The BEACH-containing protein WDR81 coordinates p62 and LC3C to promote aggrephagy

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Abbreviations used: ANOVA, analysis of variance; BEACH, beige and Chediak–Higashi; WD40, repeat-containing protein; WDR81, WD40 repeat-containing protein; p62, SQSTM1; LC3, light chain 3; BEACH, beige and Chediak–Higashi; BFA1, bafilomycin A1; eC disconnect, conditional knockout; CLR, candidate noncanonical LC3-interacting region; E, embryonic day; LR, LC3-interacting region; MEF, mouse embryonic fibroblast; NRK, normal rat kidney; P, postnatal day; P3K, phosphatidylinositol-3-kinase; TEM, transmission EM; Ub, ubiquitin.

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Autophagy-dependent clearance of ubiquitinated and aggregated proteins is critical to protein quality control, but the underlying mechanisms are not well understood. Here, we report the essential role of the BEACH (beige and Chediak–Higashi) and WD40 repeat-containing protein WDR81 in eliminating ubiquitinated proteins through autophagy. WDR81 associates with ubiquitin (Ub)-positive protein foci, and its loss causes accumulation of Ub proteins and the autophagy cargo receptor p62. WDR81 interacts with p62, facilitating recognition of Ub proteins by p62. Furthermore, WDR81 interacts with LC3C through canonical LC3-interacting regions in the BEACH domain, promoting LC3C recruitment to ubiquitinated proteins. Inactivation of LC3C or defective autophagy results in accumulation of Ub protein aggregates enriched for WDR81. In mice, WDR81 inactivation causes accumulation of p62 bodies in cortical and striatal neurons in the brain. These data suggest that WDR81 coordinates p62 and LC3C to facilitate autophagic removal of Ub proteins, and provide important insights into CAMRQ2 syndrome, a WDR81-related developmental disorder.

Introduction

Protein misfolding frequently occurs during the process of normal protein synthesis, and can be greatly exacerbated by cellular stresses such as heat shock, ER stress, and oxidative stress (Goldberg, 2003). If not properly resolved, misfolded proteins can further aggregate and impair cellular functions (Kopito, 2000; Kirkin et al., 2009b; Tyedmers et al., 2010). Clearance of aggregated protein is thus critical to cell homeostasis, defects in which are associated with many late-onset neurodegenerative disorders, including Parkinson’s disease and Huntington’s disease (Rubinsztein, 2006).

Autophagy can selectively remove ubiquitinated and aggregated proteins, thus serving as a protein quality-control system (Rubinsztein, 2006; Gamerdinger et al., 2009). Autophagic clearance of aggregated proteins, also termed “aggrephagy,” requires the recognition of ubiquitinated and aggregated proteins by receptors such as p62/SQSTM1 and NBR1 (Noda et al., 2010; Johansen and Lamark, 2011). p62/SQSTM1 is a general cargo receptor that preferentially binds Ub chains linked by K63 through the C-terminal Ub-associated domain (Seibenhener et al., 2004; Komatsu et al., 2007; Pankiv et al., 2007; Rogov et al., 2014). In addition, p62 interacts with members of the LC3 and GABARAP subfamilies through an LC3-interacting region (LIR; Bjørkøy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Because p62 recognizes a diverse range of ubiquitinated cargos and interacts with LC3 or GABARAP family members in a nonselective manner, additional receptors or scaffold/adaptor proteins cooperate with p62 to promote selective autophagy (Johansen and Lamark, 2011; Rogov et al., 2014; Khaminets et al., 2016). For example, the Huntington’s disease gene-encoded Huntington protein interacts with p62 and functions as a scaffold that recruits ULK1 but excludes mTOR, thus promoting selective autophagy of several cargos (Rui et al., 2015). ALFY/ WDFY3, a PtdIns3P-binding protein, plays an important role in aggrephagy by interacting with p62 and Atg5-12 (Clausen et al., 2010; Filimonenko et al., 2010). In the nematode Caenorhabditis elegans, EPG-7 acts as a scaffold that interacts with the p62 homologue SQST-1 and a variety of autophagy regulators to mediate clearance of aggregated proteins in embryo development (Lin et al., 2013). The cargo specificity of autophagy may be achieved by selective interaction of certain p62-interacting proteins.
scaffolds or receptors with members of the LC3 or GABARAP family. For instance, NDP52 selectively interacts with LC3C to mediate bacterial autophagy (von Muhllinen et al., 2012). In the autophagy pathway, ALFY was recently found to mainly interact with GABARAP (Lystad et al., 2014). In C. elegans, the LC3 homologues LGG-1 and LGG-2 bind to distinct groups of autophagy substrates, thus playing different roles in autophagy (Wu et al., 2015).

Although autophagy is critical to protein quality control, the mechanisms determining the selectivity of cargo recognition and autophagosome formation during autophagy are not fully understood. In this study, we identify WDR81, a BEACH (beige and Chediak–Higashi) domain– and WD40-containing protein, as an essential regulator of autophagy. WDR81 and its C. elegans homologue, SORF-2, were recently shown to function in maintaining endosomal PtdIns3P levels by forming a complex with another WD40-containing protein, WDR91 (Liu et al., 2016; Rapiteanu et al., 2016). Here, we uncover a distinct function of WDR81 in autophagy. We show that WDR81 directly interacts with p62 and promotes the recognition of ubiquitinated proteins. We further demonstrate that out of the three isoforms of the autophagosome membrane protein LC3, WDR81 preferentially interacts with LC3C to facilitate the removal of ubiquitinated proteins. We provide evidence that knocking out WDR81 results in accumulation of p62 bodies in mouse brain. These findings establish WDR81 as an essential regulator of autophagy and provide valuable insights into CAMRQ2 syndrome, a WDR81-related neuronal disorder.

**Results**

**Inactivation of WDR81 causes accumulation of ubiquitinated and aggregated proteins**

Previously, we found that WDR81 partially localizes to endosomes and forms a complex with WDR91 to regulate early-to-late endosome conversion (Liu et al., 2016). The fact that a substantial proportion of WDR81 is not located on endosomes suggests that WDR81 may have other functions in addition to a role in intracellular trafficking. To explore this possibility, we examined whether WDR81 localizes to the Golgi apparatus, ER, or mitochondria. Immunostaining revealed that WDR81 does not obviously colocalize with the Golgi protein GM130 or the ER protein Sec61β, suggesting that WDR81 does not act in the Golgi or ER (Fig. S1 A). Partial colocalization of WDR81 with MitoTracker Red staining was detected, as previously reported in mice (Fig. S1 A; Traka et al., 2013). Interestingly, we found that WDR81 substantially colocalized with ubiquitin in HeLa cells and human neuroblastoma SH-SY5Y cells (Fig. 1 A). To consolidate this observation, we treated HeLa cells with MG132, a proteasome inhibitor that induces premature release of polypeptide chains from ribosomes (Blobel, 1971). Both MG132 and puromycin induced formation of large Ub-positive focal structures that were enriched in WDR81 (Fig. 1 B). In contrast, tunicamycin or FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone), which induces ER stress or depolarizes mitochondrial membrane potential, respectively, did not induce formation of WDR81-positive foci (Fig. S1 B). These results suggest that WDR81 likely participates in a process involving ubiquitinated and misfolded proteins. To investigate this possibility, we depleted WDR81 using siRNA or CRISPR/Cas9 (Fig. 1 C and D; Liu et al., 2016) and then examined the levels of Ub proteins. In HeLa cells and SH-SY5Y cells, WDR81 siRNA (si81) greatly increased the number and size of Ub protein foci compared with control siRNA (siCtrl; Fig. 1 E and F). Some of these Ub protein foci were also positive for p62/SQSTM1, a receptor for diverse autophagy cargoes (Fig. 1, E and F; Björkty et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Similarly, large Ub protein foci that contained with p62 were frequently observed in WDR81 knockout (KO-81) HeLa cells, but not control HeLa cells (Fig. 1 G). The levels of Ub proteins and p62, as revealed by immunoblotting, were strongly elevated in si81 or KO-81 cells (Fig. 1, H–J). The increased Ub proteins in KO-81 cells contained predominantly K63-linked Ub chains (K63-Ub), whereas the levels of Ub proteins with K48-linked Ub (K48-Ub) were not changed (Fig. 1 K). Consistent with this, the MG132- or puromycin-induced WDR81 foci were mainly positive for K63-Ub but only partially costained with K48-Ub (Fig. 1 L). In addition, we evaluated the effect of si81 on polyQ aggregates. In HeLa cells inducibly expressing Htt7Q-GFP, si81 significantly increased the number of polyQ foci, comparable to that caused by knocking down several major autophagy regulators, including the p62 receptor, the autophagy scaffold protein ALFY (Björkty et al., 2005; Filimonenko et al., 2010), and the core autophagy factors Atg5 and Beclin1 (Fig. S2, A and B). Collectively, these findings suggest that WDR81 is important for preventing the accumulation of ubiquitinated and aggregated proteins.

