in the presence of NAP1(33–75) proteins] (Fig. 6, A and B). Meanwhile, there is no stable RB1CC1(1286–1594)/NDP52(1–316)/NAP1(33–75) ternary complex formation (Fig. 6C and fig. S17). In line with this biochemical result, detailed structural comparison analysis of the conformations of NDP52 SKICH in the RB1CC1–bound and the NAP1–bound forms showed obvious conformational differences of the key RB1CC1–binding interface residue Tyr97 of NDP52 SKICH in the RB1CC1–bound form and the NAP1–bound form (fig. S18A). Notably, the conformation of NDP52 Tyr97 in the NAP1–bound form is unable to interact with RB1CC1 due to the potential steric hindrance (fig. S18B). Thus, the coiled-coil domains of RB1CC1 and NAP1 are actually competitive in binding to the SKICH domain of NDP52, likely due to allosteric effects. In contrast, the NAP1(1–75) fragment, which contains both the RB1CC1 Claw–binding FIR motif and the NDP52 SKICH–binding coiled-coil, can readily form a stable ternary complex with RB1CC1(1286–1594) and NDP52(1–316) (Fig. 6, D to F, and fig. S19), highlighting the essential role of NAP1 FIR motif for the formation of the trimeric RB1CC1/NAP1/NDP52 complex.

**The FIR motif of NAP1 can selectively bind to six mammalian ATG8 orthologs**

As the sequence pattern of NAP1 FIR motif is very similar to the noncanonical LIR motif of NDP52 (Fig. 4A), which was previously demonstrated to selectively interact with LC3C (35), we wondered whether NAP1 FIR could also associate with mammalian ATG8 family proteins. As expected, using AGFC and FP assays, we elucidated that NAP1 FIR can directly bind to six mammalian ATG8 orthologs (Fig. 7, A to C, and figs. S20 and S21), similar to that of NDP52 LIR (Fig. 7C and figs. S22 and S23). However, in contrast to that of NDP52 LIR (Fig. 7C and fig. S23), the FIR motif of NAP1 preferentially binds to GABARAP, GABARAPL1, and LC3C with a binding affinity $K_d$ value of $\sim$5.1, $\sim$3.9, and $\sim$7.2 $\mu$M, respectively (Fig. 7, B and C, and fig. S21). Together, our data clearly demonstrated that NAP1 FIR is also an unconventional LIR motif, which can selectively bind to six mammalian ATG8 members.

**The structure of NAP1 FIR in complex with GABARAP**

To further elucidate the molecular mechanism underpinning the selective interactions of NAP1 FIR with ATG8 family members, we determined the crystal structure of NAP1 FIR in complex with GABARAP. As expected, in the solved NAP1 FIR/GABARAP complex structure, GABARAP adopts a typical ATG8 homolog protein architecture consisting of two N-terminal $\alpha$-helices followed by a ubiquitin-like core (Fig. 7D). The defined NAP1 FIR motif in the complex structure contains eight highly conserved residues (“DDICILNH”) (Fig. 7D and fig. S9) and adopts an extended configuration to pack against a solvent-exposed elongated groove mainly formed by the $\alpha_2$ helix, $\alpha_3$ helix, $\beta_1$ strand, and $\beta_2$ strand of GABARAP, covering a total binding interface area of $\sim$447.8 Å$^2$ (Fig. 7, D and E). Similar to that of NDP52 LIR and LC3C interaction observed in the determined NDP52 LIR/LC3C complex structure (PDB ID: 3VWV) (fig. S24), the specific interaction between NAP1 FIR and GABARAP is also mediated by both hydrophobic and polar interactions (Fig. 7, E and F). Specifically, the hydrophobic side chains of I9 and I11 residues from NAP1 FIR pack against a hydrophobic patch of GABARAP assembled by the hydrophobic side chains of GABARAP I21, P30, L50, and F104 as well as the aliphatic side chain of GABARAP K48 residue (Fig. 7, E and F). In parallel, the side chains of C10 and L12 residues of NAP1 FIR occupy a hydrophobic groove formed by the side chains of Y49, V51, P52, L55, F60, and L63 residues of GABARAP (Fig. 7, E and F). Furthermore, the negatively charged side chains of NAP1 D7 and D8 directly couple with the positively charged side chains of GABARAP K47 and K48 to form two specific charge–charge interactions, and the backbone oxygen and amide group of NAP1 C10 together with the backbone amide group of NAP1 L12 form three backbone hydrogen bonds with the K48 and L50 residues of GABARAP (Fig. 7, E and F). In addition, the NAP1 FIR/GABARAP complex is further stabilized by two weak hydrogen bonds, one of which is formed between the side chain of NAP1 C10 and the positively charged side chain of GABARAP K46, while the other is mediated by the backbone oxygen group of NAP1 L12 and the positively charged side chain of GABARAP R28 residue (Fig. 7, E and F). All these key interface residues of NAP1 and GABARAP are highly conserved during evolution (figs. S9 and S25A). Intriguingly, detailed amino acid sequence alignment analysis revealed that several key binding interface residues vary significantly among the six ATG8 orthologs (fig. S25B). For instance, the residue corresponding to the positively charged K47 in GABARAP is a polar Thr residue in the LC3 subfamily, and the residues corresponding to the bulk hydrophobic L55 and F60 in GABARAP are two much smaller Val and Leu residues in LC3A and LC3B (fig. S25B). The identification of those nonconserved interface residues among different ATG8 family proteins rationalizes the selective binding of NAP1 FIR to different ATG8 family proteins.

