Autophagy Receptor NDP52 Regulates Pathogen-Containing Autophagosome Maturation

Highlights

- The autophagy receptor NDP52 plays a dual function during xenophagy
- NDP52 also promotes pathogen-containing autophagosome maturation
- NDP52 independently regulates targeting of bacteria to autophagosomes and maturation
- NDP52 controls autophagy maturation by interacting with ATG8 orthologs and MYOSIN VI

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In Brief

Xenophagy is the degradation of intracellular microorganisms by autophagy. Selective targeting of pathogens to autophagosomes is accomplished by specific autophagy receptors such as NDP52. Verlhac et al. show that, in addition, NDP52 ensures subsequent pathogen degradation by regulating maturation of the pathogen-containing autophagosome for efficient xenophagy.
Autophagy Receptor NDP52 Regulates Pathogen-Containing Autophagosome Maturation

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SUMMARY

Xenophagy, an essential anti-microbial cell-autonomous mechanism, relies on the ability of the autophagic process to selectively entrap intracellular pathogens within autophagosomes to degrade them in autolysosomes. This selective targeting is carried out by specialized autophagy receptors, such as NDP52, but it is unknown whether the fusion of pathogen-containing autophagosomes with lysosomes is also regulated by pathogen-specific cellular factors. Here, we show that NDP52 also promotes the maturation of autophagosomes via its interaction with LC3A, LC3B, and/or GABARAPL2 through a distinct LC3-interacting region, and with MYOSIN VI. During Salmonella Typhimurium infection, the regulatory function of NDP52 in autophagosome maturation is complementary but independent of its function in pathogen targeting to autophagosomes, which relies on the interaction with LC3C. Thus, complete xenophagy is selectively regulated by a single autophagy receptor, which initially orchestrates bacteria targeting to autophagosomes and subsequently ensures pathogen degradation by regulating pathogen-containing autophagosome maturation.

INTRODUCTION

Macroautophagy, hereafter referred to as autophagy, is a lysosomal-related vesicular catabolic process essential for the maintenance of cell homeostasis, as it ensures the constant elimination of end-life or dysfunctional cellular contents. Autophagy is also a response to several sources of cellular stress to avoid accumulation of otherwise deleterious components. Among targets of autophagy are intracellular pathogens (Huang and Brumell, 2014; Levine et al., 2011). To avoid excessive endogenous bystander damage, cells express autophagy receptors, which can selectively detect intracellular pathogens and target them toward the autophagy machinery for degradation, a process known as xenophagy (Levine, 2005; Randow and Youle, 2014).

Three autophagy receptors for effective xenophagy are known: SQSTM1/p62, NDP52 (nuclear dot protein 52 kDa, also known as CALCOCO2), and OPTINEURIN (Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009). They bind to ubiquitinated intracellular bacteria via an ubiquitin (Ub)-binding domain and bring bacteria to nascent autophagosomes through their interaction with an autophagosomal membrane-anchored member of the ATG8 family (LC3 and GABARAP family members in mammals) via a so-called LC3 interaction region (LIR) (Randow and Youle, 2014). To mediate bacteria targeting to autophagosomes, NDP52 has the additional property to detect pathogens through the binding of GaLECTIN 8, which is recruited on bacteria-containing damaged vacuoles (Thurston et al., 2012). Furthermore, among the LC3 family, NDP52 binds selectively to LC3C via a non-canonical LIR (CLIR) motif, with no or very weak affinity for other members of the LC3 family (von Muhlen et al., 2012).

Once sequestered within a completed autophagosome, pathogens can be destroyed within the acidic environment produced subsequently to the fusion of the bacteria-containing autophagosome with a lysosome (Levine et al., 2011). The molecular regulation of this so-called autophagosome maturation step remains largely unknown, but the motor protein MYOSIN VI was recently shown to contribute to this process by docking TOM-1-expressing endosomes with autophagy receptor-linked autophagosomes. MYOSIN VI has the potency to interact with NDP52, OPTINEURIN, and T6BP, and either the individual depletion in MYOSIN VI or TOM-1, or the simultaneous extinction of the three autophagy receptors affected autophagosome maturation (Tumbarello et al., 2012). However, the individual contribution of these receptors to this process as well as the possible mechanisms involved remained totally unknown, especially in the context of bacterial infections.

