

Nix is a selective autophagy receptor for mitochondrial clearance

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Autophagy is the cellular homeostatic pathway that delivers large cytosolic materials for degradation in the lysosome. Recent evidence indicates that autophagy mediates selective removal of protein aggregates, organelles and microbes in cells. Yet, the specificity in targeting a particular substrate to the autophagy pathway remains poorly understood. Here, we show that the mitochondrial protein Nix is a selective autophagy receptor by binding to LC3/GABARAP proteins, ubiquitin-like modifiers that are required for the growth of autophagosomal membranes. In cultured cells, Nix recruits GABARAP-L1 to damaged mitochondria through its amino-terminal LC3-interacting region. Furthermore, ablation of the Nix:LC3/GABARAP interaction retards mitochondrial clearance in maturing murine reticulocytes. Thus, Nix functions as an autophagy receptor, which mediates mitochondrial clearance after mitochondrial damage and during erythrocyte differentiation.

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INTRODUCTION

Mounting evidence suggests that selective degradation of bulky cytosolic substrates, such as protein aggregates, damaged organelles and intracellular microbes, is mediated by macroautophagy (autophagy hereafter; Kirkin *et al*, 2009b). Its main hallmark is the formation of double-membrane vesicles—autophagosomes—that engulf cargo and deliver it to lysosomes for degradation (Xie & Klionsky, 2007; Nakatogawa *et al*, 2009). Ubiquitination has been proposed as a signal for selective autophagy (Kirkin *et al*, 2009b). Receptor proteins, p62 and neighbour of BRCA1 gene 1 (NBR1), which interact with both ubiquitin (Ub) and autophagosome-specific Ub-like (UBL) proteins, Atg8-family proteins LC3/GABARAP, have been identified (Komatsu *et al*, 2007; Pankiv *et al*, 2007; Kirkin *et al*, 2009a).

Specific elimination of damaged mitochondria by autophagy (mitophagy) has been demonstrated convincingly (Elmore *et al*, 2001; Priault *et al*, 2005; Kim *et al*, 2007; Narendra *et al*, 2008). In mammals, mitophagy is important in programmed mitochondrial clearance during differentiation of reticulocytes and T lymphocytes (Schweers *et al*, 2007; Kundu *et al*, 2008; Sandoval *et al*, 2008; Zhang *et al*, 2009b). The mitochondrion-localized proteins Bcl2/E1B 19 kDa-interacting protein 3-like protein (BNIP3) and Nix (also known as BNIP3L) have been implicated in the removal of mitochondria during an autophagic response (Zhang & Ney, 2009). Thus, hypoxia-induced autophagy depends on both BNIP3 and Nix, and can be mimicked by overexpression of their respective BH3 domains (Bellot *et al*, 2009). Furthermore, Nix is indispensable for programmed elimination of mitochondria during reticulocyte maturation. Interestingly, in Nix-deficient reticulocytes autophagy *per se* is functional, but mitochondria fail to enter the autophagosomes (Schweers *et al*, 2007; Sandoval *et al*, 2008). Moreover, knockout of autophagy-specific genes (*Ulk1*, *Atg5* and *Atg7*) does not abolish fully programmed mitochondrial clearance in reticulocytes (Matsui *et al*, 2006;

Kundu *et al*, 2008; Zhang *et al*, 2009b), suggesting the existence of autophagy-independent pathways for programmed mitochondrial clearance in this cell lineage.

Selective autophagy implies the targeted formation of autophagosomes around a particular substrate (for example, mitochondrion). At the molecular level, selectivity can be mediated by the binding of autophagy receptors to membrane-anchored, autophagy-specific UBL proteins. This idea has been supported by the identification of several receptors that bind to UBL proteins, coupling their substrate conjugation to a biological response (Kirkin & Dikic, 2007). The common binding site for Atg8-family proteins contains the W/YxxL/I core motif and is referred to as the LC3-interacting region (LIR; Komatsu *et al*, 2007; Pankiv *et al*, 2007; Kirkin *et al*, 2009a). Here, we describe the identification of Nix as a mammalian mitophagy receptor.

RESULTS

Nix interacts with Atg8/LC3/GABARAP proteins

To search for proteins that interact with autophagy-specific UBL proteins, we used *Saccharomyces cerevisiae* (Sc)Atg8 as bait in yeast two-hybrid screens. One Atg8-interacting clone encoded the full-length Nix protein previously linked to autophagy (Zhang & Ney, 2009). This interaction was confirmed by transformation of the isolated Nix-encoding vector into yeast strains expressing ScAtg8 (Fig 1A). Whereas ATG4A, ATG4B and p62 interacted preferentially with LC3B in this assay, Nix, similarly to NBR1 (Kirkin *et al*, 2009a), interacted strongly with Atg8 but not LC3B (Fig 1A). The Nix:Atg8 interaction was specific as Nix failed to interact with Atg12 and small ubiquitin-like modifier 1 (SUMO1) in this assay (Fig 1A).