**WDR81 acts in the autophagy pathway for clearance of ubiquitinated and aggregated proteins**

Given that proteins with K63-linked polyubiquitination are mainly degraded through autophagy (Newton et al., 2008; Tan et al., 2008; Kirkin et al., 2009b; Wong and Cuervo, 2010; Matsumoto et al., 2011; Rui et al., 2015), we investigated whether WDR81 promotes Ub protein clearance in an autophagy-dependent manner. Although si81 or KO-81 HeLa cells accumulated much higher levels of Ub proteins and p62 than cells with siControl treatment further enhanced the levels of Ub proteins and p62 in these cells (Fig. 2, A and B). Because MG132 causes inhibition of the ubiquitin–proteasome system (Kawaguchi et al., 2003), the additive effect of si81 and MG132 suggests that WDR81 does not act in proteasome-dependent clearance of Ub proteins. Next, we knocked down WDR81 in both wild-type and ATG5−/− mouse embryonic fibroblast (MEF) cells (Kuma et al., 2004). As shown in Fig. 2 C, Ub proteins accumulated similarly in ATG5−/− MEF cells or si81 MEF cells; nevertheless, si81 treatment did not further enhance the levels of Ub proteins in ATG5−/− cells. Likewise, the elevation in p62 protein levels in ATG5−/− MEF cells was not enhanced with si81 treatment (Fig. 2 D). These results suggest that WDR81 acts through autophagy to promote clearance of Ub proteins. Consolidating this conclusion, we found that WDR81 and Ub proteins colocalized to large focal structures in ATG5−/− MEF cells, and the fluorescence intensities of endogenous WDR81 staining were significantly higher in ATG5−/− MEF cells and ATG12−/− normal rat kidney (NRK) cells (Figs. 2 E and S2 C). Furthermore, ectopically expressed BFP-WDR81 colocalized with Myc-Ub and the autophagosome-decorating GFP-LC3B to punctate...
structures, which became even more obvious when cells were treated with MG132 (Fig. 2 F).

To investigate if WDR81 is degraded through autophagy, we knocked down the core components of the autophagy machinery or used bafilomycin A1 (BFA1) to inhibit lysosomal activity. siRNA of ATG5 or Beclin1 strongly increased Flag-WDR81 that was stably expressed in HeLa cells (Fig. 2 G). Levels of stably expressed Flag-WDR81 were similarly enhanced with BFA1 treatment (Fig. 2 H). These results suggest that WDR81 acts in autophagic clearance of Ub proteins and can be degraded through autophagy. Interestingly, MG132 treatment also enhanced Flag-WDR81 levels (Fig. 2 H), suggesting that the ubiquitin-proteasome system may contribute to WDR81 stability. In addition, we investigated whether WDR81 is required for general autophagy by examining the LC3 forms in KO-81 cells. The LC3-II/LC3-I ratio was the same in KO-81 and control cells (Fig. S2 D). Starvation (Earle’s Balanced Salt Solution treatment) or lysosomal inhibition (BFA1) did not alter this ratio either (Fig. S2 D). Consistent with this, KO-81 cells did not show a significant increase in GFP-LC3 foci compared with control cells when cultured normally or treated with Earle’s Balanced Salt Solution or BFA1 (Fig. S2 E). Using the RFP-GFP-LC3 fusion protein as an indicator, we found that the numbers of autophagosomes and autolysosomes were similar in control cells and KO-81 cells without or with starvation or BFA1 treatment (Fig. S2 F). Altogether, these findings suggest that WDR81 is unlikely to play a role in general autophagy.

The function of WDR81 in aggrephagy is independent of the endosomal WDR91–WDR81 complex

Because WDR81 and WDR91 form a complex to promote endosome maturation (Liu et al., 2016; Rapiteanu et al., 2016), we asked whether knockout of WDR91 also induced accumulation of Ub protein and p62 foci. Unlike KO-81 cells, WDR91 knockout (KO-91) cells do not contain enlarged foci of Ub proteins and p62 (Fig. S3 A). In addition, puromycin- or MG132-induced p62 foci were negative for WDR91 (Fig. S3 B). Given that inactivation of WDR81 or WDR91 similarly impairs endosome conversion (Liu et al., 2016; Rapiteanu et al., 2016), these results suggest that the elevation in Ub proteins and p62 in KO-81 cells do not result from defective endosome maturation. Supporting this conclusion, coexpression of GFP-WDR81 with mCherry-WDR91 (mCh-WDR91) led to perfect colocalization of these proteins with obvious endosomal structures, suggesting that WDR91 promotes the recruitment of WDR81 to endosomes (Fig. 3, A and B); however, MG132 or puromycin induced formation of distinct GFP-WDR81 foci separate from mCh-WDR91 (Fig. 3 A). Similarly, whereas GFP-WDR81 colocalized fairly well with the late endosome–specific marker Rab7 tagged with mCherry (mCh-Rab7), GFP-WDR81 no longer colocalized with mCh-Rab7 after MG132 or puromycin treatment (Fig. 3 B). In contrast, MG132 or puromycin did not change the colocalization of GFP-WDR91 with mCh-Rab7 (Fig. 3 C). These findings suggest that the function of WDR81 in aggrephagy is independent of the endosomal WDR81–WDR91 complex. In addition, whereas WDR81 and WDR91 form a complex with Beclin1 and their knockout enhanced Beclin1-dependent endosomal phosphatidylinositol-3-kinase (PI3K) activity (Liu et al., 2016; Fig. 3 D), neither protein was found in the Atg14L–Beclin1–Vps34 complex, and knockout of either WDR81 or WDR91 did not affect autophagic PI3K activity (Fig. 3 D). This suggests that WDR81 does not fulfill its role in aggrephagy by affecting autophagic PI3K activity.

To understand the requirement for WDR81 in aggrephagy, we performed transmission EM (TEM) analysis of control and KO-81 HeLa cells with MG132 treatment. The total number of autophagic vesicles (autophagosomes and autolysosomes) in control and KO-81 cells was not significantly different (Fig. 3, E and F), confirming that loss of WDR81 did not affect global autophagy. Remarkably, the autophagic vesicles in control cells predominantly contained darkly stained proteinaceous material and had intact membranes (Fig. 3, E and H). KO-81 cells, however, contained many proteinaceous structures in the cytosol that were more darkly stained and were either not enclosed by membranes or only partially surrounded by membranes (Fig. 3, E, G, and H). These results suggest that loss of WDR81 impaired the delivery or sequestration of aggregated proteins within autophagosomes.