We asked here whether, in addition to its activity in the targeting of bacteria to autophagy, NDP52 could regulate the maturation of bacteria-containing autophagosomes. We found an unexpected ability of this autophagy receptor to complete, via dual but independent mechanisms, the two essential steps for xenophagy, meaning the targeting of bacteria to nascent autophagosomes and the promotion of the degradative activity of these vesicles upon efficient maturation.
RESULTS

The MYOSIN VI Binding Domain of NDP52 Is Required for Autophagy-Mediated Intracellular Bacteria Degradation, but Not for Bacteria Targeting to Autophagosomes

Cellular depletion in MYOSIN VI, a non-conventional myosin motor that can travel toward the minus end of actin filaments, strongly compromises the maturation of autophagosomes and, therefore, autophagy substrate degradation (Tumbarello et al., 2012). Interestingly, NDP52 binds to MYOSIN VI, and the NDP52 C425 residue is instrumental in such an interaction (Morriswood et al., 2007). We therefore asked whether the interaction between NDP52 and MYOSIN VI was required for bacterial xenophagy. To this aim, we monitored the intracellular proliferation of Salmonella Typhimurium in HeLa cells treated with short interference RNA (siRNA) reducing NDP52 expression (Figure S1A). As previously shown, siNDP52 treatment facilitated S. Typhimurium intracellular proliferation (Thurston et al., 2009), what we found to be rescued by the ectopic expression of NDP52 (Figures 1A, S1B, and S1C). Interestingly, however, the ectopic expression of a NDP52 C425A mutant unable to bind to MYOSIN VI (Morriswood et al., 2007) did not rescue the ability of cells to restrict S. Typhimurium proliferation in the absence of NDP52 expression (Figures 1A, S1B, and S1C). Similarly, a NDP52 construct lacking the CLIR motif (NDP52 ΔSKICHΔCLIR, see Figure S3A for all the constructs used in this study) was equally unable to control S. Typhimurium proliferation (Figures 1A and S1B). Thus, these results suggest that the interactions of NDP52 with MYOSIN VI and LC3C are both crucial for cells to fight intracellular S. Typhimurium growth.

Compromised restriction of intracellular bacteria through autophagy could result from either an inefficient targeting of pathogens to autophagosomes, an essential function known for NDP52, or a defective maturation of bacteria-containing autophagosomes, or a combination of both. We wondered whether the NDP52 C425A mutant impacted the targeting of S. Typhimurium to autophagosomes by looking at intracellular ubiquitinated bacteria co-localization with LC3+ autophagosomes in infected GFP-LC3-expressing HeLa cells. As expected, when compared with control cells, NDP52-deficient cells showed reduced GFP-LC3+ ubiquitinated bacteria, what was rescued by the ectopic expression of NDP52 (Figure 1B). However, whereas the NDP52 C425A did not control S. Typhimurium proliferation, this mutant still allowed the targeting of ubiquitinated bacteria to autophagosomes (Figure 1B). As expected, the CLIR-lacking NDP52 construct with no potency at controlling S. Typhimurium proliferation (Figure 1A) was not able to address bacteria to autophagosomes (Figure 1B). Thus, these results indicate that NDP52 interaction with MYOSIN VI has no role in bacteria targeting to autophagosomes but suggest a potential role in completion of the autophagy process.

NDP52 Contributes to the Regulation of the Autophagy Process

In light of our results, we analyzed autophagy in siNDP52-treated HeLa cells and measured first the conversion of the cytosol-located LC3-I form into the autophagosome-anchored lipidated LC3-II form (Klionsky et al., 2012). In contrast to the silencing of the autophagy essential ATG5 gene (Figure S1A), which limits LC3-I conversion, we found that depletion in NDP52 led to an increased expression of LC3-II (Figure 1C). Furthermore, the reduced expression of NDP52 did not impair LC3-II accumulation upon treatment of the cells with chloroquine, a potent inhibitor of autophagosome recycling (Figure 1D). These results were confirmed when we analyzed autophagy through both confocal microscopy and flow cytometry-based assays (Klionsky et al., 2012) for the formation of GFP-LC3 dots or for the intensity of GFP-LC3 expression upon saponin treatment in siNDP52-treated GFP-LC3 HeLa cells, respectively (Figures 1E and S1D). Similar results were observed in 293T cells (not shown). Moreover, as observed by others (Newman et al., 2012; Tumbarello et al., 2012), we found co-localization of NDP52 with GFP-LC3+ autophagosomes. However, such co-localization events were very rare in control healthy cells, whereas increased co-localization was observed in cells treated with chloroquine (Figure 1F). Together, these results indicate that NDP52 has an impact on the autophagy process, but not on autophagosome biogenesis, and that it could play a role in the maturation step of the autophagosomes.