The *in vitro* interaction of Nix with the mammalian Atg8 protein family was investigated further in biochemical assays. Flag-Nix appeared on SDS-polyacrylamide gel electrophoresis gels as two distinct protein species of approximately 40 and 70 kDa (monomeric and dimeric forms; Fig 1B; Imazu *et al*, 1999). Both Nix forms interacted with Atg8-family proteins, but not with Ub in glutathione-S-transferase (GST) pulldown assays (Fig 1B). The Nix transmembrane (TM) domain is responsible for dimerization (Sulistijo & MacKenzie, 2006; Bocharov *et al*, 2007). Recombinant Nix lacking a TM (Nix Δ TM) was used to show direct interaction of Nix with human Atg8 homologues (Fig 1C). In both assays, Nix interacted strongly with all tested LC3/GABARAP proteins except for LC3B, which showed much weaker binding.

To confirm the *in vivo* interaction between Nix and LC3/GABARAP, we performed co-immunoprecipitation experiments with endogenous proteins. We found that Nix co-precipitated weakly with LC3 under normal growth conditions (Fig 1D), but interacted more strongly with LC3 under conditions that induce mitochondrial stress (rotenone or carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment) or block autophagy (bafilomycin-A1 treatment; Fig 1D).

Nix contains LIRs

The known autophagy receptors bind to Atg8 family members through a linear sequence motif known as the LIR. Its core consensus sequence is W/YxxL/I (Kirkin *et al*, 2009b). By using multiple species alignment of Nix and its relative BNIP3, we predicted several putative LIR sequences in Nix (Fig 2A). We found one WxL motif near the amino-terminal of Nix (amino

acids 35–38 in murine Nix) that is well conserved between Nix and BNIP3 (Fig 2A,B). This motif closely resembles the LIR found in ScAtg19 protein (Noda *et al*, 2008). The other potential LIR is located adjacent to an atypical BH3-like domain (Fig 2A,B). It has low homology to the W/YxxL/I core LIR consensus, but instead contains a double DW motif (amino acids 138–143) that is similar to p62 (DDWTHL) and NBR1 (EDYIILL; Pankiv *et al*, 2007; Kirkin *et al*, 2009a). To distinguish between the two putative LIR sequences, we refer to the N-terminal motif as LIR-W35 and to the BH3 domain-specific one as LIR-W139/143 (Fig 2B).

To determine whether Nix binding to Atg8-family proteins depends on one of the putative LIR motifs, we performed a series of GST pulldown experiments with Nix mutants (Fig 2C). Mutation of the conserved W35 residue in the N-terminal LIR to an alanine ablated most of Nix binding to LC3A (Fig 2C). Furthermore, the L38A mutation also abolished binding (Fig 2C). Trp:Ala replacements in the W139/W143 motif impaired the interaction only slightly (Fig 2C). In addition, removal of WVLE either alone (Δ 35–38 mutant) or in the context of the larger N-terminal deletion (Δ 39 mutant) led to ablation of Nix:LC3A binding (Fig 2C). These results suggest that LIR-W35 is the main Atg8-family protein-interaction site in Nix, whereas LIR-W139/143 has a minor role.

Characterization of Nix-LIR binding to LC3 proteins

Next, we characterized the interaction of the putative Nix-LIRs with Atg8-family proteins by isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR). We used short synthetic peptides, corresponding to LIR-W35 (Nix-W35) and LIR-W139/143 (Nix-W139/143; supplementary Fig S1A online). A peptide corresponding to p62 LIR (p62-LIR) was used as reference (Ichimura *et al*, 2008; Noda *et al*, 2008). LC3B showed the lowest affinity in the binding assays (Fig 1), so we chose this protein to establish the basal level of Atg8-family interactions with Nix-LIRs. For comparison, we assessed the interaction of Nix-LIRs with LC3A, as a representative of the Atg8 protein family with stronger Nix binding (Fig 1). First, the ITC experiments showed that the Nix-W35 peptide interacted with both LC3B and LC3A, albeit with differing affinities: K_D of 91 and 28 μ M, respectively (Table 1). Second, an ITC comparison of the interaction between LC3 proteins and Nix-W35 versus Nix-W139/143 peptides showed that LC3 proteins interacted with Nix-W35 much more strongly than with Nix-W139/143: K_D values of 91 μ M (LC3B) and 28 μ M (LC3A) versus 670 μ M (LC3B) and 130 μ M (LC3A), respectively. Experiments with the reference peptide (p62-LIR) showed that the Nix-LIRs interact much more weakly with Atg8-family proteins than p62 does (K_D of 1.5 μ M for the p62-LIR:LC3B interaction; Table 1). NMR titration experiments with 15 N-labelled proteins confirmed the ITC results (supplementary Fig S1B,C online).