WDR81 functions together with p62

To pinpoint how WDR81 functions in aggrephagy, we investigated whether it acts in complex with p62. In ATG5−/− MEF cells, WDR81 and p62 colocalized to large focal structures, suggesting that these two factors likely act at the same stage during aggrephagy (Fig. 4 A). Likewise, puromycin or MG132 treatment induced colocalization of WDR81 and p62 to large focal structures (Fig. S4 A). Because p62 overexpression results in formation of p62 bodies (Bjørkly et al., 2005; Szeto et al., 2006; Pankiv et al., 2007; Clausen et al., 2010), we tested if such p62 bodies could recruit WDR81. As shown in Fig. 4 B, GFP-WDR81 coexpressed with GFP-p62 localized exclusively to p62 bodies. Likewise, overexpressed GFP-p62 recruited endogenous WDR81 to focal structures in both HeLa cells and SH-SY5Y cells. Insets show magnified (2×) views of the box in the merged images. [B] Colocalization of endogenous WDR81 and Ub proteins (arrows) to large focal structures in HeLa cells treated with 1 μg/ml puromycin or 10 μM MG132 for 12 h. Arrows indicate the enlarged foci contained with WDR81 and Ub. [C] WDR81 siRNA effectively knocked down WDR81. Left: HeLa cells stably expressing GFP-WDR81 and Ub. (D) WDR81 siRNA knockdown leads to collagen type I degradation in HeLa cells. (E) WDR81 and Ub foci are present in a population of WDR81 and Ub coexpressing cells. (F) WDR81 and Ub foci are present in a population of WDR81 and Ub coexpressing cells. (G) WDR81 and Ub foci are present in a population of WDR81 and Ub coexpressing cells. (H) WDR81 and Ub foci are present in a population of WDR81 and Ub coexpressing cells. (I) Colocalization of endogenous WDR81 with K63- or K48-linked Ub proteins (K63Ub or K48Ub) in HeLa cells treated with 1 μg/ml puromycin or 10 μM MG132 for 12 h. Insets show images of WDR81 foci (green) and K63Ub or K48Ub foci (red) indicated by arrows in the main panels. Quantification of Ub proteins and WDR81 is shown in the right panel. ≥40 WDR81 foci were examined for each Ub antibody. Bars: (all images) 10 μm; (insets) 5 μm. For all quantifications, data (mean ± SEM) were from three independent experiments and analyzed using ANOVA. **, P < 0.01; ***, P < 0.001. Fold changes of Ub proteins or p62 (H–K) were normalized to control and are indicated at the bottom.
and SH-SY5Y cells (Figs. 4 B and S4 B). GFP-tagged NBR1 (GFP-NBR1), a p62-like cargo receptor (Kirkin et al., 2009a), also formed obvious foci when overexpressed. Nevertheless, these GFP-NBR1 foci failed to recruit either ectopically expressed mCh-WDR81 or endogenous WDR81 (Fig. S4 C). In addition, mCherry-tagged NDP52, optineurin, and TAX1BP1, which are adaptors for xenophagy or mitophagy (Wild et al., 2011; von Muhlinen et al., 2012; Lazarou et al., 2015), localized to large Myc-Ub–positive focal structures in cells treated with MG132 (Fig. S4 D). However, they did not colocalize with GFP-WDR81 to focal structures in cells without MG132 treatment (Fig. S4 E). Together, these findings suggest that WDR81 is preferentially recruited by p62. In addition, colocalization of BFP-WDR81 with Myc-Ub and GFP-LC3B in HeLa cells treated without (Ctrl) or with MG132. In the Ctrl group, insets show magnified (1.8 x) views of the boxed region in the merged image. In the MG132 group, arrows indicate the large foci containing all three tagged proteins. Bars: (main images) 10 µm; (insets) 5 µm. (G) WDR81 is degraded by the autophagy pathway. Hela cells stably expressing Flag-WDR81 were treated with siAtg5 or siBeclin1. Knockdown efficiency, determined by immunoblotting, is shown in the left panel. The siRNA-induced change in Flag-WDR81 levels is shown in the right panel. (H) Immunoblotting of Flag-WDR81 cells treated with 0.4 µM BFA1 and 10 µM MG132 for the indicated times. In G and H, fold changes of Flag-WDR81 were normalized to control and are indicated at the bottom. The treatment of cells with puromycin or MG132 was performed as in Fig. 1 (except in H).
Figure 3. **WDR81 acts in aggrephagy independently of the endosomal WDR81-WDR91 complex.** (A) Colocalization of GFP-WDR81 with mCh-WDR91 in HeLa cells treated with or without puromycin or MG132 (left). Quantification of WDR91-vesicles positive for WDR81 (right). (B) Colocalization of GFP-WDR81 with mCh-Rab7 in HeLa cells treated with or without puromycin or MG132 (left). Arrows indicate WDR81-positive Rab7 vesicles; arrowheads indicate WDR81 foci that do not localize to Rab7 vesicles. Quantification of Rab7-vesicles positive for WDR81 (right). (C) Colocalization of GFP-Rab7 with mCh-WDR91 in HeLa cells treated with or without puromycin or MG132 (left). Quantification of WDR91-vesicles positive for Rab7 (right). (D) Western blot analysis of Vps34, WDR81, WDR91, Beclin1, and Atg14L in HeLa cells treated with or without puromycin or MG132 (left). Quantification of Vps34 activity (right). (E) Electron microscopy images of HeLa cells treated with or without puromycin or MG132 (left). Quantification of AV number per section (right). (F) Quantification of AV number per section in intact and KO-81 cells (left). Quantification of AV number per section in intact and KO-81 cells (right).
with truncated WDR81 proteins fused with GFP, we found that p62 foci efficiently recruited WDR81 truncations containing the N-terminal 1–650 aa (WDR81(1–650)), which include the BEACH domain. Other WDR81 truncations lacking this region failed to colocalize with p62 (Fig. 4 D). Using immunoprecipitation (IP), we found that HA-p62 physically interacted with Flag-tagged full-length WDR81 and WDR81(1–650), but not the WD40-repeat region (aa 1,637–1,940, WDR81(WD40); Fig. 4, E–G). In vitro, GST-fused p62, but not GST, efficiently pulled down either full-length WDR81 or WDR81(1–650), but not WDR81(WD40) (Fig. 4 H). GST-p62 also pulled down 35S-labeled full-length WDR81 (Fig. 4 I). Altogether, these findings suggest that WDR81 and p62 directly interact with one another and that the BEACH-containing N terminus (1–650) of WDR81 is sufficient and necessary for this interaction.

We then investigated which domain in p62 is required for the interaction with WDR81 by coexpressing GFP-WDR81(1–650) with a series of mCh-p62 truncations. We found that p62 fragments containing the PB1 domain (aa 1–120), including aa 1–120, 1–180, and 1–385, formed foci to which GFP-WDR81(1–650) was recruited (Fig. 4 J). Longer fragments had a stronger effect on WDR81(1–650) recruitment. In contrast, p62 truncations without the PB1 domain did not show obvious colocalization with WDR81 (Fig. 4 J). Given that WDR81 promotes cargo recognition by interacting with Ub proteins, we investigated whether there is a functional connection between WDR81 and Ub proteins by changing p62 phosphorylation.

To understand how the interaction of WDR81 may affect p62 function, we investigated the binding of p62 with Ub proteins in KO-81 cells. To this end, we immunoprecipitated p62 and examined the coprecipitated Ub proteins. Whereas the levels of total Ub proteins and K63-Ub proteins were strongly enhanced in KO-81 cells, the amount of total Ub proteins or K63-Ub proteins coprecipitated with p62 was much lower in KO-81 cells than in control cells (Fig. 5, A and B). K48-Ub proteins were unaffected (Fig. 5 C). Consistent with this, the proportion of Ub protein foci positive for p62 in KO-81 cells, regardless of proteotoxic stress (e.g., MG132 treatment), was significantly decreased compared with that in control HeLa cells treated with MG132 (Fig. 5 D). In contrast, p62-associated total Ub and K63-Ub proteins, but not K48-Ub proteins, were greatly increased in HEK293 cells overexpressing Flag-WDR81 (Fig. 5, E–G). These data suggest that WDR81 facilitates the interaction of p62 with K63-Ub proteins. To corroborate this point, we coexpressed Flag-WDR81 and HA-p62 with Myc-Ub(K63) or Myc-Ub(K48) in HEK293 cells and immunoprecipitated HA-p62. Co-expression of Flag-WDR81 strongly increased the association with HA-p62 of Myc-Ub(K63)-labeled, but not Myc-Ub(K48)-labeled, proteins (Fig. 5, H and I). KO-81 HeLa cells expressing Flag-WDR81 did not show an obvious change in p62 phosphorylation at Ser 403, though this phosphorylation was increased by BFA1 treatment as reported previously (Fig. 5 J; Matsumoto et al., 2011; Lim et al., 2015). Thus, WDR81 did not facilitate p62 interaction with K63-Ub proteins by changing p62 phosphorylation.