**TBK1–mediated phosphorylation of T137 in NDP52 LIR enhances the binding of NDP52 LIR to ATG8 family proteins**

Previously, our and other people’s reports revealed that, in addition to associating with NDP52 through its N-terminal coiled-coil domain, NAP1 can also directly bind to TBK1 through its C-terminal TBD domain (Fig. 1A), thereby NAP1 can function as an adaptor to recruit the TBK1 kinase for NDP52 (7, 36, 40, 41). The recruited active TBK1, in turn, can phosphorylate NDP52 and regulate NDP52–mediated selective autophagy processes (7, 32, 43). Using in vitro phosphorylation assays, we confirmed that TBK1 can directly phosphorylate NDP52 (fig. S26). Our mass spectrometry (MS) analysis revealed that the conserved T137 residue within the NDP52 LIR region can be readily phosphorylated by TBK1 (Fig. 8A). On the basis of the determined NDP52 LIR/LC3C complex structure, the
T137 residue of NDP52 is adjacent to the positively charged K36 residue of LC3C (fig. S27, A and B), and the corresponding residue in other ATG8 family proteins is also a positively charged Lys or Arg residue (figs. S25 and S27B), suggesting that the phosphorylation of NDP52 T137 might strengthen the interactions of NDP52 LIR with ATG8 family proteins. Consistent with our structure and sequence analyses showing the protein components of corresponding fraction A11 to B11 collected from the analytic gel filtration chromatography experiments of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with increasing molar ratios of NAP1(33-75) fragment in (A) or NAP1(1-75) fragment in (D), (B and E) SDS-PAGE combined with Coomassie blue staining analyses showing the protein components of corresponding fraction A11 to B11 collected from the analytic gel filtration chromatography experiments of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with increasing molar ratios of NAP1(33-75) fragment in (A), (B, or NAP1(1-75) fragment in (D), (E). Overlay plot of the sedimentation velocity data of NAP1(33-75) fragment (black), RB1CC1(1286-1594) fragment (blue), the NDP52(1-316)/NAP1(33-75) complex (green), and the fraction B12 collected from the analytic gel filtration chromatography experiment of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with 1:4 molar ratio of NAP1(33-75) fragment in (A). (F) Overlay plot of the sedimentation velocity data of NAP1(1-75) fragment (black), NDP52(1-316)/NAP1(1-75) mixture (green), the RB1CC1(1286-1594)/NAP1(1-75) mixture (blue), and the fraction A12 collected from the analytic gel filtration chromatography experiment of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with 1:4 molar ratio of NAP1(1-75) fragment in (D).