Nix recruits GABARAP-L1 to depolarized mitochondria

Previous studies showed that Nix localizes to the outer mitochondrial membrane where it affects mitochondrial integrity (Zhang & Ney, 2009). To see if Nix recruits LC3/GABARAP to stressed mitochondria, we performed a series of colocalization studies of cells cultured under normal growing conditions or treated with mitochondrial poison, CCCP, to induce mitochondrial stress and autophagy (Chen *et al*, 2007). For these studies we used GABARAP-L1 because of its strong interaction with Nix in our previous assays (Fig 1B).

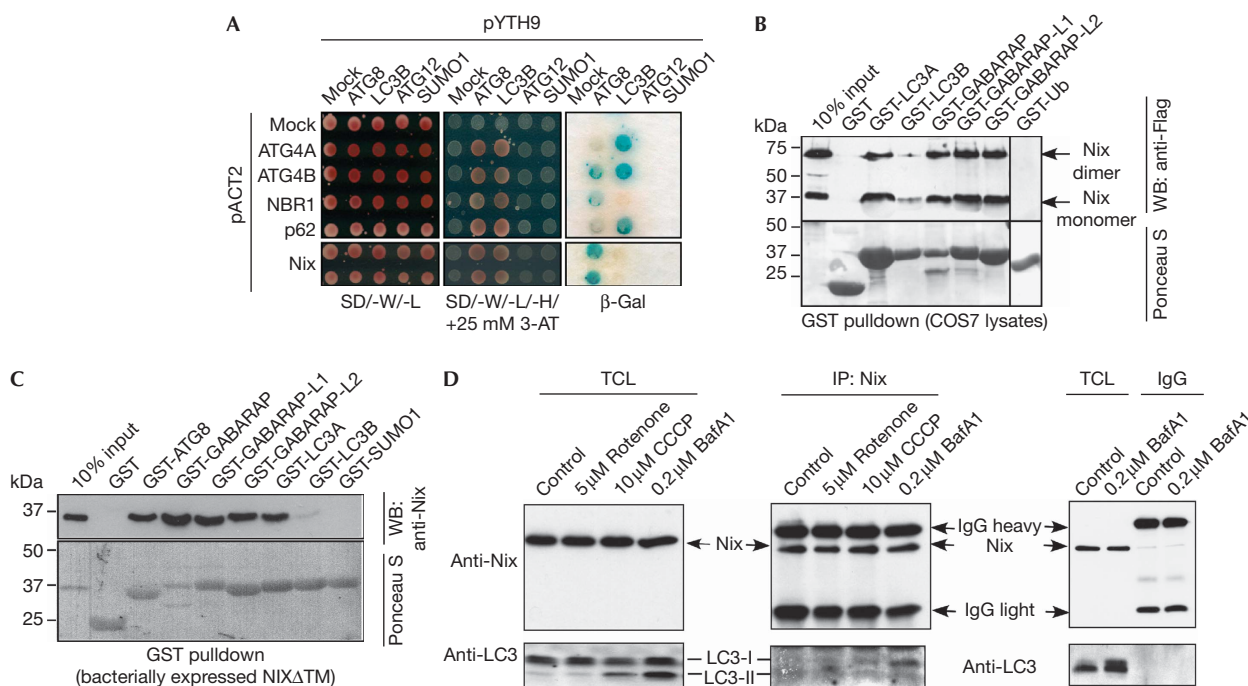


Fig 1 | Nix interacts with Atg8/LC3/GABARAP proteins. (A) Yeast clones harbouring empty bait vector (pYTH9, mock) or those encoding ScAtg8, LC3B, Atg12 and SUMO1 were transformed with empty prey vector (pACT2, mock) or those encoding fragments of ATG4A, ATG4B, NBR1, p62 and full-length Nix. Interaction was assessed by yeast growth on the SD/-W/-L/-H medium with a β -gal assay. (B) GST pull-down assays using cell extracts of Flag-Nix-expressing COS7 cells and immobilized GST or the indicated GST fusions. Coprecipitated Nix was detected with Flag antibodies. (C) Purified GST-tagged Nix Δ TM was cleaved from the GST moiety by thrombin, purified and used for precipitation by GST fusion proteins. Precipitated proteins were analysed by western blotting (WB) with Nix antibodies. Ponceau S staining was used to visualize GST-fusion proteins. (D) HeLa cells were treated as indicated and endogenous proteins were immunoprecipitated (IP) from cell extracts using Nix antibody and analysed by using Nix and LC3 antibodies. β -gal, β -galactosidase; BafA1, bafilomycin-A1; CCCP, carbonyl cyanide m-chlorophenyl hidrazone; GST, glutathione-S-transferase; IgG, immunoglobulin G; Nix Δ TM, recombinant Nix lacking a TM; SUMO1, small ubiquitin-like modifier 1; TCL, total cell lysate; TM, transmembrane domain.

First, we examined the effect of CCCP on enhanced green fluorescent protein (EGFP)-GABARAP-L1 colocalization with mitochondria in reconstituted *Nix*^{-/-} primary mouse embryonic fibroblasts (MEFs). *Nix*^{-/-} MEFs were reconstituted with either wild-type (wt) Nix (Flag-wt-Nix) or the W35A mutant of Nix (Flag-Nix-W35A). Importantly, we observed that both versions of Nix colocalized with mitochondria, indicating that the W35A mutation of Nix does not disrupt its recruitment to mitochondria (Fig 3A,B).