We also examined whether WDR81 associates with Ub proteins in the absence of p62. For this purpose, we expressed Flag-WDR81 in wild-type or p62−/− immortalized baby mouse kidney epithelial cells (Guo et al., 2011a) and then performed IPs. We found that Ub proteins were communoprecipitated with Flag-WDR81 in wild-type cells (Fig. 5 K). The association of Ub proteins with WDR81 is probably noncovalent, because very low levels of Ub proteins were coprecipitated with WDR81 when IPs were performed with SDS-containing RIPA buffer (+RIPA), which is known to prevent noncovalent interactions (Seibenhener et al., 2004). In p62−/− cells, the amount of Ub proteins coprecipitated with Flag-WDR81 was also markedly reduced, suggesting that p62 is important for the association of WDR81 with Ub proteins (Fig. 5 K). Collectively, these findings suggest that WDR81 cooperates with p62 to promote recognition of Ub proteins for degradation through autophagy.

WDR81 is critical for LC3C recruitment by Ub proteins

Given that WDR81 promotes cargo recognition by interacting with p62 during aggrephagy, we further asked if WDR81 plays a role in facilitating the recruitment of major autophagy factors to Ub protein aggregates. The recruitment of ULK1, ATG12, Vps34, or ATG14L by Ub protein foci was similar in MG132-treated control HeLa cells and in KO-81 cells with or without MG132 treatment (Fig. S5 A). However, the proportion of Ub protein foci that were positive for LC3 family proteins in KO-81 cells was significantly reduced compared with that in control cells treated with MG132 (Fig. 6 A), suggesting that WDR81 is important for recruitment of LC3 family members by Ub proteins. To test this hypothesis, we investigated whether WDR81 interacts with individual members of the LC3 or GAB ARAP subfamilies. Whereas no obvious coprecipitation of mCh-LC3B or mCh-LC3A with Flag-WDR81 was detected, mCh-LC3C clearly communoprecipitated with Flag-WDR81 (Fig. 6, B and C). In addition, mCh-tagged GABARAP and GABARAPL1, but not GABARAPL2, showed very weak association with Flag-WDR81 (Fig. 6 C). These results indicate that WDR81 preferentially interacts with LC3C. Consistent
Figure 4. WDR81 interacts with p62. (A) Immunostaining of p62 and WDR81 in wildtype (WT) and ATG5−/− MEF cells. Insets show a magnified view (1.8×) of the boxed area in the merged images. (B) Images of GFP-WDR81 and GFP-p62 coexpressed in HeLa cells (top row); and images of overexpressed GFP-p62 and immunostained endogenous WDR81 (bottom). Dashed lines indicate the cell outline. (C) Colocalization of BFP-WDR81 with GFP-p62 (green arrows) or mCh-WDR91 (red arrows) in HeLa cells. (D) Colocalization of GFP-tagged WDR81 truncations with mCherry-p62 (mCh-p62) in HeLa cells. Schematic representations of the WDR81 truncations are shown in the top panel; the colocalizations are shown in the bottom panel. [E–G] Co-IP of HA-p62 with Flag-WDR81 (E), of HA-p62 with Flag-WDR81(1–650) (F), and of HA-p62 with Flag-WDR81(WD40) (G) in HEK293 cells. IPs were performed...
with this notion, coexpressed BFP-WDR81 and GFP-LC3C colocalized to Myc-Ub foci, and this colocalization became more obvious after exposure to MG132 (Fig. S5 B). In KO-81 cells, however, a significant proportion of Myc-Ub foci were negative for GFP-LC3C, whereas the colocalization of GFP-LC3B with Ub protein foci was unchanged regardless of MG132 treatment (Fig. 6, D–F). Collectively, these findings suggest that WDR81 preferentially interacts with LC3C and promotes its recruitment by Ub proteins.

Using quantitative RT-PCR or a human LC3C–specific antibody, we found that LC3C is expressed in both control and KO-81 HeLa cells, as well as in HEK293 and SH-SY5Y cells (Fig. S5, C–E). Intriguingly, no information about the LC3C gene was found in the mouse genome database. Nevertheless, an LC3C-like protein comparable to human LC3C was detected in both wild-type and ATG5−/− MEF cells with the same LC3C-specific antibody, and ATG5−/− MEF cells seemed to have a higher level of this LC3C-like protein (Fig. S5 F). In addition, whereas siLC3A and siLC3B specifically knocked down LC3A and LC3B in ATG5−/− MEF cells, respectively, the level of the LC3C-like protein was not changed by these siRNA treatments (Fig. S5 G). Like ATG5−/− MEF cells, the brain of WDR81 conditional knockout (cKO) mice (see below) also has a higher level of the LC3C-like protein than the wild type (Fig. S5 H). These findings suggest that mice probably have an LC3C-like protein that might act similarly to human LC3C in selective autophagy. In HeLa cells, siRNA of LC3C, which did not affect other LC3 isoforms (Fig. 6, G and H), led to formation of Ub protein focal structures that contained with endogenous p62 or WDR81 and were immunostained more intensely than the small puncta in control cells (Fig. 6, I and J). Immunoblotting analysis confirmed that the levels of Ub proteins and p62 were indeed greatly elevated after siLC3C treatment (Fig. 6 K). Similarly, siLC3C strongly increased the protein level of stably expressed Flag-WDR81 (Fig. 6 L). Thus, LC3C is essential for clearance of Ub proteins, p62, and WDR81, suggesting that LC3C plays a critical role in aggrephagy.

WDR81 interacts with LC3C through LIRs

We next investigated which domain in WDR81 is responsible for interaction with LC3C. Using co-IPs, we found that GFP-tagged WDR81(1–650), but not other regions, coprecipitated with mCh-LC3C (Fig. 7 A). Further co-IP analysis revealed that the BEACH domain (WDR81(341–650)) was sufficient and necessary for WDR81 to interact with LC3C (Fig. 7 B). Based on these results, we sought to identify amino acid residues in this region that are required for interaction with LC3C. Interestingly, this region contains two putative canonical LIRs, which are found in several autophagy receptors such as p62, NBR1, and FUNDC1 (Pankiv et al., 2007; Kirkin et al., 2009a; Liu et al., 2012). These putative LIRs are also found in WDR81 homologues in multiple species (Fig. S5 I). In addition, two candidate noncanonical LIRs (CLIRs), which are similar to that in NDP52, are also found in this region (von Muhlenen et al., 2012; Fig. 7 C). We thus mutated key residues in these putative LIRs or CLIRs and examined the interaction with LC3C. As shown in Fig. 7 D, mutations in LIR1 (W544A/L547A, LIR1AA) and LIR2 (S577A/Y578A/V581A, LIR2AA), but not the putative CLIRs, abrogated the interaction of WDR81(341–650) with mCh-LC3C in co-IP assays. Similarly, whereas full-length Flag-WDR81 clearly coimmunoprecipitated with mCh-LC3C, Flag-WDR81 containing LIR1AA, LIR2AA, or both, failed to associate with mCh-LC3 in co-IP assays (Fig. 7 E). Using GST pull-down assays, we confirmed that GST-LC3C, but not GST, pulled down full-length Flag-WDR81 but not Flag-WDR81 containing LIR mutations (Fig. 7 F). Altogether these findings suggest that WDR81 interacts with LC3C through canonical LIRs.