DISCUSSION
In this study, we elucidated that, in addition to the previously reported NDP52 SKICH and RB1CC1 coiled-coil interaction (Fig. 1), the LIR motif of NDP52 can directly bind to the Claw domain of

Fig. 6. The FIR motif of NAP1 is essential for the stable RB1CC1/NAP1/NDP52 ternary complex formation. (A and D) AGFC analyses of the purified NDP52(1-316)/RB1CC1(1286-1594) complex titrated with increasing molar ratios of NAP1(33-75) (A) or NAP1(1-75) (D). (B and E) SDS-PAGE combined with Coomassie blue staining analyses showing the protein components of corresponding fraction A11 to B11 collected from the analytic gel filtration chromatography experiments of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with increasing molar ratios of NAP1(33-75) fragment in (A) (B), or NAP1(1-75) fragment in (D) (E). (C) Overlay plot of the sedimentation velocity data of NAP1(33-75) fragment (black), RB1CC1(1286-1594) fragment (blue), the NDP52(1-316)/NAP1(33-75) complex (green), and the fraction B12 collected from the analytic gel filtration chromatography experiment of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with 1:4 molar ratio of NAP1(33-75) fragment in (A). (F) Overlay plot of the sedimentation velocity data of NAP1(1-75) fragment (black), NDP52(1-316)/NAP1(1-75) mixture (green), the RB1CC1(1286-1594)/NAP1(1-75) mixture (blue), and the fraction A12 collected from the analytic gel filtration chromatography experiment of the NDP52(1-316)/RB1CC1(1286-1594) complex titrated with 1:4 molar ratio of NAP1(1-75) fragment in (D).
RB1CC1 (Fig. 3). Therefore, there are dual interaction sites between NDP52 and RB1CC1, in contrast to previous reports (44, 45). Since NDP52 and RB1CC1 form homodimers (Fig. 3B), this dual interaction mode may not only increase the binding efficiency between NDP52 and RB1CC1 but also produce multiple binding sites between the dimeric NDP52 and RB1CC1, thereby facilitating the formation of large polymeric NDP52/RB1CC1 binary complex, in line with our aforementioned biochemical data (Fig. 3, A and B). Notably, the crucial role of the specific interaction between NDP52 SKICH and RB1CC1 coiled-coil for the recruitment of ULK complex by NDP52 during NDP52-mediated selective autophagy processes had been well established (44, 45). Unfortunately, because of our current technological limitations, the potential functional roles contributed by the newly identified NDP52 LIR/RB1CC1 Claw interaction in relevant NDP52-mediated selective autophagy still await further investigation.

Our analytical ultracentrifugation analyses showed that, in contrast to that of the NDP52(1-316)/RB1CC1(1286-1594)/NAP1(1-75) ternary complex, the purified NDP52(1-316)/RB1CC1(1286-1594) binary complex has a very large and broad sedimentation coefficient distribution (Fig. S28, A to C), suggesting that it forms an inhomogeneous binary complex likely with different oligomeric states. Consistently, further structure modeling analysis showed that NDP52(1-316) and RB1CC1(1286-1594) is unlikely to form a stable 2:2 stoichiometric heterotetramer, as NDP52(1-316) forms a coiled-coil–mediated dimer but the C termini of the two NDP52 molecules in the NDP52(10-126)/RB1CC1(1286-1395) complex structure point to opposite directions and are far away from each other (fig. S28D). Meanwhile, we also calculated and summarized the friction ratio values, which contain conformational information, from our analytical ultracentrifugation data (fig. S28C). The isolated NAP1(1-75), NDP52(1-316) and RB1CC1(1286-1594) as well as the NAP1(1-75)/NDP52(1-316) complex have larger friction ratio values of about 1.548, 2.143, 1.725, and 2.313, respectively (fig. S28D), in line with the fact that all of them contain a large number of dimeric coiled-coil regions, which have highly asymmetric rather than globular shapes. Although with a very larger sedimentation coefficient value, the NDP52(1-316)/RB1CC1(1286-1594) binary complex has a very small friction ratio value of 1.348 (fig. S28C). In sharp contrast, the NDP52(1-316)/RB1CC1(1286-1594)/NAP1(1-75) ternary complex shows a relatively smaller sedimentation coefficient value and has a very large friction ratio value of 1.939 (fig. S28C), which indicates that the binding of NAP1(1-75) may not only affect the oligomerization state of the NDP52(1-316)/RB1CC1(1286-1594)
complex but also induce conformation changes of the NDP52(1-316)/RB1CC1(1286-1594) complex.