In *Nix*^{-/-} MEFs cotransfected with EGFP-GABARAP-L1 and Flag-wt-Nix, GABARAP-L1 colocalized infrequently with Nix in mitochondria (Fig 3A); however, on treatment with CCCP there was an increase in puncta with colocalization of GABARAP-L1 and Nix, indicating recruitment of GABARAP-L1 to Nix in mitochondria (Fig 3A,C). Following treatment with CCCP, the ability of Nix-W35A to recruit EGFP-GABARAP-L1 to stressed mitochondria was impaired but not lost completely (Fig 3B,C). Quantification of GABARAP-L1 and Nix (wt or W35A) colocalization showed that there is approximately a 50% reduction in GABARAP-L1 recruitment to Nix-W35A mitochondria, as compared with wt Nix (Fig 3C). We obtained similar results with transfected HeLa cells (supplementary Fig S2A–D online). We also

assessed the recruitment of EGFP-GABARAP-L1 to mitochondria in *Nix*^{+/+} and *Nix*^{-/-} MEFs, and observed its impairment in the absence of Nix (supplementary Fig S3 online). These results suggest that recruitment of EGFP-GABARAP-L1 to mitochondria, under stress conditions, is partly dependent on the Nix-W35 LIR motif.

Mitochondrial clearance is partly mediated by LIR-35

Nix deficiency in mice leads to pronounced impairment in mitochondrial clearance during reticulocyte maturation (Schweers et al, 2007; Sandoval et al, 2008). We asked whether mitochondrial clearance in these cells depends on Nix LIR sequences, and hence on the binding of Nix to LC3/GABARAP proteins. To investigate this we used *Nix*^{-/-} mice (Schweers et al, 2007). We expressed wt-Nix or LIR mutants of Nix in *Nix*^{-/-} reticulocytes by using retrovirus-mediated gene transfer into *Nix*^{-/-} bone marrow, followed by transplantation into lethally irradiated recipients. In unstressed mice, reconstitution with either wt-Nix or its LIR mutants (W35A, W139/143A and W35/139/143A) rescued mitochondrial clearance (data not shown). We speculated that mitochondrial clearance was partly defective, but that erythrocytes *in vivo* could clear their mitochondria, given enough