To determine the requirement for the WDR81-LC3C interaction in degradation of p62, we examined whether wild-type or mutant WDR81 rescued the elevation of p62 in KO-81 cells. Expression of Flag-WDR81 reduced the accumulation of p62 foci and the elevation of p62 in KO-81 cells to a level comparable to that in control HeLa cells (Fig. 7, G and H). However, overexpression of Flag-WDR81 carrying LIR1AA, LIR2AA, or both did not obviously rescue the accumulation of p62 in KO-81 cells (Fig. 7, G and H). These results suggest that the interaction of WDR81 with LC3C is required for p62 degradation.

Interaction of LC3C with WDR81 is essential for aggrephagy

We next investigated the requirement for LC3C to interact with WDR81 in aggrephagy. Given that LC3 family members normally interact with LIR-containing proteins through a hydrophobic pocket containing a “W-site” and an “L-site” (Noda et al., 2010) and that WDR81 interacts with LC3C, but not LC3B, we mutated the critical W-site and L-site residues in LC3C to those in LC3B and tested the interaction with WDR81 (von Muhlenen et al., 2012). Our co-IP results showed that neither the K32Q/F33H mutations, which affect the W-site, nor the L64V/F69L mutations, which affect the L-site, had an obvious effect on the interaction of mCh-LC3 with Flag-WDR81 (Fig. 7 I). However, the K32Q/F33H/L64V/F69L mutations, which changed both sites in LC3C to those of LC3B, greatly reduced the interaction of LC3C with WDR81 (Fig. 7 I). In contrast, the same mutations strongly increased the interaction of LC3C with p62 compared with wild-type LC3C (Fig. S5 J). These results suggest that LC3C interacts with WDR81 through the W-site and L-site. Furthermore, overexpression of the K32Q/F33H/L64V/F69L LC3C mutant failed to rescue the accumulation of p62 induced by siRNA of LC3C, in sharp contrast to the full rescuing effect of wild-type LC3C (Fig. 7 J). Collectively, these results suggest that the interaction with WDR81 is important for the functional role of LC3C in aggrephagy.

using Flag antibody, and precipitated proteins were detected with the indicated antibodies. (H) Interaction of GST-p62 with Flag-WDR81. Purified GST and GST-p62 (left) immobilized on glutathione-Sepharose beads were incubated for 12–16 h at 4°C with lysates of HEK293 cells expressing Flag-WDR81, Flag-WDR81(1–650), or Flag-WDR81(1–650) (right). After extensive washing, bound proteins were subjected to immunoblotting (IB) with antibodies to Flag (left). (I) In vitro interaction of GST-p62 with 35S-labeled WDR81. (J) Co-IP of mCh-tagged p62 truncations with Flag-WDR81(1–650) in HEK293 cells. IPs were performed using Flag antibody, and precipitated proteins were detected with antibodies to Flag and mCherry. Bars: [main images] 10 μm; [A, insets] 5 μm.

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Figure 5. WDR81 promotes the binding of p62 with Ub proteins. (A–C) IP of endogenous p62 in Ctrl or KO-81 cells with anti-p62 antibodies. Coprecipitated Ub proteins were detected with antibodies to Ub (A), K63-Ub (B), and K48-Ub (C). The ratios of Ub proteins/p62 in the precipitates are indicated at the bottom. (D) Immunostaining of p62 and K63-Ub in Ctrl or KO-81 cells treated without or with 10 µM MG132 (12 h). Insets show magnified (1.8×) views of the box in the merged images. Bars: (main images) 10 µm; (insets) 5 µm. Quantification of p62-positive Ub foci is shown in the right panel. Data (mean ± SEM) were derived from three independent experiments and analyzed using ANOVA. *, P < 0.05. (E–G) IP of endogenous p62 in HEK293 cells...
**WDR81 deficiency leads to accumulation of p62 bodies in mouse brain**

In mice, WDR81 expression was detected in several tissues and was highest in the brain (Fig. 8 A). This is consistent with a previous study on human WDR81 (Gulsuner et al., 2011). To investigate whether WDR81 promotes aggrephagy in mouse brain as in human cells, we generated mice (WDR81<sup>+/−</sup>) with loxP sites flanking exons 2–4 of the WDR81 gene, and then crossed them with mice expressing the Cre recombinase driven by the promoter of the brain-abundant Nestin gene (Nestin-Cre). This led to deletion of exons 2–4, resulting in a frameshift and a premature stop codon in exon 5 of the Nestin gene (Nestin-Cre). By the promoter of the brain-abundant Nestin-Cre. Mice were crossed with mice expressing the Cre recombinase driven by the promoter of the brain-abundant Nestin gene to generate Nestin-Cre mice (hereafter referred to as WDR81<sup>+/−</sup>;Nestin-Cre mice; Fig. 8 B).

WDR81<sup>+/−</sup> cKO mice were born at the expected Mendelian frequency, but most of them died quickly within a few hours after birth (Fig. 8 C), apart from a few escapers that grew into adults. The death of WDR81<sup>+/−</sup> cKO mice might result from the unexpectedly high recombination efficiency of Cre controlled by the Nestin promoter (Haigh et al., 2003). To investigate the effect of WDR81 inactivation on protein aggregates in the brain, we examined brain sections and performed immunostaining of p62 and ubiquitin. Intriguingly, no obvious brain abnormalities were observed in WDR81<sup>+/−</sup> cKO mice, unlike the defective development of the corpus callosum, third ventricle, fourth ventricle, and cerebellum in human CAMRQ2 patients carrying a WDR81 mutation (Gulsuner et al., 2011). However, p62 foci strongly accumulated in the cortex of both dead and surviving WDR81<sup>+/−</sup> cKO mice at postnatal day 0 (P0), and the dead mice had higher levels of accumulation of p62 foci (Fig. 8 D). Both Ub-positive and Ub-negative p62 foci were observed (Fig. 8 E). Accumulation of p62 bodies was also observed in the developing cortex at embryonic day 14.5 (E14.5) and E18.5 (Fig. 8, F and G). In surviving adult WDR81<sup>+/−</sup> cKO mice at P180, p62 foci were found not only in the cortex but also in the striatum, whereas the brain did not show any obvious abnormalities (Fig. 8 H).

To determine the cell types in which WDR81 functions, we performed immunohistochemistry analysis and found that WDR81 is mainly expressed in neurons, but not astrocytes, in the brain (Fig. 9, A–C). Consistent with this, we found that p62 foci were mainly present in NeuN-positive neurons, but not GFAP-positive astrocytes (Fig. 9, D and E). These data suggest that WDR81 is required for preventing protein aggregate accumulation in neurons. To consolidate this conclusion, we induced WDR81 inactivation in the striatum of adult WDR81<sup>+/−</sup> mice by stereotaxically grafting lentivirus expressing a GFP-fused Cre (Cre-GFP) or a GFP-fused Cre truncation (ΔCre-GFP) that has no recombinase activity (Fig. 9 F). Grafting of lentivirus expressing Cre-GFP effectively ablated WDR81 expression (Fig. 9 F). Consistent with this, accumulation of p62 foci was detected over time in the NeuN-positive neurons, but not GFAP-positive astrocytes, in the brain regions infected by viruses expressing Cre-GFP, whereas no p62 foci were observed in the contralateral regions infected with ΔCre-GFP-expressing viruses (Fig. 9, G and H). Collectively, these findings provide further evidence that WDR81 plays an important role in removing aggregated proteins in neuronal cells in mice. Of note, these results do not exclude the possibility that WDR81 participates in other selective autophagy processes in mice, as the accumulated p62 or Ub foci in WDR81 cKO mouse brain may include other selective autophagy cargoes.

