Developmental Cell

NIPSNAP1 and NIPSNAP2 Act as "Eat Me" Signals for Mitophagy

Graphical Abstract



Highlights

- The mitochondria proteins NIPSNAP1 and NIPSNAP2 bind to autophagy-related proteins
- NIPSNAP1 and NIPSNAP2 recruit autophagy receptors to depolarized mitochondria
- NIPSNAP1 and NIPSNAP2, acting redundantly, are required for PARKIN-dependent mitophagy
- Nipsnap1-deficient zebrafish larvae display parkinsonism

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In Brief

Abudu and coworkers show that the mitochondrial proteins NIPSNAP1 and NIPSNAP2 are needed for PARKINdependent mitophagy, by facilitating recruitment of the autophagy machinery required for clearance of damaged mitochondria. Nipsnap1-deficient zebrafish larvae have a parkinsonian phenotype including accumulation of reactive oxygen species, reduced dopaminergic neurons, and a locomotion defect.



Developmental Cell Article

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SUMMARY

The clearance of damaged or dysfunctional mitochondria by selective autophagy (mitophagy) is important for cellular homeostasis and prevention of disease. Our understanding of the mitochondrial signals that trigger their recognition and targeting by mitophagy is limited. Here, we show that the mitochondrial matrix proteins 4-Nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (NIPSNAP1) and NIPSNAP2 accumulate on the mitochondria surface upon mitochondrial depolarization. There, they recruit proteins involved in selective autophagy, including autophagy receptors and ATG8 proteins, thereby functioning as an "eat me" signal for mitophagy. NIPSNAP1 and NIPSNAP2 have a redundant function in mitophagy and are predominantly expressed in different tissues. Zebrafish lacking a functional Nipsnap1 display reduced mitophagy in the brain and parkinsonian phenotypes, including loss of tyrosine hydroxylase (Th1)-positive dopaminergic (DA) neurons, reduced motor activity, and increased oxidative stress.

INTRODUCTION

Macroautophagy (hereafter autophagy) involves sequestration of cytoplasmic cargo into autophagosomes that fuse with lysosomes for degradation (Lamb et al., 2013). Autophagy is needed for survival upon cellular stress and has an important housekeeping function by selective removal of damaged or dysfunctional components. Selective autophagy employs specific autophagy receptors that recognize the cargo to become degraded (Rogov et al., 2014; Stolz et al., 2014). Sequestosome-1 (hereafter referred to as p62), the best studied autophagy receptor, is implicated in clearance of many types of ubiquitinated cargos, including aggregate-prone proteins (Bjørkøy et al., 2005), mitochondria (Geisler et al., 2010; Zhong et al., 2016), bacteria (Zheng et al., 2009), and midbody remnants (Isakson et al., 2013; Pohl and Jentsch, 2009). Autophagy receptors contain a light chain 3 (LC3)-interacting region (LIR) mediating their interaction with microtubule-associated protein 1 LC3 and/or GABA type A receptor-associated protein (GABARAP) family proteins in the autophagy membrane (Birgisdottir et al., 2013; Pankiv et al., 2007). Selective autophagy may also require autophagy adaptor proteins, which possess an LIR but are themselves not degraded by autophagy (Stolz et al., 2014). One such adaptor protein is the large scaffolding protein autophagy-linked FYVE (ALFY), which binds GABARAP and phosphatidylinositol-3phosphate (PtdIns3P) (Lystad et al., 2014; Simonsen et al., 2004), as well as p62 and neighbor of BRCA1 gene 1 (NBR1) (Clausen et al., 2010; Isakson et al., 2013), and is important for selective clearance of protein aggregates (Filimonenko et al., 2010; Lystad et al., 2014), midbody remnants (Isakson et al., 2013), and viral particles (Mandell et al., 2014).

Turnover of mitochondria through autophagy (mitophagy) is important for cellular homeostasis, particularly in post-mitotic and slow dividing cells, such as neurons and cardiomyocytes. Causative mutations in two proteins involved in mitophagy, the E3 ubiquitin ligase PARKIN and PTEN-induced putative kinase 1 (PINK1), are linked to Parkinson's disease (PD) (Pickrell and Youle, 2015). PINK1 is stabilized on the outer mitochondrial membrane (OMM) after loss of mitochondrial membrane potential, where it phosphorylates ubiquitin (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014) and PARKIN (Kondapalli et al., 2012), leading to PARKIN activation and further K63-linked poly-ubiquitination of mitochondrial substrate(s). This is followed by the recruitment of autophagy receptors, including optineurin (OPTN) and nuclear dot protein 52 (NDP52) (also called CALCOCO2) (Lazarou et al., 2015). While p62 and NBR1 seem dispensable for PARKIN-dependent mitophagy in HeLa cells (Lazarou et al., 2015), p62 is essential for PARKIN-dependent mitophagy in macrophages treated with inflammasome NLRP3 agonists (Zhong et al., 2016), suggesting cell- or context-specific variations in employment of autophagy receptors in mitophagy. Little is known