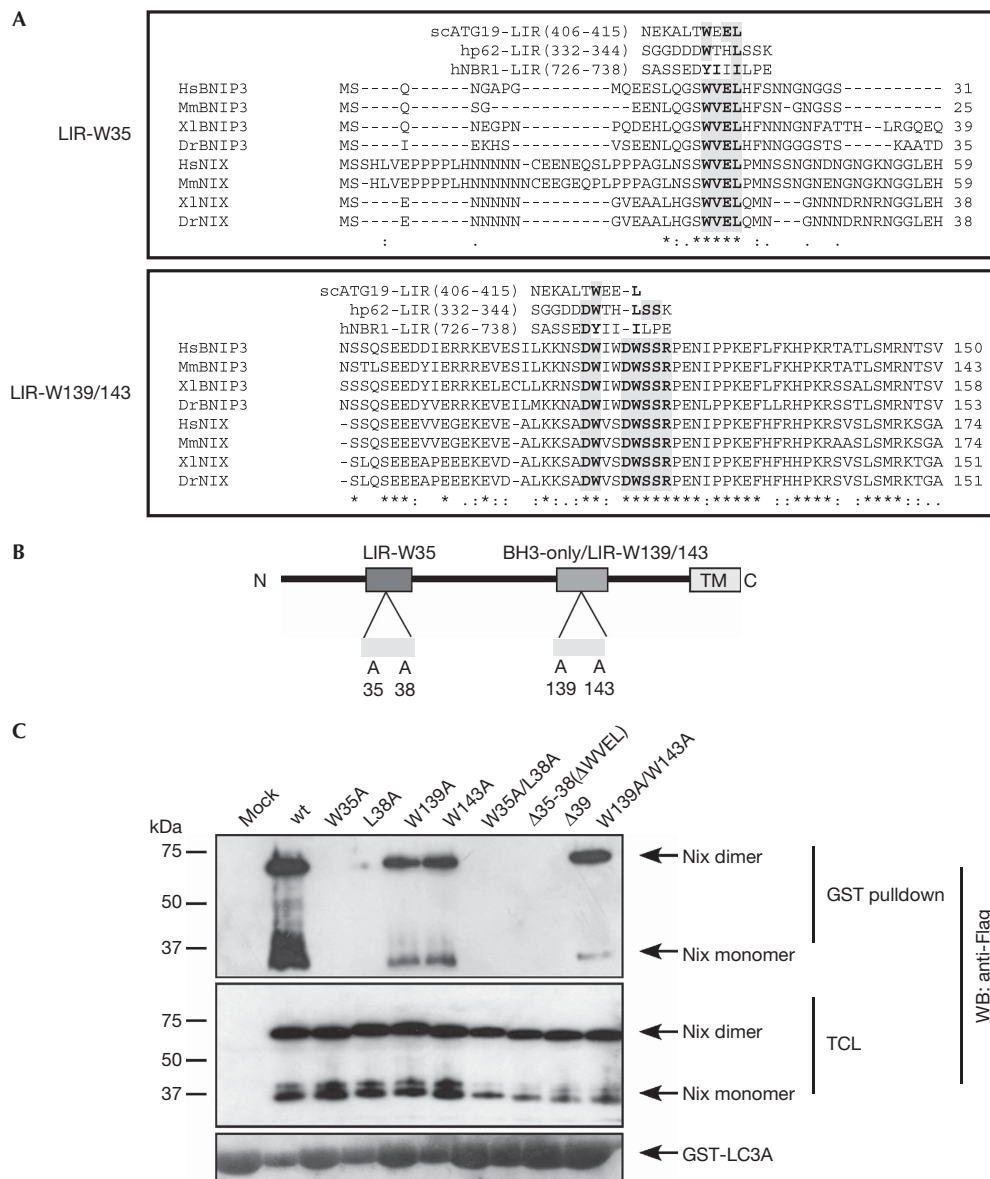


Fig 2 | Identification of Nix-LIRs. (A) Amino-acid sequences of Nix and related BNIP3 protein from the indicated species were aligned using the ClustalW2 program (EMBL-EBI, Cambridge, UK). The published LIR sequences were aligned manually alongside Nix/BNIP3 for comparison. The shaded regions indicate highly conserved residues in the two putative LIRs. Consensus symbols * (identical residues), : (conserved substitution) and . (semi-conserved substitution) are valid for the Nix/BNIP3 alignment only. (B) A schematic of Nix showing its domain organization and the point mutations used in this study to identify LIRs. (C) Lysates of COS7 cells transfected with Flag-wt-Nix or the indicated mutants were incubated with immobilized GST-LC3A. The coprecipitated proteins were detected by western blotting (WB) with Flag antibodies. BNIP3, Bcl2/E1B 19 kDa-interacting protein 3-like protein; Dr, *Danio reio*; GST, glutathione-S-transferase; Hs, *Homo sapiens*; LIR, LC3-interacting region; Mm, *Mus musculus*; wt, wild type; Xl, *Xenopus laevis*.

time. To investigate this possibility, we examined mitochondrial clearance in reticulocytes cultured *ex vivo*. We induced reticulocytosis in transplant recipients with phenylhydrazine (Vannucchi *et al*, 2001), harvested the reticulocyte-enriched blood and monitored the mitochondrial clearance over three days. Under these conditions, wt-Nix rescued mitochondrial clearance to the same levels seen in *Nix*^{+/+} mice. By contrast, LIR-W35A-mutant Nix showed impaired ability to rescue mitochondrial clearance (Fig 4A,B). Western blot analysis of Nix

protein and the W35A mutant confirmed similar expression levels of reconstituted Nix protein in transduced erythroid cells (Fig 4C). These results are consistent with a role for LC3/GABARAP binding to Nix in programmed mitochondrial clearance in reticulocytes.