Intriguingly, using solid biochemical analyses, we found that although the N-terminal coiled-coil domain of NAP1 alone can compete with RB1CC1 for binding to the NDP52 SKICH domain, however, in the presence of NAP1 FIR motif, NAP1, NDP52, and RB1CC1 actually can work together to form a stable ternary complex (Fig. 6). Given that there are dual interactions between NDP52 and RB1CC1, although NAP1(33-75) can compete with RB1CC1 coiled-coil for binding to the NDP52 SKICH domain, RB1CC1 may still interact with the LIR region of NDP52 through its Claw domain, thereby associating with NDP52 to form a NAP1(33-75)/NDP52/RB1CC1 ternary complex. Therefore, it is puzzling why NAP1(1-75) but not NAP1(33-75) can form a stable ternary complex with RB1CC1 and NDP52. When we aligned a NDP52 SKICH molecule in the NAP1(33-75)/NDP52 SKICH complex structure (PDB ID: 5Z7L) with a selected NDP52 SKICH-LIR molecule from the NDP52 SKICH-LIR/LC3C complex (PDB ID: 3VVW), which is further superimposed with a structure model of the NDP52 LIR/RB1CC1 Claw complex through their NDP52 LIR regions, we can clearly find that it is impossible for NDP52 to simultaneously interact with NAP1(33-75) and RB1CC1, owing to a potential steric hindrance between the NAP1(33-75) coiled-coil helices and the dimeric RB1CC1 Claw (fig. S29A). Therefore, consistent with our biochemical results (Fig. 6), our structural analyses further confirmed that NAP1(33-75) cannot form a stable ternary complex with RB1CC1 and NDP52. As for the interactions in the NDP52/NAP1(1-75)/RB1CC1 ternary complex, on the basis of the quantitative data obtained in this study together with our previous ITC characterization (36), the FIR motif of NAP1 has a roughly 10-fold stronger binding ability toward RB1CC1 Claw than that of NDP52 LIR (Fig. 4C), and the NAP1 coiled-coil has a 10-fold stronger binding ability to NDP52 SKICH than that of the RB1CC1 coiled-coil ($K_d \sim 1.2 \mu M$ versus $K_d \sim 12.5 \mu M$). Meanwhile, the FIR motif of NAP1 is essential for the formation of the NDP52/NAP1/RB1CC1 ternary complex (Fig. 6). Therefore, the NAP1 FIR/RB1CC1 Claw and NAP1 coiled-coil/NDP52 SKICH interactions must exist in the NDP52/NAP1/RB1CC1 ternary complex. However, we cannot exclude the possibility that there are additional weak interactions of RB1CC1 with NDP52 and/or NAP1 in the NDP52/NAP1/RB1CC1 ternary complex due to synergistic effects. Nevertheless, on the basis of the current biochemical and structural results, we drew a simplified model to describe the formation of the NDP52/NAP1/RB1CC1 ternary complex (fig. S29B). In this model, the dimeric NDP52, NAP1, and RB1CC1 form a stable 2:2:2 stoichiometric ternary complex mediated by the specific NAP1 FIR/RB1CC1 Claw and NAP1 coiled-coil/NDP52 SKICH interactions (fig. S29B). In the near future, we hope to solve the trimeric NDP52/NAP1/RB1CC1 complex structure to elucidate the precise molecular mechanism governing the assembly of this ternary complex.

Notably, recent biochemical and electron microscopy–based structural studies showed that the engagement of NAP1 with RB1CC1 can allosterically stimulate the membrane-binding ability of RB1CC1 as well as the ULK complex and is required for the membrane recruitment of ULK complex (53). Our structural study showed that the binding of NAP1 FIR to the RB1CC1 Claw can cause a large conformational reorganization of the dimeric RB1CC1 Claw domains (fig. S10). Whether the conformational changes of the dimeric RB1CC1 Claw induced by NAP1 FIR or likely NDP52 LIR might potentially regulate the membrane-binding ability of RB1CC1 remains an open question that is worthwhile to be addressed in the future.