(legend on next page)

about the mitochondrial signals that trigger recruitment of autophagy receptors and mitophagy. Recently, the inner mitochondrial membrane protein, prohibitin 2 (PHB2) was found to bind LC3 upon OMM rupture and function as a receptor for PARKINdependent mitophagy (Wei et al., 2017). In this study, we identify the mitochondrial matrix proteins NIPSNAP1 and NIPSNAP2 as "eat me" signals for damaged mitochondria via their recruitment of autophagy receptors and show that NIPSNAP1 and NIPSNAP2 have redundant roles in PARKIN-dependent mitophagy and a neuroprotective function *in vivo*.

RESULTS

NIPSNAP1 and NIPSNAP2 Interact with hATG8 Proteins, ALFY, and Autophagy Receptors

To identify new ALFY and/or p62 interactors, cell lysates of ALFY^{+/+} and ALFY^{-/-} mouse embryonic fibroblasts (MEFs) (Dragich et al., 2016) or HEK293 cells stably expressing Enhanced Green Fluorescent Protein (EGFP)-p62 were immunoprecipitated with anti-ALFY antibody or GFP-TRAP, respectively, followed by mass spectrometry analysis of precipitates. The homologous proteins NIPSNAP1 and NIPSNAP2 (also called GBAS) were identified as unique interactors of both ALFY and p62 (Figures 1A and 1B).

In addition to NIPSNAP1 and NIPSNAP2, the highly conserved NIPSNAP-domain protein family contains NIPSNAP3A and NIPSNAP3B (Figure S1A). These proteins contain a putative mitochondrial targeting signal (MTS) in the N terminus, followed by two dimeric alpha-beta-barrel (DABB) domains, the second also referred to as a NIPSNAP domain (Figure S1B). The expression of NIPSNAP1 and NIPSNAP2 in mice was limited to a few organs rich in mitochondria and only partially overlapping (Figure 1C). NIPSNAP1 was almost exclusively expressed in brain, kidney, and liver, while NIPSNAP2 was most expressed in heart but also expressed in brain, kidney, liver, muscle, and brown adipose tissue. This is in line with human mRNA levels of *NIPSNAP1* and *NIPSNAP2* (gtexportal.org), suggesting these proteins may have similar functions in different tissues.

ALFY and p62 are known to interact (Clausen et al., 2010), but their interactions with NIPSNAP1 were independent, as endogenous ALFY and p62 both interacted with NIPSNAP1-EGFP in U2OS cells depleted of either transcript (Figure 1D) or in the respective knockout (KO) MEFs (Figure S1C). Several autophagy receptors were found to co-purify with NIPSNAP1and NIPSNAP2-MYC stably expressed in HeLa cells, including NBR1, NDP52, and Tax1-binding protein 1 (TAX1BP1) (Figure S1D). Direct interactions between p62, NBR1, NDP52, and TAX1BP1 with NIPSNAP1 or NIPSNAP2 were confirmed by glutathione S-transferase (GST) pulldown assays of *in-vitro* translated proteins (Figures 1E and S1E).

NIPSNAP1 and NIPSNAP2 were previously identified as binding partners of LC3 and GABARAP proteins (Behrends et al., 2010; Rigbolt et al., 2014). Hence, both endogenous NIPSNAP1 and *in-vitro* translated NIPSNAP1 and NIPSNAP2 interacted with all human LC3 and GABARAP proteins overexpressed as EGFPtagged proteins in HeLa cells (Figure S1F) or expressed as recombinant GST-tagged proteins (Figure 1F). Taken together, we have identified NIPSNAP1 and NIPSNAP2 as binding partners of proteins involved in selective autophagy, including autophagy receptors p62, NBR1, NDP52 and TAX1BP1, ALFY, and human ATG8 proteins.