DISCUSSION

Here we identify Nix as an interaction partner for autophagy-specific UBL proteins, LC3/GABARAP, implicated in mitophagy. Nix binds to LC3/GABARAP through two potential LIRs

Fig 3 | Nix recruits EGFP-GABARAP-L1 to stressed mitochondria in reconstituted *Nix*^{-/-} MEFs. *Nix*^{-/-} MEFs cotransfected with EGFP-GABARAP-L1 and wt-Nix (A), or Nix-W35A (B), were incubated with and without mitochondrial poison CCCP for 3 h. Cells were then fixed and analysed for colocalization between EGFP-GABARAP-L1 and Nix (stained with Flag antibodies). The final panel in each row shows colocalization between Flag-Nix-Cy3 and GABARAP-L1 using the ‘colocalization’ highlighter plug-in for ImageJ software (NIH, Bethesda, MA, USA). (C) Quantification was achieved by counting the number of complete colocalizations, EGFP/Cy3 per cell for approximately 30 cells per condition. Results are expressed as a percentage of colocalization (EGFP: Cy3) in control and CCCP-treated cells. Error bars in (C) show the standard deviation (s.d.) of three independent experiments. Statistical analysis was performed using the Mann-Whitney *t*-test. CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; MEF, mouse embryonic fibroblast; wt, wild type.

Table 1 | K_D values acquired by ITC analyses of the interaction of Nix-LIR peptides with LC3 proteins

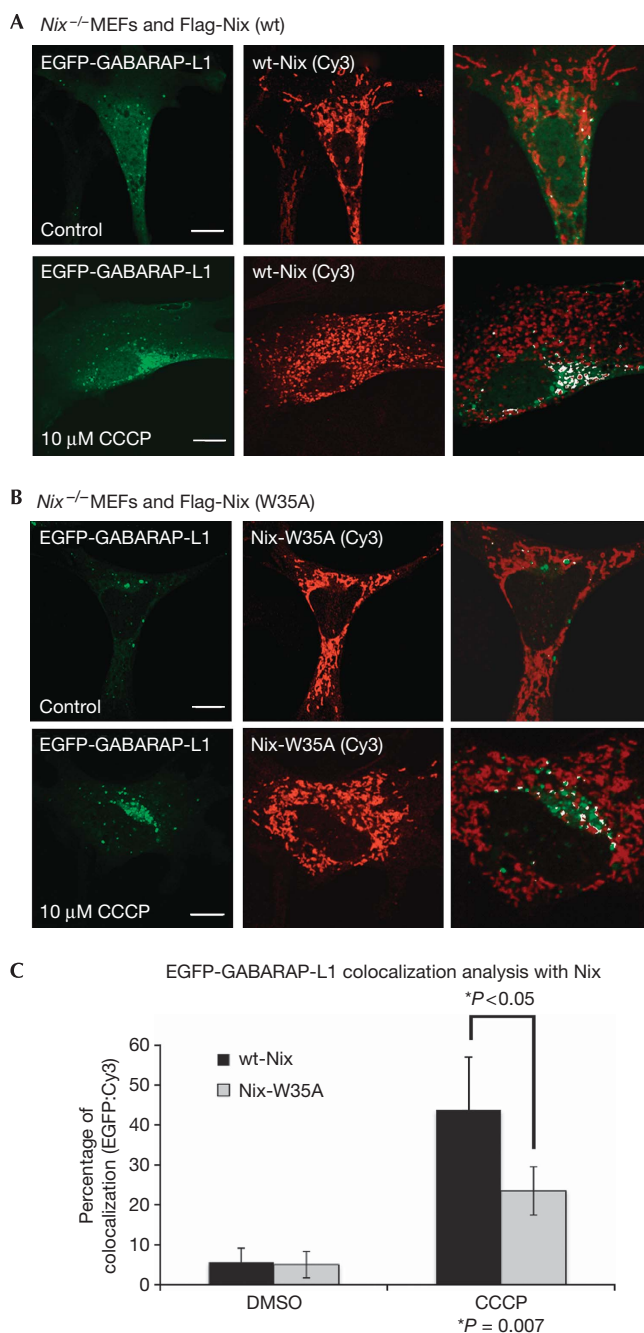
	LC3B	LC3A
p62-LIR	1.5 μ M	NA
Nix-W35	91 μ M	28 μ M
Nix-W139/W143	670 μ M	130 μ M

ITC, isothermal titration calorimetry; LIR, LC3-interacting region; NA, not available.

that share selected features with the published LIR sequences in other proteins. Of the two LIRs characterized in this study, LIR-W35 is particularly important as its mutation or deletion abolishes Nix:LC3/GABARAP binding *in vitro* and *in vivo*. Importantly, while this paper was in preparation, Schwarten *et al* reported the identification of Nix as a GABARAP-interacting protein. A Nix-derived peptide identical to LIR-W35 was implicated in the GABARAP-Nix interaction and a mutation of W35 to an alanine abolished this binding (Schwarten *et al*, 2009).