In this work, we uncovered that the unconventional LIR motif of NDP52 and the FIR motif of NAP1 are very similar, both of which can directly interact with the Claw domain of RB1CC1 and ATG8 family proteins (Fig. 4, A to C, and Fig. 7, A to C). In the future, it will be interesting to know whether NAP1/SINTBAD could function as an autophagy receptor to mediate unknown selective autophagy processes. Structural comparison analyses revealed that the FIR motif of NAP1 adopts essentially the same key residues to interact with RB1CC1 Claw and the ATG8 family member GABARAP (Figs. 5A and 7F). Therefore, RB1CC1 and ATG8 family protein should be competitive in binding to NDP52 LIR and NAP1 FIR, and the recruitments of ULK complex and ATG8 family proteins by
NDP52 are likely to be mutually exclusive, similar to that of P62 (48). Our AGFC coupled with SDS-PAGE assays showed that NDP52 alone can readily interact with LC3C, while the NDP52/RB1CC1 binary complex and the NDP52/NAP1/RB1CC1 ternary complex display a poor ability to recruit LC3C (fig. S30). As the TBK1-mediated phosphorylation of T137 in the LIR of NDP52 can specifically enhance the interactions between NDP52 and ATG8 family proteins (fig. 8B), this TBK1-mediated phosphorylation event of NDP52 T137 may facilitate the downstream association of NDP52 with ATG8 family proteins. Definitely, more work is required to elucidate the precise relationship between ATG8 family proteins, ULK complex, and TBK1 complex in associating with NDP52 in the future.

Notably, previous functional studies demonstrated that the recruitments of ULK and TBK1 complexes by NDP52 are independent, and the NDP52 SKICH/RB1CC1 coiled-coil interaction rather than the NAP1 FIR/RB1CC1 Claw interaction is essential for the recruitment of RB1CC1 to invading S. Typhimurium (48). Further considering that there is a direct competition between RB1CC1 and NAP1 in binding to the SKICH domain of NDP52 (fig. 6, A and B), therefore, it is highly likely that NDP52 should first recruit the ULK complex and then interact with NAP1/SINTBAD to recruit the TBK1 complex during selective autophagy. Accordingly, on the basis of our studies together with other group reports, we proposed a model depicting the recruitments of ULK complex, TBK1 complex, and ATG8 family proteins by autophagy receptor NDP52 during the initiation of autophagosome formation in NDP52-mediated selective autophagy (fig. S31). In this model, the ubiquitin proteins decorated on the autophagic cargo, such as invading pathogen or dysfunctional mitochondria, was first recognized by NDP52, which, in turn, recruits the RB1CC1-containing ULK complex mediated by the dual interaction sites between NDP52 and RB1CC1, forming high-order clusters to activate the ULK complex (fig. S31). Meanwhile, the TBK1 complex was also recruited by NDP52 through the adaptor protein NAP1/SINTBAD, which, in turn, could further rearrange the association of NDP52 with ULK complex to form a stable complex with the NDP52 and ULK complex (fig. S31). Subsequently, the activated ULK complex would in situ initiate autophagosome formation by promoting the recruitment of downstream autophagic machinery, which eventually leads to the phosphatidylethanolamine (PE) conjugation and the enrichment of ATG8 family proteins on the nascent phagophore membrane (fig. S31). Meanwhile, the activated TBK1 complex would directly phosphorylate NDP52 and promote its ability to interact with ATG8 family protein (fig. S31). Then, the enriched PE-modified ATG8 family proteins can more easily compete with RB1CC1 for binding to the phosphorylated NDP52, thereby facilitating the forming of autophagosome around the autophagic cargo targeted by NDP52 (fig. S31).

**MATERIALS AND METHODS**

**Plasmids and peptides**

The full-length human NDP52, RB1CC1, and TBK1 were obtained from J. Han from the School of Life Sciences, Xiamen University, Xiamen, China. The synthetic fluorescein isothiocyanate (FITC)-labeled peptides 5-FITC-ENEELIVpTTQG (phos-NDP52 LIR), 5-FITC-ENEELIVVTTQG (NDP52 LIR), and 5-FITC-EDDICILNHEK (p-NAP1 LIR), where pT corresponds to phosphorylated threonine, were purchased from the China Peptides company, and the purities of the commercially synthesized peptides were >95%.