**Discussion**

In this study, we identify WDR81 as an essential factor in autophagy-dependent clearance of Ub proteins. Loss of WDR81 function leads to accumulation of Ub proteins and the autophagy receptor p62 in cells and mouse brains. WDR81 associates with ubiquitinated and aggregated proteins, where it acts in complex with p62. WDR81 inactivation significantly reduced, whereas its overexpression strongly enhanced, the association of p62 with Ub proteins. This suggests that the interaction of WDR81 with p62 promotes cargo recognition for aggrephagy. In addition, loss of WDR81 specifically reduced the recruitment of LC3C to ubiquitinated proteins. Our data further indicate that WDR81 interacts with LC3C, but not other LC3 subfamily members. The canonical LIRs in WDR81 mediate the interaction with LC3C through the LC3C-specific hydrophobic pocket. These findings collectively suggest that WDR81 coordinates p62-dependent recognition of Ub proteins with LC3C-mediated assembly of autophagosomes, thereby facilitating Ub protein clearance through autophagy.

Although WDR81 was found to act in complex with WDR91 to regulate endosome maturation (Liu et al., 2016), the findings in this study suggest that neither WDR81 nor WDR91 affects the activity of the autophagic PI3K complex. The function of WDR81 in aggrephagy does not require WDR91. This is supported by the fact that WDR81, but not WDR91, is recruited by Ub protein foci and p62 bodies and that KO-91 cells do not accumulate enlarged foci of Ub proteins and p62. In addition, WDR81 separately interacts with p62 and WDR91. Thus, WDR81 plays dual functions in aggrephagy and endosome maturation by forming complexes with p62/LC3C or WDR91, respectively.

Previously it has been shown that LC3C interacts with NDP52 to mediate antibacterial autophagy. This interaction, conferred by a noncanonical LIR in NDP52, executes selective autophagic removal of bacteria (von Muhlinen et al., 2012). Our findings about the WDR81–LC3C interaction establish the essential role of LC3C in aggrephagy. We found that LC3C interacts through its hydrophobic pocket with the canonical LIRs of WDR81. Interestingly, the hydrophobic pocket residues in LC3C are more closely related to those in GABARAP subfamily members. For example, K32, L64, and...
Figure 6. **WDR81 promotes LC3C recruitment to Ub proteins.** (A) Immunostaining of LC3 and Ub in Ctrl or KO-81 HeLa cells treated without or with 10 μM MG132 (12 h; left). The LC3 antibody reacts with LC3A, B, and C. Insets show images of LC3 foci (green) and Ub proteins (red) indicated by arrows in the main panels. Bars: (main images) 10 µm; (insets) 5 µm. Quantification of LC3-positive Ub foci (right). Data (mean ± SEM) were derived from three independent experiments. Comparisons were made using ANOVA. **, P < 0.01. (B) Co-IP of Flag-WDR81 with LC3B in HEK293 cells without or with 10 μM MG132 treatment (12 h). IPs were performed with Flag antibody, and precipitated proteins were detected with antibodies to mCherry and Flag. (C) Co-IP of Flag-WDR81 with individual members of the LC3 or GABARAP subfamilies in HEK293 cells. IPs were performed with Flag antibody, and precipitated...
F69 in LC3C are conserved in GABARAP members, but not in LC3A or LC3B (von Muhlen et al., 2012). Nevertheless, WDR81 strongly interacts with LC3C but weakly with GAB ARAPs, whereas no interaction with LC3A and LC3B was observed. Thus, other residues in the hydrophobic pocket in LC3C may also contribute to the preferential interaction with WDR81. In addition, because loss of WDR81 does not completely abrogate LC3C recruitment to Ub proteins, other LC3C-interacting adaptor proteins, for example ALFY or NDP52, likely act redundantly with WDR81 in aggrephagy. Our results, together with those reported previously, suggest that the specific role of LC3C in selective autophagy is achieved by interacting with different adaptors/receptors.

Our findings suggest that the BDCP (BEACH domain–containing protein) family members WDR81 and ALFY play distinct roles in aggrephagy (Cullinane et al., 2013). Both WDR81 and ALFY facilitate aggrephagy by interacting with p62. Nevertheless, ALFY appears to act as a scaffold during the interaction of the autophagy factors Atg5–12 with the phagophore membrane without being degraded (Clausen et al., 2010; Filimonenko et al., 2010). In contrast, WDR81 does not obviously affect assembly of core autophagy factors but is degraded together with autophagy cargos and the p62 receptor. In addition, a recent study revealed that ALFY directly interacts with GABARAPs, but it interacts with LC3C much more weakly (Lystad et al., 2014). In our study, we found that WDR81 strongly interacts with LC3C but weakly with GABARAPs. Thus, the preferential interactions of WDR81 or ALFY with LC3 or GABARAPs likely play different roles in formation of autophagosomes during aggrephagy. Consistent with this notion, it has been shown that LGG-1 and LGG-2, the ATG8 homologues in C. elegans, bind to different substrates or ATG protein complexes in autophagic degradation of protein aggregates during C. elegans development (Wu et al., 2015).

A human WDR81 mutation was found to be associated with CAMRQ2 syndrome, which manifests as quadrapedal locomotion, mental retardation, and cerebrocerebellar hypoplasia (Gulsuner et al., 2011). In this study, we revealed a distinct role of WDR81 in removing ubiquitinated and aggregated proteins. This is strongly supported by the fact that WDR81 knockout leads to a strong accumulation of p62 bodies and aggregated proteins in the striatum and cortex of mouse brains. WDR81 may also participate in clearance of damaged mitochondria, because mice carrying a WDR81 point mutation accumulate aberrant mitochondria in Purkinje cell dendrites (Traka et al., 2013). Although no LC3C gene information was found in the mouse genome database, an LC3C-like protein, which seems to be degraded in a WDR81- and ATG5-dependent manner (Fig. S5, F and H), might be involved in aggrephagy or other selective autophagy processes. It is possible that WDR81 acts together with this LC3C-like protein in mice, similar to the WDR81–LC3C interaction in human cells. Future studies will determine the identity of the LC3C-like protein and how it functions. In addition, although WDR81 cKO mice did not phenocopy the defective brain development in CAMRQ2 patients, the accumulation of aggregated proteins or damaged mitochondria may impair the function of neurons and render them sensitive to induction of cell death, a process that is implicated in neurodegenerative disease (Filimonenko et al., 2007; Moscat and Díaz-Meco, 2009; Menzies et al., 2015). Moreover, we and others recently showed that WDR81 is important for early-to-late endosome conversion, a key step in intracellular trafficking (Liu et al., 2016; Rapiteanu et al., 2016). Given the multifunctional properties of WDR81, CAMRQ2 syndrome may result from diverse defective cellular processes that lead to developmental defects. Importantly, mutations in several other BDCP family members are found to be associated with diseases, including Chediak–Higashi syndrome, immune deficiency, autism, gray platelet syndrome, and systemic lupus erythematosus (Cullinane et al., 2013). It will therefore be of great interest to explore whether these BDCP proteins act similarly to WDR81 or ALFY in regulating intracellular trafficking or autophagy-related cellular processes.

Materials and methods

Cell culture, reagents, and transfection

Cells were cultured at 37°C with 5% CO2 in DMEM supplemented with 10% FBS (HyClone), 100 U/ml penicillin, and 100 mg/ml streptomycin. ATG5−/− MEF cells were provided by N. Mizushima (University of Tokyo, Tokyo, Japan). p62−/− immortalized baby mouse kidney cells were provided by E. White (Rutgers University, New Brunswick, NJ). Wild-type and ATG12−/− NRK cells were gifts from L. Yu (Tsinghua University, Beijing, China). Transfections were performed with Lipofectamine 2000 (Invitrogen) or Vigofect (Vigorous Biotechnology Beijing Co., Ltd) according to the manufacturer’s instructions. BFA1, puromycin, MG132, basticidin, and zeocin were obtained commercially.