NDP52 Promotes Autophagosome Maturation

Accumulation of LC3-II+ autophagosomes as observed in siNDP52-treated cells could result either from an increase in

Figure 1. NDP52 Activity in Xenophagy and Autophagy

(A and B) HeLa cells (A) or GFP-LC3 HeLa cells (B) were first transfected with control siRNA or siNDP52 for 48 hr before a second transfection with the indicated construct-encoding plasmid, and 24 hr later cells were infected. (A) For each condition, intracellular S. Typhimurium viability is presented as the ratio of alive intracellular bacteria at t = 6 hr/t = 2 hr post infection (pi), compared to siRNA-control condition. (B) HeLa cells expressing GFP-LC3 (green) were infected for 1 hr, fixed, and labeled bacteria (blue) and ubiquitin (red) detection was performed by confocal microscopy. Representative infected cells are shown, and the percentage of labeled intracellular S. Typhimurium co-localized with GFP-LC3 vesicles and ubiquitin is represented on a graph.

(C) HeLa cells transfected with the indicated siRNAs for 48 hr were lysed, and anti-LC3 or anti-ACTIN western blots were performed. Representative results are shown along with a graph representing the intensity of LC3-I/LC3-I bands normalized to the control condition.

(D) HeLa cells transfected with the indicated siRNAs for 48 hr were lysed and eventually treated the last 4 hr with chloroquine or kept with vehicle. Western blot and analysis were carried out as in (C).

(E) GFP-LC3 HeLa cells transfected with the indicated siRNAs for 48 hr were fixed for analysis. The number of GFP+ vesicles per cell section was assessed by confocal microscopy. Representative profiles are shown along with a graph expressing the relative fold induction of dot number compared with control cells.

(F) GFP-LC3 HeLa cells were treated with chloroquine for 4 hr, or kept with vehicle (Mock), and fixed for confocal microscopy. Endogenous NDP52 was stained (red), and co-localization with GFP-LC3 (green) was assessed using Mander’s coefficient. Representative pictures of the results are shown along with a graph displaying the percentage of GFP-LC3 dots co-localized with NDP52 (white histograms), or the percentage of NDP52 labeling co-localized with GFP-LC3 dots (black histograms).

For (A)-(F), means ± SD of three independent experiments (A, B, and F were each carried out in triplicates). See also Figure S1.
the de novo formation of autophagy vesicles or from the accumulation of autophagosomes due to impaired recycling (Klionsky et al., 2012). To investigate the role of NDP52 in the autophagy flux, we first looked at the expression level of SQSTM1/p62, a long-lived protein mainly degraded through autophagy catabolism; its accumulation in cells indicates an aborted autophagy flux. Interestingly, when compared to the control condition, the level of expression of SQSTM1/p62 was significantly higher in both siNDP52- and siATG5-treated HeLa cells or 293T cells (Figure 2A and data not shown). These results suggest that NDP52 is an active component of the autophagy process.