Nix binds relatively weakly to LC3B in comparison with LC3A and GABARAP proteins (Fig 1; Table 1; supplementary Fig S1 online; Schwarten *et al*, 2009). This suggests that LC3A and GABARAP proteins, rather than LC3B, have a leading role in Nix-dependent mitophagy. Further, Nix recruits GABARAP-L1 to stressed mitochondria in a LIR-W35A-dependent manner, suggesting involvement of previously uncharacterized Atg8-family proteins in the clearance of damaged mitochondria. In line with this, ATG4D, an isoform of the LC3/GABARAP-specific cysteine protease, which is essential for LC3/GABARAP function, was shown recently to have increased specificity to GABARAP-L1 and to localize to mitochondria (Betin & Lane, 2009).

The sequence conservation of Nix LIRs across the Chordata phylum suggests that the interaction between Nix and the autophagic machinery has been essential in the course of evolution. Recently, a novel mitochondrial protein, Atg32, was characterized as a selective autophagy receptor for autophagic degradation of stressed mitochondria in yeast (Kanki *et al*, 2009; Okamoto *et al*, 2009). As no homologue of Atg32 in higher organisms could be identified, we propose that Nix, and possibly



BNIP3, might fulfill its function in higher organisms. Atg32 shares several features with Nix: (i) a carboxy-terminal TM domain that is essential for the targeting of the protein to the outer mitochondrial membrane; (ii) an LIR responsible for binding to Atg8; and (iii) Atg32 is induced by stress cues and recruits Atg8 to stressed mitochondria. Interestingly, the LIR of Atg32 is also only partly required for mitophagy as Atg32 interacts with the Cvt pathway adaptor Atg11 independently of its LIR domain. This suggests that several signals might be essential for efficient mitophagy in yeast.

Programmed mitochondrial clearance in reticulocytes is Nix-dependent (Schweers *et al*, 2007; Sandoval *et al*, 2008) and