Antibodies

WDR81 antibodies were generated in guinea pigs and rabbits by injecting purified recombinant GST-WDR81(332–604). WDR91 antibody was generated in mouse by injecting GST-WDR91(406–730). GFP and mCherry antibodies were generated in guinea pigs or rabbits by injecting recombinant proteins. Rabbit polyclonal antibodies to p62 (PM045), LC3 (PM036), and Beclin1 (PD017) and rat antibody to p-p62(S403) (D343-3) were purchased from Medical & Biological Laboratories. Rabbit polyclonal antibodies to human LC3A (ab62720) and LC3C (ab168813) were purchased from Abcam. Rabbit polyclonal antibodies to human LC3B (NB100-2220) were purchased from Novus.

Proteins were detected with antibodies to mCherry and Flag. (D–F) Colocalization of Myc-Ub with GFP-LC3B (D) or GFP-LC3C (E) in Ctrl and KO-81 cells treated without or with 10 μM MG132 (12 h). Insets show images of GFP-LC3B or GFP-LC3C foci (green) and Myc-Ub foci (red) in the boxes in the main panels. Bars: [main images] 10 μm; [insets] 5 μm. Quantification of Myc-Ub foci positive for GFP-LC3B or GFP-LC3C is shown in F. Data (mean ± SEM) were derived from three independent experiments. Comparisons were made using ANOVA. **, P < 0.01. (G) Quantitative RTPCR analysis of the specificity and efficiency of siLC3C. The mRNA levels of LC3A, LC3B, and LC3C were normalized to siCtrl treatment; error bars represent SEM derived from three independent experiments. Comparisons were made using ANOVA. **, P < 0.01. (H) Western blot analysis of LC3C and LC3A in HeLa cells treated with siCtrl or siLC3C. (I) Colocalization of endogenous Ub proteins with p62 or WDR81 in HeLa cells treated with siCtrl or siLC3C. (J) Quantifications of enlarged Ub protein foci positive for p62 or WDR81 [left] and the intensity of Ub protein or WDR81 staining [right] in HeLa cells treated with siCtrl or siLC3C. ≥100 cells were analyzed for each treatment. Data representing mean ± SEM were derived from three independent experiments. Comparisons were made using ANOVA. **, P < 0.01. (K) Immunoblotting of U proteins [top] and p62 [bottom] in siCtrl- or siLC3C- treated HeLa cells. Fold changes in Ub proteins or p62 were determined by normalizing to the control and are indicated at the bottom. (L) Immunoblotting of Flag-WDR81 that was stably expressed in HeLa cells treated with siCtrl or siLC3C. The fold change in Flag-WDR81 was determined by normalizing to the control and is indicated at the bottom.
Figure 7. WDR81 interacts with LC3C through two LIRs to facilitate autophagy. (A and B) Co-IP of GFP-tagged WDR81 fragments with mCh-LC3C. Individual GFP-WDR81 fragments were coexpressed with mCh-LC3C in HEK293 cells. IPs were performed with mCherry antibody, and precipitated proteins were detected with antibodies to GFP and mCherry. (C) Alignment of canonical and noncanonical LIRs in WDR81 with those in p62, NBR1, FUNDC1, or NDP52. Conserved residues are shown in red. (D) Co-IP of mCh-LC3C with wild-type (WT), LIR, or CLIR mutants of GFP-WDR81(341–650) in HEK293 cells. WDR81 mutations are shown in the box. IPs were performed with mCherry antibody and precipitated proteins were detected with antibodies to GFP and mCherry. (E) Co-IP of mCh-LC3C with WT or the indicated LIR mutants of Flag-WDR81 full-length protein in HEK293 cells. IPs were performed as in A. (F) Pull-down of Flag-WDR81 by GST-LC3C. Purified recombinant GST and GST-LC3C immobilized on glutathione-Sepharose beads were incubated overnight at 4°C with lysates of HEK293 cells expressing Flag-WDR81 or the indicated Flag-WDR81 mutants. After extensive washing, bound proteins were subjected to Western blot with Flag antibody. (G) KO-81 HeLa cells were transfected with Flag-WDR81 or the indicated Flag-WDR81 mutants. 48 h later, cells were subjected to immunostaining (G) or immunoblotting (H) with p62 antibody. Quantification (mean ± SEM) of p62 in (G) is shown in the right panel. *** P < 0.001. Bars, 10 µm. (I) Co-IP of Flag-WDR81 with WT or the indicated hydrophobic pocket mutants of LC3C in HEK293 cells. IPs were performed with Flag antibody, and precipitated proteins were detected with antibodies to Flag and mCherry. (J) Immunoblotting of p62 in siCtrl or siLC3C cells expressing RNAi-resistant LC3C (WT) or the LC3C (K32Q/F33H/L64V/F69) mutant in HEK293 cells. In H and J, fold changes in p62 levels were normalized to the control and are indicated at the bottom.
Figure 8. WDR81 deficiency causes accumulation of p62-positive aggregates in mouse brain. (A) RT-PCR analysis of WDR81 expression in different mouse tissues. (B) Graphic representation of the scheme for generating WDR81 conditional knockout (WDR81f/f cKO) mice by crossing WDR81f/f mice with Nestin-Cre mice. (C) WDR81f/f cKO mice display perinatal lethality at P0. (D, left) Immunostaining of p62 in brain sections from WDR81f/f and WDR81f/f cKO mice at P0 (left). Nuclei are stained with DAPI. Boxed cortex regions in each image of whole brain sections (10x objective, top row) were observed under 60x objective and are shown in the bottom row (bars, 10 µm). (right) p62 intensities in the cortex were quantified and normalized to WDR81f/f. 20 sections from four mice for each genotype were examined. Data (mean ± SEM) were analyzed using ANOVA. **, P < 0.01; ***, P < 0.001. (E) Immunostaining of p62 and Ub in brain sections from WDR81f/f and WDR81f/f cKO mice at P0 (left). Nuclei are stained with DAPI. Insets show magnified (2×) views of the dotted (Ub-positive) or solid (Ub-negative) box in the merged images. Bars: (main images) 10 µm; (insets) 5 µm. Quantification of p62 foci...
were examined for p62 foci, and data (mean ± SEM) were analyzed using ANOVA. ***, $P < 0.001$.

For views of whole-brain sections in D, F, and H, multiple images (10x) were stitched together to generate the composite images. (F) p62 immunostaining and DAPI labeling in whole-brain sections from WDR81 f/f mice at P180 (left). Boxed regions in the whole brain sections (top) were observed under 60x objective and are shown in the bottom row (bars, 10 µm). H1 and H3 indicate cortex regions. H2 and H4 indicate striatum regions. Right: >500 cells in 10 sections from two mice for each genotype were examined for p62 foci, and data (mean ± SEM) were analyzed using ANOVA. ***, $P < 0.001$.

**Western blotting and IP**

Cells were lysed in ice-cold Triton X-100 buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and 1 mM PMSF) or RIPA buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1 mM PMSF) containing Complete Protease Inhibitor Cocktail (Roche). Cell lysates were spun down at 12,000 rpm for 10 min, and 50 µg supernatants were resolved on SDS-PAGE and blotted with the indicated antibodies. The amount of β-actin was used as the loading control. Blots were developed with an ECL Select Western Blotting Detection system (GE Healthcare) and imaged with the Minichemi machine (Sagecreaction). Quantification of Western blots was performed using ImageJ software.

To test the direct interaction of two proteins tagged with different epitopes, the corresponding expression vectors were cotransfected in HEK293 cells. 48 h later, cells were lysed in regular IP buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 1 mM PMSF, and 1% glycerol) containing Complete Protease Inhibitor Cocktail. Cell lysates were spun down at 12,000 rpm for 10 min at 4°C, and the supernatants were incubated with Flag antibody (M2)–conjugated or HA antibody–conjugated beads (∼5 µg antibody for each sample) overnight at 4°C. The beads were centrifuged and washed three times with IP buffer. Precipitated proteins were resolved by SDS-PAGE, blotted, and probed with different antibodies.