To further confirm a role of NDP52 in autophagosome maturation, we used mRFP-GFP-LC3-expressing HeLa cells, allowing for the discrimination of autophagosomes (expressing both RFP and GFP fluorochromes) from acidified autolysosomes (expressing only GFP due to rapid GFP quenching in autolysosomes) (Klionsky et al., 2012). Similarly to a treatment with chloroquine, an inhibitor of autolysosome acidification leading to the accumulation of non-degradative autophagic vesicles, the reduced expression of NDP52 significantly inverted the autophagosomes/autolysosomes ratio when compared to normal cells or cells treated with rapamycin, an inducer of the autophagy flux (Figures 2B–2E). This modulation of the autophagy flux was dependent on ATG5, as the co-silencing of ATG5 together with NDP52 abrogated the accumulation of autophagy vesicles (Figure 2B). Moreover, the ectopic expression of NDP52 in siNDP52-treated cells restored an autophagosomes/autolysosomes ratio similar to that of control cells, uncovering the specific function of NDP52 in the maturation of autophagosomes (Figures 2D and 2E). Thus, NDP52 seems involved in autophagosome maturation in uninfected cells since the absence of NDP52 leads to the accumulation of autophagosomes due to impaired recycling.

**NDP52 Promotes Autophagosome Maturation via a LIR Motif**

In order to determine how NDP52 regulates autophagosome maturation, we thought to delineate the portion(s) of NDP52 that could be involved in this activity. Human NDP52 consists of an NH2-terminal skeletal muscle and kidney-enriched inositol phosphatase carboxyl homology (SKICH) domain (aa 1–127), a coiled-coil (CC) domain (aa 145–350), and an intermediate region between a COOH-terminal Zinc finger (Zn) domain (aa 420–446) (Figure 3A). We first found that when we reconstituted siNDP52-treated cells with an NDP52 construct expressing only the SKICH domain and the CLIR motif (NDP52 ΔCCΔZn), a normal phenotype of autophagosome maturation could not be restored (Figures 3B and 3C). This indicated that the LC3C-binding motif was not sufficient for NDP52 to trigger maturation of autophagosomes. In contrast, an NDP52 construct lacking the SKICH domain and the CLIR motif (NDP52 ΔSKICHΔCLIR) did rescue autophagy maturation otherwise impaired in siNDP52-treated cells (Figures 3B and 3C). Thus, although providing a specific interaction with an autophagosomal membrane-linked protein, LC3C, and being essential to targeting pathogen to autophagosomes (von Mühlner et al., 2012), the CLIR motif is dispensable for the NDP52-mediated autophagosome maturation.

Although the CLIR motif was not involved in the process, we thought that NDP52 could prompt autophagosome maturation via interaction with other LC3 family members. Supporting this hypothesis, we found that although NDP52 interacted strongly with LC3C, a weak interaction with LC3B was detected in co-affinity experiments (Figures 3D and S2A). Interestingly, the NDP52 ΔSKICHΔCLIR construct lacking the CLIR motif still interacted efficiently with LC3B, but only marginally with LC3C (Figure 3D). Looking at the sequence remaining in the NDP52 ΔSKICHΔCLIR construct, we identified a region at position 203–208 that differs from the canonical LIR motif only by the absence of an expected hydrophobic residue in position X3 (T207 or E208 instead of an I/V/L) (Johansen and Lamark, 2011). Interestingly, the mutation of aa 203–206 to alanine totally abrogated the interaction of NDP52 with LC3B, without affecting the marginal interaction with LC3C (Figures 3E and S2A). Moreover, NDP52 interactions with LC3A, GABARAP, GABARAL1, and GABARAPL2 were also detected, but only LC3A and GABARAPL2 interactions with NDP52 were affected by the introduction of alanines within the LIR-like motif (Figures S2B and S2C). We therefore asked whether this LIR-like motif could intervene in the capacity of NDP52 to regulate autophagy maturation. Strikingly, a LIR-like mutant of NDP52 (NDP52 LIR-like null) was found defective in its ability to rescue autophagosome maturation in cells silenced expression of NDP52 (Figures S3A–S3D). Furthermore, when we individually silenced each of the ATG8 orthologs using specific siRNA, a similar modulation of p62 expression was detected, suggesting redundancy between ATG8 orthologs in autophagy (Figures S3E and S3F). Thus, these data identify another LIR motif in NDP52, which is involved in an efficient completion of the autophagy process.