**Protein expression and purification**

Following procedures previously described in (51), we performed the protein expression and purification experiments. The different DNA fragments encoding human RB1CC1, NDP52, NAP1, and other related mutants were cloned into the pET-32M3C vector (a modified version of pET28a vector containing an N-terminal His6-tag and Trx-tag or Trx-tag) or the pET-NanoLuc vector (a modified version of pET28a vector containing an N-terminal Nano-tag and His6-tag) for recombinant protein expressions. Recombinant proteins were expressed in BL21 (DE3) *Escherichia coli* cells induced by 100 μM isopropyl-β-D-thiogalactopyranoside at 16°C. The bacterial cell pellets were resuspended in the binding buffer [50 mM tris, 500 mM NaCl, and 5 mM imidazole (pH 7.9)] and then lysed by the ultra-high-pressure homogenizer FB-110XNANO homogenizer machine (Shanghai Litu Machinery Equipment Engineering Co. Ltd.). Then, the lysis was spun down by centrifugation at 16,500 rpm (35,000 g) for 30 min to remove the pellet fractions. His6-tagged proteins were purified by Ni2+-NTA agarose (GE Healthcare) affinity chromatography. Each recombinant protein was further purified by size exclusion chromatography. The N-terminal Trx-tag or Nano-tag was cleaved by HRV3C protease and then further removed by size exclusion chromatography or Q sepharose Fast Flow (HiTrapQFF) ion-exchange chromatography. Uniformly 15N-labeled NDP52 fragment proteins were prepared by growing bacteria in M9 medium using 15NH4Cl (Cambridge Isotope Laboratories Inc.) as the sole nitrogen source.

**Analytical gel filtration chromatography**

Following procedures previously described in (51), we performed the AGFC analyses. Specifically, purified proteins were loaded on to a Superose 200 increase 10/300 GL column (GE Healthcare) equilibrated with a buffer containing 20 mM tris-HCl (pH 7.9), 100 mM NaCl, and 1 mM dithiothreitol (DTT). AGFC was carried out on an AKTA FPLC system (GE Healthcare). The fitting results were further output to the Origin 9.0 software and aligned with each other.

**ITC assay**

Following procedures previously described in (36), we performed the ITC measurements on a MicroCal PEAQ-ITC calorimeter or an automated system (Malvern) at 25°C. All protein samples were in the same buffer containing 50 mM tris (pH 7.5), 100 mM NaCl, and 1 mM DTT. The titration processes were performed by injecting 40 μl of aliquots of the syringe sample into the cell sample at time intervals of 2 min to ensure that the titration peak returned to the baseline. The titration data were analyzed using the Malvern MicroCal PEAQ-ITC analysis program and fitted using the one-site binding model.

**NMR spectroscopy**

Following procedures previously described in (51), we performed the NMR analyses. The 15N-labeled protein samples for NMR titration experiments were concentrated to ~0.2 mM. All the protein samples for NMR studies were prepared in the 50 mM potassium phosphate buffer containing 50 mM NaCl and 1 mM DTT (pH 6.5), and NMR spectra were acquired at 25°C on an Agilent 800 MHz spectrometer equipped with an actively z gradient shielded triple.
resonance cryogenic probe at the Shanghai Institute of Organic Chemistry.

**Analytical ultracentrifugation**

Following procedures previously described in (51), we performed sedimentation velocity experiments on a Beckman XL-I analytical ultracentrifuge equipped with an eight-cell rotor under 42,000 rpm at 20°C. The partial specific volume of different protein samples and the buffer density were calculated using the program SEDNTERP (www.rasmb.org/). The final sedimentation velocity data were analyzed and fitted to a continuous sedimentation coefficient distribution model using the program SEDFIT (54). The fitting results were further output to the Origin 9.0 software and aligned with each other.

**Fluorescence polarization assay**

Following procedures previously described in (51), we performed fluorescence anisotropy binding assays on the SpectraMax i3x Multi-Mode Detection Platform from Molecular Devices, using a 485-nm excitation filter and a 535-nm emission filter. Peptides were labeled with FITC isomer I at the N terminus. In this assay, the FITC-labeled peptide (~0.25 μM) was titrated with increasing amount of testing proteins in a 20 mM tris (pH 7.9) buffer containing 100 mM NaCl and 1 mM DTT at 25°C. The K_d values were obtained by fitting the titration curves with the classical one-site binding model using GraphPad Prism 8 software.