**Immunostaining and confocal microscopy**

Cells grown on coverslips were fixed for 15 min at RT in 4% PFA followed by permeabilization for 10 min at RT with 0.05% saponin. After extensive washing with PBS, cells were incubated with primary antibodies in PBS containing 5% BSA at 4°C overnight. Cells were washed extensively again and incubated with secondary antibodies for 1 h at RT. After another round of thorough washing, cells were mounted with VECTASHIELD (H-1000; Vector Laboratories) and sealed on slides for microscopy analysis. For live-cell imaging, cells were grown in confocal dishes (Glass Bottom Dish; In Vitro Scientific). Samples were examined with an inverted FV1000 confocal microscope system (IX81: Olympus) using a 60x 1.42-NA oil objective. Excitation was achieved using gas-state 488-nm and solid-state 595-nm lasers. All images were taken at RT and analyzed with FV10-ASW 4.0a Viewer.

**Expression vectors**

Mammalian and bacterial expression vectors were constructed using standard protocols and are listed in Table S1. The following vectors were provided by other scientists: pCMV-GFP-p62, pCMV-GFP-NBR1, and pCMV-HA-p62 (Y. Chen, Tsinghua University, Beijing, China); pCMV-Myc-UB, pET28a-UB(K63) (K63 only) and pET28a-UB(K48) (K48 only; W. Li, Institute of Zoology, Chinese Academy of Sciences, Beijing, China); and pCDNA3.1-His97Q-GFP (X. Li, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China).

**Recombinant proteins and GST pull-down**

Recombinant GST, GST-p62, GST-p62(PB1), and GST-LC3C were expressed in Escherichia coli BL21 (DE3) cells and purified with glutathione-Sepharose beads (GE Healthcare) according to the instructions provided by the supplier. For GST pull-down of proteins expressed in mammalian cells, cell lysates containing Flag-tagged WDR81 proteins was prepared with Triton X-100 buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and 1 mM PMSF) as described in the Western blotting and IP section. 35S-labeled WDR81 and WDR81(1–650) proteins were prepared by in vitro translation following the protocol provided by the supplier (Promega). Purified GST, GST-p62, GST-p62(PB1), or GST-LC3C proteins (5 µg each) immobilized on glutathione-Sepharose beads were incubated with cell lysates containing WDR81 or 35S-labeled WDR81 or 35S-labeled WDR81(1–650) proteins at 4°C overnight and then washed extensively with the same buffer. Bound proteins were resolved by SDS-PAGE and visualized by Western blot and autoradiography.

**siRNAs**

RNA oligos used for siRNA are listed in Table S2. Cells were transfected with 100 pmol siRNA oligos twice at an interval of 24 h using Lipofectamine 2000. Cells were subjected to further analysis 24 h after the last transfection.
Quantitative RT-PCR

Total RNA was extracted using Trizol (Invitrogen) and chloroform. 2 µg RNA was used as template to generate cDNAs using the ImProm-II Reverse Transcription system (Promega). Quantitative RT-PCR reactions were performed on an MX3000P system (Agilent Technologies).

Generation of cell lines stably expressing Tet-on Htt97Q-GFP

HeLa cells stably expressing pcDNA6/TR (provided by Q. Chen, Institute of Zoology, Chinese Academy of Sciences, Beijing, China) were transfected with pcDNA4-Htt97Q-GFP. 24 h later, cells were subjected to selection for 2–3 wk in medium supplemented with blasticidin (5 µg/ml) and zeocin (100 µg/ml). Single colonies were picked and propagated.
and 1 µl 10 mM ATP in 30 µl reaction buffer for 30 min at RT. Conver-
sion of ATP to ADP was measured with an ADP-GloTM kinase assay
kit (Promega) following the instructions provided by the manufacturer.

Site-directed mutagenesis
WDR81 and LC3C mutants were generated by PCR-mediated muta-
genesis with pairs of oligos listed in Table S3.

Immunohistology
Mice
WDR81f/f mice were generated by BIO CYT OGEN. WDR81f/f and Nes-
tin-Cre mice were bred onto the C57/B/L6 background and housed at the
animal facility at the Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences. All procedures and husbandry were per-
formed according to protocols approved by the Institutional Animal Care
and Use Committee at Institute of Genetics and Developmental Biology.

Statistical analysis
Data were analyzed with Prism (GraphPad Software) to generate bar
graphs. Error bars represent SEM. The two-tailed unpaired \( t \) test was
used for statistical comparisons of two groups of samples. Analysis of
variance (ANOVA) with a Newman–Keuls posttest was used for sta-
tistical comparisons of multiple groups of samples. \( P > 0.05 \) was con-
sidered not significant.

Acknowledgments
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Li, and X.J. Li for expression vectors and Drs. E. White, N. Mizushima, and L. Yu for p62, ATG5, and ATG12 knockout

Online supplemental material
Fig. S1 describes the subcellular localization of WDR81. Fig. S2 shows
that WDR81 is important for clearance of polyQ aggregates, but not es-
sential for general autophagy. Fig. S3 demonstrates that WDR91 is
not required in aggrephagy. Fig. S4 shows that WDR81 is recruited by p62, but not other autophagy receptors or adaptors. Fig. S5 char-
acterizes the recruitment of major autophagy regulators to Ub foci,
the expression of LC3C in different cell types and mouse brain, the
WDR81 LIR motifs, and the interaction of p62 with LC3C mutants.
Table S1 summarizes the mammalian expression vectors used in the
study. Table S2 lists oligos used for siRNA. Table S3 lists oligos used
for site-directed mutagenesis.

For immunohistological analysis, brain sections were washed
extensively with TBS (50 mM Tris-HCl, pH 7.2, and 100 mM NaCl)
and blocked with 5% donkey serum containing 0.05% Triton X-100 for
1 h at room temperature. Brain sections were then incubated with pri-
mary antibodies in TBS containing 5% donkey serum at 4°C overnight.
After extensive washing, the sections were incubated with secondary
antibodies for 1 h at room temperature. After staining, sections were
mounted, coverslipped, and maintained at 4°C in the dark until analysis.

Lentivirus production and lentiviral grafting
Lentivirus production and lentiviral grafting were performed as de-
scribed previously (Guo et al., 2011b). In brief, lentiviral transfer vec-
tor DNA and packaging plasmid DNA were cotransfected into 293T
 cells. The medium was collected and pooled at 40, 64, and 88 h and
then filtered through a 0.2-μm filter. Viruses were concentrated by ul-
tracentrifugation at 19,000 rpm for 2 h at 20°C using a SW27 rotor
(Beckman Coulter). To determine the titer of the viruses, the virus
preparation was made in 10-fold serial dilutions. 2 µl of each dilution
was used to infect 100,000 HeLa cells/well in six-well plates. 48 h later,
cells were collected and analyzed by flow cytometry. The percentage of
GFP-positive cells between 1% and 10% was used to calculate virus
titer (infection units per microliter) using the following formula: titer
(infection units per microliter) = [(percentage of GFP-positive cells)
× 100,000 × dilution factor)/2. For in vivo lentiviral grafting, 4-wk-
old C57/B/L6 male mice were anesthetized, and viruses (≥108 viruses/
µl in 1.5 µl) were injected stereotaxically into the striatum using the
following coordinates relative to bregma: anteroposterior, 0 mm; lat-
eral, ±2 mm; ventral, −2 mm (from dura). Every 2 weeks after viral
grafting, mice were deeply anesthetized with avertin and perfused with
saline followed by 4% PFA.

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Author contributions: C. Yang, X. Liu, Y. Li, and W. Guo conceived and designed the research. X. Liu, Y. Li, and W. Guo performed most of the biochemical, cell biology, and animal experiments. X. Wang performed TEM analysis. K. Liu, Q. Gan, Z. Gao, and Y. Jian generated some antibodies and plasmids. X. Liu, Y. Li, S. Luo, W. Guo, and C. Yang analyzed and interpreted the experimental data. C. Yang, X. Liu, Y. Li, and W. Guo wrote the paper, and all authors commented on the manuscript.

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References