**The LIR-like Motif and the MYOSIN VI-Binding Domain of NDP52 Are Instrumental for Xenophagy**

On one hand, MYOSIN VI was reported to facilitate autophagosome maturation, and on the other hand, it can interact with...
NDP52 (Tumbarello et al., 2012; Morriswood et al., 2007). Moreover, we found that the NDP52 C425A mutant, impaired in its capacity to bind MYOSIN VI, did not restrict S. Typhimurium intracellular growth (Figure 1A). These observations suggested that the NDP52 C425A mutant fails to promote maturation of autophagosomes. Indeed, we found that the NDP52 C425A mutant did not rescue a normal autophagy phenotype in siNDP52-treated cells (Figures S3A–S3D). Thus, we found that the MYOSIN VI binding site of NDP52 is essential for both S. Typhimurium clearance and autophagosome maturation (Figures 1A and 3A–3D). We therefore asked whether the LIR-like motif, which is essential for the NDP52-mediated autophagosome maturation, is also crucial for xenophagy. Interestingly, reminiscent of what we observed for the NDP52 C425A mutant, we found that the NDP52 LIR-like null mutant did not restrict intracellular proliferation of S. Typhimurium, indicating that this NDP52 motif is essential for an efficient xenophagy (Figure 4A). However, the NDP52 LIR-like null mutant was still able to target bacteria to autophagosomes, as evaluated by confocal microscopy (Figure 4B), indicating that this motif is not involved in this function, which is reserved to the CLIR motif (Figure 1B; von Muhlinen et al., 2012).

To extend these results, we used mRFP-GFP-LC3-expressing HeLa cells to look for S. Typhimurium in autophagic vesicles (Figure 4C). Whereas bacteria were found in both autophagosomes and autolysosomes in control infected cells, as expected, the reduced expression of NDP52 restricted the number of intracellular bacteria addressed to autophagic vesicles. However, the complementation of siNDP52-treated cells with either the wild-type NDP52, the NDP52 C425A mutant, or the NDP52 LIR-like null mutant restored the targeting of bacteria to autophagic vesicles. Nevertheless, in cells reconstituted with either the NDP52 C425A mutant or the NDP52 LIR-like null mutant, bacteria were mainly found in non-matured autophagosomes as opposed to cells reconstituted with wild-type NDP52 in which bacteria were also found in acidic autolysosomes (Figure 4C). In support of these results, we also found that the reconstitution of siNDP52-treated cells with either the NDP52 C425A mutant or the NDP52 LIR-like null mutant did not rescue a normal level of S. Typhimurium co-localization with the lysosomal marker LAMP1, contrarily to the ectopic expression of wild-type NDP52 (Figure 4D). These results strongly support a contribution for NDP52 in the regulation of the fusion of bacteria-containing autophagosomes with lysosomes, which involves both the MYOSIN VI binding motif and the LIR-like domain of NDP52. Importantly, all NDP52 constructs that displayed default in bacterial xenophagy retained their ability to interact with GALECTIN 8 (Figure S4A), suggesting that the NDP52-GALECTIN 8 interaction has no role in autophagosome maturation.

Interestingly, NDP52 constructs with no CLIR and no LIR-like motifs (NDP52 ΔSKICHA CLIR/LIR-like null) or no CLIR motif and no MYOSIN VI binding motif (NDP52 ΔSKICHA CLIR/C425A) were nonetheless unable to protect cells from S. Typhimurium proliferation, but seemed to facilitate even more the bacterial growth, possibly by competing for bacteria binding with residual endogenous NDP52 in siNDP52-treated cells (Figure 4A). To determine the respective contribution of NDP52 in pathogen targeting to autophagosome and in the subsequent degradation, we monitored the intracellular proliferation of S. Typhimurium in siNDP52-treated cells co-reconstituted with two distinct NDP52 mutants that were individually defective to restrict bacteria intracellular proliferation. The co-expression of the NDP52 construct defective for the bacteria targeting to autophagy (NDP52 ΔSKICHA CLIR) with a NDP52 mutant defective for an efficient bacteria-containing autophagosome maturation (NDP52 C425A or NDP52 LIR-like null) did reconstitute the “wild-type” function of NDP52, as it allowed for the control of intracellular proliferation of S. Typhimurium (Figures 4E and S4B). Similarly, the intracellular proliferation of S. Typhimurium was restricted in siNDP52-treated cells co-expressing the two NDP52 mutants, NDP52 C425A + NDP52 LIR-like null, which individually were able to target bacteria to autophagosome but unable to restrict the proliferation (Figures 4E and S4B). Thus, our results suggest that xenophagy is completed by NDP52, which is crucial for both bacterial targeting to autophagosomes and autophagosome maturation via independent interactions for efficient xenophagy. When we looked at the contribution of other autophagy receptors known for selective intracellular pathogen restriction via autophagy, we found that the reduced expression of OPTINEURIN, but not the one of SQSTM1/p62, partially blocked the autophagy flux, as observed for NDP52 (Figures S4C–S4E). Thus, other autophagy receptors might operate similarly to NDP52 in regulating xenophagy from the targeting of the pathogen to autophagy up to its degradation via independent but functionally complementary domains.