**Protein crystallization and structural elucidation**

Crystals of RB1CC1(1286-1395)/NDP52(10-126) complex, NAP1(6-16) fused with RB1CC1(1490-1594), and NAPI(6-16)/GABARAP complex were obtained using the sitting-drop vapor-diffusion method at 16°C. The fresh purified RB1CC1(1286-1395)/NDP52(10-126) protein [10 or 20 mg/ml in 20 mM tris-HCl, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA (pH 7.9)] was mixed with equal volumes of reservoir solution containing 0.1 M MgCl_2, 0.1 M MES (pH 6.0), 8% PEG-6000 (polyethylene glycol, average molecular weight 6000 DA). The fresh purified NAPI(6-16) fused with RB1CC1(1490-1594) protein was mixed with equal volumes of reservoir solution containing 0.1 M phosphate/citrate (pH 4.2) and 40% PEG-300. The fresh purified NAPI(6-16)/GABARAP complex was mixed with equal volumes of reservoir solution containing 0.3 M sodium nitrate, 0.3 M sodium phosphate dibasic, 0.3 M ammonium sulfate, 0.1 M bicine-tris (pH 8.5), 25% (v/v) MPD, 25% PEG-1000, and 25% (v/v) PEG-3350. Before diffraction experiments, glycerol was added as the cryo-protectant. A 2.6-Å resolution x-ray dataset for NAP1 FIR/ RB1CC1(1286-1395) complex, a 2.1-Å resolution x-ray dataset for NAP1 FIR/RB1CC1 Claw complex, and a 2.6-Å resolution x-ray dataset for NAP1 FIR/GABARAP complex were collected at the beamline BL17U or BL19U1 of the Shanghai Synchrotron Radiation Facility (55). The diffraction data were processed and scaled using HKL2000 (36).

**MS and data analysis**

The phosphorylation protein sample used for the protein identification by liquid chromatography (LC)/tandem MS (MS/MS) was obtained by in vitro phosphorylation of Trx-NDP52 by purified Trx-TBK1(1-674). Following procedures previously described in (51), we performed the MS analyses. In particular, proteins were precipitated by acetone and then dissolved in 8 M urea [100 mM tris-HCl (pH 8.5)]. Tris(2-carboxyethyl)phosphine (TCEP) (final concentration is 5 mM) (Thermo Fisher Scientific) and iodoacetamide (final concentration is 10 mM) (Sigma-Aldrich) for reduction and alkylation. The protein mixture was digested with trypsin at 1:50 (w/w) (Promega) overnight. The digestion was stopped by adding 5% formic acid (final concentration) and desalted by a MonoSpin C18 column (GL Sciences). The peptide mixture was analyzed by a homemade 30-cm-long pulled-tip analytical column (75 μm of inner diameter packed with ReproSil-Pur C18-AQ 1.9-μm resin; Dr. Maisch GmbH), and the column was then placed in line with an Easy-nLC 1200 nano HPLC (Thermo Fisher Scientific, San Jose, CA) for MS analysis. The analytical column temperature was set at 55°C during the experiments. The mobile phase and elution gradient used for peptide separation were as follows: 0.1% formic acid in water as buffer A and 0.1% formic acid in 80% acetonitrile as buffer B, 0 to 95 min, 2 to 35% buffer B; 95 to 103 min, 35 to 60% buffer B; 103 to 104 min, 60 to 100% buffer B; and 104 to 120 min, 100% buffer B. The flow rate was set as 300 nl/min. Data-dependent MS/MS analysis was performed with a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Peptides eluted from the LC column were directly electrosprayed into the mass spectrometer with the application of a distal 2.5-kV spray voltage. A cycle of one full-scan MS spectrum [mass/charge ratio (m/z) from 300 to 1800] was acquired followed by top 20 MS/MS events, sequentially processed by X!Tandem (version 1.17.0) and searched through the Uniprot database (30-35). The final refinement statistics of solved structures in this study were listed in table S1. All the structural diagrams were prepared using the program PyMOL (www.pymol.org/).
generated on the 1st to the 20th most intense ions selected from the full MS spectrum at a 28% normalized collision energy.

The acquired MS/MS data were analyzed against a UniProtKB Human (database released on 30 September 2018) using Proteome Discoverer 2.4 (Thermo Fisher Scientific). Mass tolerances for precursor ions were set at 20 parts per million and for MS/MS were set at 0.02 Da. Trypsin was defined as a cleavage enzyme; cysteine alkylation by iodoacetamide was specified as fixed modification with a mass shift of 57.02146. Methionine oxidation and serine/threonine/tyrosine phosphorylation were set as variable modification. To accurately estimate peptide probabilities and false discovery rates, we used a decoy database containing the reversed sequences of all the proteins appended to the target database (51).

**Cell culture, transfection, and co-IP assay**

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cotransfections of green fluorescent protein (GFP)–NDP52 and mCherry-RB1CC1 or related with 10% fetal bovine serum (Invitrogen). Cotransfections of green

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/33/eabi6582/DC1

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