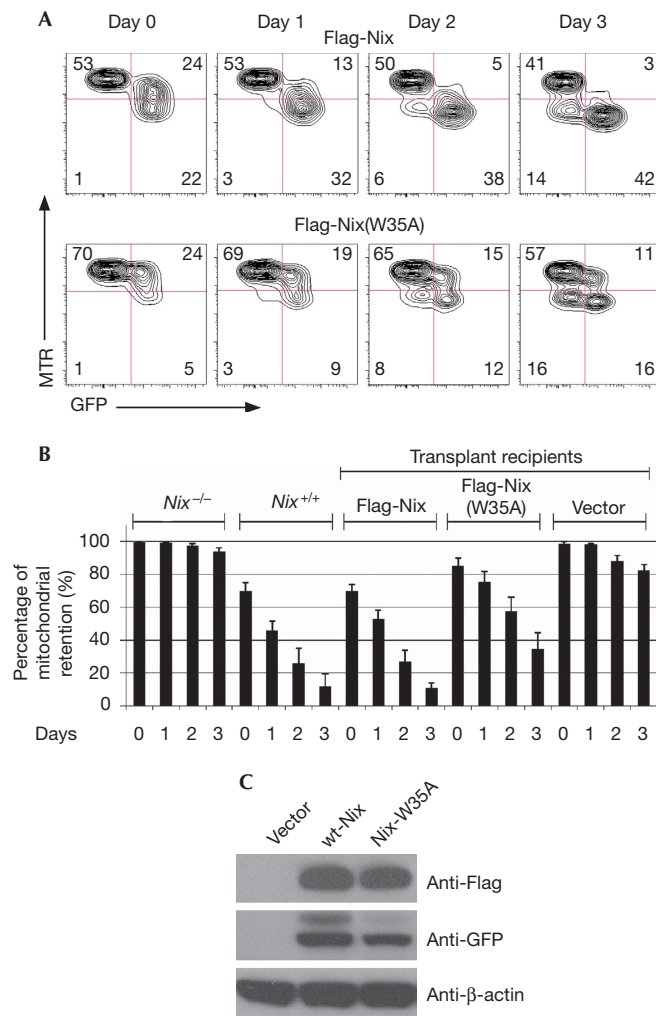


Fig 4 | Mitochondrial clearance is impaired in *Nix*^{-/-} reticulocytes transduced with Nix-W35A. (A) Flow cytometry of reticulocyte-enriched blood, cultured *in vitro* for 3 days, stained with MTR. The blood was taken from mice transplanted with *Nix*^{-/-} bone marrow and reconstituted with wt-Nix and Nix-W35A viruses. Reticulocytes were induced by phenylhydrazine treatment. The viruses also express GFP. (B) Mitochondrial clearance in the GFP-positive fraction of the reticulocyte-enriched blood, as described in panel (A). Mice were reconstituted with wt-Nix, LIR-W35A or empty vector viruses. Data from *Nix*^{-/-} and *Nix*^{+/+} mice are shown for comparison. (C) Anti-Flag, anti-GFP and anti-β-actin western blots of fetal liver cells transduced with Flag-Nix and Flag-W35A-Nix viruses, normalized to total protein. GFP, green fluorescent protein; LIR, LC3-interacting region; MTR, MitoTracker Red; wt, wild type.

Nix binding to LC3/GABARAP through LIR-W35 might contribute to this process during reticulocyte maturation (Fig 4). The effect of the LIR mutation on Nix-mediated mitochondrial clearance *in vivo* is partial, indicating that other properties of Nix are also important in this process. Similarly, defects in general autophagy caused by gene targeting of Ulk1, Atg7 and Atg5 also had a partial effect on mitochondrial clearance in reticulocytes (Matsui *et al*, 2006; Kundu *et al*, 2008; Zhang *et al*, 2009b). As, however, ATG5/

ATG7-independent autophagy has now been described, more studies are required to understand the role of autophagy in reticulocyte maturation (Nishida *et al*, 2009).

We envisage that Nix-dependent recruitment of LC3/GABARAP proteins associated with autophagosomes to mitochondria might be one factor in mediating membrane tethering and/or hemifusion of mitochondria with autophagosomes. However, other signals—for example, ubiquitination of mitochondrial proteins or interactions with lysosomal components—might mediate the full incorporation of mitochondria into autophagosomes (supplementary Fig S4 online). This could be the reason why mitochondria align to autophagosomes, but are not incorporated in *Nix*^{-/-} reticulocytes. Several E3 ligases associated with mitochondria, such as mitochondrial E3 ubiquitin ligase, membrane-associated ring finger 5 or LISTERIN, might participate in ubiquitination of mitochondrial membrane proteins and contribute to mitochondrial clearance through mitophagy (Karbowski *et al*, 2007; Li *et al*, 2008; Chu *et al*, 2009). Furthermore, a recent work showed that translocation of E3 ligase Parkin and its interacting partner PTEN-induced putative kinase 1 to damaged mitochondria is required for efficient mitophagy (Narendra *et al*, 2008; Dagda *et al*, 2009).

Nix might mediate mitochondrial clearance in an LC3/GABARAP-independent or autophagy-independent manner. Our model (supplementary Fig S4 online) could explain why a deficiency in autophagy rather than a deficiency in Nix generally has a more subtle effect on mitochondrial clearance: lack of Nix would block both autophagy-dependent and autophagy-independent mechanisms, whereas failure of Nix to interact with autophagosome-localized LC3/GABARAP proteins would only inhibit autophagy-dependent mitochondrial clearance.

METHODS

Detailed experimental procedures are described in the supplementary information online.

Protein purifications and biochemical assays. Yeast transformation assay and interaction between recombinant, purified proteins and assays of cellular proteins were performed as described in the supplementary information online.

ITC and NMR spectroscopy. ITC experiments were performed at 25 °C using a VP-ITC calorimeter (MicroCal Inc., Northampton, MA, USA) and analysed with the ITC-Origin software (MicroCal Inc.) based on the assumption of one-site binding reactions.

Laser-scanning microscopy. Images of fixed and stained cells expressing fusions of fluorescent proteins cells were acquired by the LSM 510 META laser-scanning microscope (Carl Zeiss MicroImaging, Jena, Germany).

Retrovirus-mediated gene transfer of Nix into *Nix*^{-/-} mice and analysis of mitochondrial clearance. Retrovirus-mediated gene transfer into *Nix*^{-/-} mice was performed as described in the supplementary information online. Reticulocytosis was induced by phenylhydrazine treatment and reticulocyte-enriched blood was cultured and stained with MitoTracker Red, as previously described (Zhang *et al*, 2009a).

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

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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