**DISCUSSION**

Xenophagy implies the recognition of pathogens, their targeting within nascent autophagosomal membranes, and finally the completion of autophagy to destroy sequestered microorganisms. Here, we report that the autophagy receptor NDP52 can also complete autophagosome maturation, which optimizes the anti-microbial activity of autophagy.
(legend on next page)
NDP52 is an essential autophagy receptor to fight infections (Huett et al., 2012; Shahnazari et al., 2010; Thurston et al., 2009, 2012; Watson et al., 2012). Strikingly, several pathogens evolved strategies to target NDP52, possibly to escape from autophagic degradation, which further highlights the importance of NDP52 in the control of intracellular pathogens (Garnett et al., 2013; Grégoire et al., 2011; Judith et al., 2013). By concomitantly silencing the expression of NDP52, T6BP, and OPTINEURIN, it was proposed that these receptors have a role in autophagosome biogenesis and maturation (Tumbarello et al., 2012). However, our results indicate that NDP52 does not play a major role in autophagosome biogenesis. It is however possible that cells with a deficiency for several autophagy receptors prevent autophagosome biogenesis due to limited targeting of cellular cargos toward autophagosomal membranes. Thus, the unique absence of NDP52 would be compensated by the activity of other autophagy receptors. Interestingly, we detected few LC3+ autophagosomes, which co-localized with NDP52, whereas most NDP52+ dots did co-localize with autophagosomes. Thus, NDP52 might bring specific substrates to only a fraction of nascent autophagosomal membranes and then complete the maturation of those specific autophagosomes. Our observation that OPTINEURIN also contributes to autophagosome maturation suggests that other autophagy receptors might share the dual function of NDP52 by targeting substrates to autophagosomes and by regulating autophagic degradation. This might be a selective advantage in the context of cellular immunity toward intracellular pathogens.

NDP52 could bring substrates to autophagosomal membranes via distinct molecular mechanisms, and the CLIR motif is essential to this achievement. The LIR-like motif in the coiled-coil region identified here is essential to promote autophagosome maturation, together with the MYOSIN VI-binding domain. In contrast to most canonical LIR motifs that allow interaction with all members of the LC3 family as well as LC3 homologs GABARAP, GABARAPL1, and GABARAPL2, the CLIR motif only binds to LC3C (Johansen and Lamark, 2011; von Mühlinen et al., 2012). This interaction gives NDP52 the unique property to potentially select LC3C-containing autophagosomal membranes for degradation of selective substrates. However, after being recruited to LC3C-associated autophagosomal membrane, other members of LC3 family and GABARAPs proteins are recruited to bacteria (von Mühlinen et al., 2012). Here, we identify another domain in NDP52, which supports the interaction with several ATG8 orthologs, LC3A, LC3B, and GABARAPL2, but not with LC3C. This motif does not fit perfectly with the canonical LIR motif (aromatic residue-X-X-hydrophobic residue), since a hydrophobic residue is missing (Johansen and Lamark, 2011). Thus, in addition to CLIR, NDP52 expresses another non-conventional LIR motif whose function is not to connect NDP52-linked substrates to phagophores, but to promote autophagosome maturation. It is possible that once bound to LC3C, NDP52 uncovers the LIR-like motif, otherwise masked within the coiled-coil domain, and therefore interacts with other members of LC3s or GABARAPL2 to achieve autophagy maturation in order to degrade the entrapped bacteria. Alternatively, the LIR-like motif could be accessible independently of the NDP52-LC3C interaction but, due to low affinity for its interacting partners, only functional when NDP52 is stabilized within nascent autophagosomes. Finally, since NDP52 forms homodimers (Kim et al., 2013; Sterndorf et al., 1997), it would be possible that bacteria-bound NDP52 recruits bacteria-free NDP52 whose function is the completion of the substrate degradation. We found that the co-expression of a NDP52 mutant unable to target bacteria to autophagosomes together with a NDP52 mutant unable to regulate autophagosome maturation restored the bacterial restriction of wild-type NDP52, indicating that distinct proteins with single function contribute to functional xenophagy. The use of a unique molecule to complete pathogen targeting to autophagy and promotion of autophagy maturation could have the double advantage to concentrate the autophagy degradation on a selective substrate, which could limit the inappropriate degradation of cellular bystander contents, and also to shorten the delay of fusion of bacteria-containing autophagosomes with lysosomes (Figure S4F). A very rapid acidification of these vesicles could be instrumental to counteract the ability of intracellular bacteria to adapt within intra-autophagosomal environment in order to either escape or hijack autophagy to their own benefit (Deretic and Levine, 2009).

Thus, we revealed an unexpected role for human NDP52 in autophagosome maturation, which further highlights its function in cell-autonomous responses and innate immunity triggered against invading bacteria (Randow and Youle, 2014). Indeed, in addition to being a scaffold protein for innate response toward pathogens (Thurston et al., 2009), NDP52 plays two independent but complementary functions in anti-microbial autophagy: the targeting of pathogens to autophagy ensured by its selective autophagy receptor activity and the autophagosome-lysosome...
fusion ensured by its regulatory function in autophagy. Both functions are instrumental in xenophagy to optimize efficient bacterial degradation and could be shared by other xenophagy receptors.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**

HeLa, mRFP-GFP-LC3 HeLa, GFP-LC3 HeLa, and 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 50 μg/ml of Gentamicin. Additional 500 μg/ml of Genticin was added for mRFP-GFP-LC3 HeLa, GFP-LC3 HeLa cell cultures. Cell transfections were performed as follows: the day before transfection with siRNA, the cells were seeded in 6-well plates with 1 × 10⁵ cells per well in OPTI-MEM complemented with 10% FBS, 2 mM of L-glutamine, 50 μg/ml of Gentamycin, 0.1 mM non-essential amino acid, 0.1 mM pyruvate sodium, and 0.1 g/L bicarbonate sodium. The cells were transfected with 100 pmol of siRNA using Lipofectamine RNAiMAX from Invitrogen according to the manufacturer’s instructions. During rescue experiments, the cells were transferred 24 hr after siRNA transfection in a 24-well plate at 5 × 10⁴ cells per well. 24 hr after, 250 ng total of DNA vector were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). The expression of individual constructs was confirmed by western blot (Figure S1B). For flux analysis with mRFP-GFP-LC3-expressing cells, at least 100 cells per individual experiment were numerated by confocal microscopy (see Supplemental Information). The references of antibodies and reagents used in this study are available in the Supplemental Information.

**Molecular Cloning, Co-Affinity Purification, and siRNA**

Human NDP52 encoding vector (Invitrogen) was used as matrix to generate all NDP52 mutants and constructs used in this study by QuickChange Lightning Site-Directed Mutagenesis kit (Agilent #210518) according to the manufacturer’s instructions. The mutations/constructs were engineered as indicated in the Supplemental Information as well as the co-affinity purification experiments. The references of the used siRNA are in the Supplemental Information.

**S. Typhimurium Infections**

Salmonella enterica serovar Typhimurium (strain 12023) was grown overnight in LB broth (Sigma), and infections were carried out as indicated in the Supplemental Information, as previously reported (Thurston et al., 2009). For confocal microscopy (see Supplemental Information), the cells were fixed using cold acetone for 5 min after the indicated period of time of infection (50–100 bacteria in infected cells were analyzed).

**Statistical Analysis**

All p values were calculated using a two-tailed Welch t test (Student’s t test assuming non-equal variances of the samples), except for the results of Figures 1A and 4A, for which a one-tailed Welch t test was applied; *p < 0.05, **p < 0.01, ***p < 0.001.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2015.02.008.

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