NIPSNAP1 and NIPSNAP2 Are Mitochondrial Proteins

In line with previous identification of NIPSNAP1 as a mitochondrial protein in rat liver (Nautiyal et al., 2010), human NIPSNAP1and NIPSNAP2-EGFP co-localized extensively with mitochondrial markers in U2OS and HeLa cells (Figures S2A and S2B). NIPSNAP1 co-purified with mitochondrial matrix protein pyruvate dehydrogenase (PDH) and translocase of outer mitochondrial membrane 20 (TOMM20) in the mitochondrial fraction (Figure 2A). NIPSNAP1 and NIPSNAP2 were protected upon proteinase K (PK) treatment of isolated mitochondria, even upon osmotic shock, similar to PDH but in contrast to TOMM20 and translocase of inner mitochondrial membrane 23 (TIMM23) (Figures 2B and S2C). Similarly, PK treatment in the presence of increasing amounts of digitonin to perforate mitochondrial membranes, showed partial protection of NIPSNAP1 and NIPSNAP2 along with mitochondrial matrix protein superoxide dismutase 2 (SOD2), while TOMM20 and TIMM23 were degraded at the lowest concentration of digitonin (Figure S2D). The protected NIPSNAP1 and NIPSNAP2 bands migrated faster upon SDS-PAGE than full-length proteins, suggesting an exposed part of the protein is efficiently cleaved off by PK. Mitochondrial NIPSNAP1 remained extractable by alkaline sodium carbonate (Na₂CO₃), indicating that it is not membrane integrated (Figure 2C). We conclude that NIPSNAP1 and NIPSNAP2 are mitochondrial matrix proteins.

Figure 1. NIPSNAP1 and NIPSNAP2 Bind to Autophagy-Related Proteins

⁽A and B) Schematic representation of co-immunoprecipitation experiments. Endogenous ALFY was immunoprecipitated from wild-type (WT) or ALFY^{-/-} MEF lysates (A). Stably transfected EGFP or EGFP-tagged p62 were immunoprecipitated from HEK293 cells (B), followed by mass spectrometry identification of interacting proteins. Only proteins showing specific interaction with ALFY or p62 are shown.

⁽C) Immunoblotting for NIPSNAP1 and NIPSNAP2 in different mouse tissues. BAT, brown adipose tissue; eWAT and sWAT, epididymal and visceral white adipose tissue.

⁽D) ALFY and p62 both co-immunoprecipitate with NIPSNAP1 independent of each other. EGFP or NIPSNAP1-EGFP was pulled down from U2OS cells transiently transfected with the indicated siRNA and plasmids. Protein levels in cell lysates (input) and immunoprecipitates were visualized by immunoblotting.

⁽E) NIPSNAP1 and NIPSNAP2 interact with selective autophagy receptors. MYC-tagged autophagy receptors were *in vitro* translated in the presence of [³⁵S]methionine and binding to GST-tagged NIPSNAP1 and NIPSNAP2 immobilized on glutathione-sepharose beads analyzed. Bound proteins were detected by autoradiography (AR) and GST proteins by Coomassie brilliant blue (CBB).

⁽F) NIPSNAP1 and NIPSNAP2 interact with all human ATG8 proteins. GST-tagged hATG8 proteins immobilized on glutathione-sepharose beads were incubated with *in-vitro* translated full-length MYC-NIPSNAP1 and NIPSNAP2. Bound proteins were detected by autoradiography (AR) and GST-tagged proteins by CBB staining. Densitometry was done using Science Lab. Image gauge (Fujifilm) from three independent experiments. Values are mean \pm SD ***p < 0.001, **p < 0.005 *p < 0.01; one-way ANOVA. See also Figure S1.



Figure 2. NIPSNAP1 and NIPSNAP2 Are Mitochondrial Matrix Proteins

(A) NIPSNAP1 and NIPSNAP2 co-purify with PDH and TOMM20 in the mitochondrial fraction. Subcellular fractions isolated using QProteome mitochondria isolation kit (Quiagen) from HeLa cells were subjected to SDS-PAGE and immunoblotting.

NIPSNAP1 and NIPSNAP2 Have Two MTSs

Overexpression in HeLa cells (Figure 2D) and an in-vitro mitochondrial import assay (Figure 2E), revealed that NIPSNAP1 is efficiently imported into mitochondria. Fusion of the N-terminal 20 or 19 amino acids (aas) of NIPSNAP1 or NIPSNAP2 to EGFP effectively targeted EGFP inside mitochondria, while deletion of the N-terminal 58 aas abolished mitochondrial localization (Figure 2D). Hence, the N-terminal parts of NIPSNAP1 and NIPSNAP2 are both sufficient and essential for intramitochondrial localization. Interestingly, while NIPSNAP1 and NIPSNAP2 lacking the first 23 or 21 aas, respectively, were not imported into mitochondria, they were recruited to the mitochondrial surface (Figures 2F and S2F), and remained sensitive to PK (Figure 2E). NIPSNAP1 (aa 24-64)-EGFP localized to the mitochondrial surface (Figure 2F), indicating that this region contains an internal MTS. In line with this, when full-length NIPSNAP1mCherry was co-expressed with NIPSNAP1(24-284)-EGFP, the two proteins showed distinct mitochondrial localization, showing that accumulation on the mitochondrial surface was not an overexpression artefact (Figures 2G and S2E). Thus, the N termini of NIPSNAP1 and NIPSNAP2 contain one signal for import into mitochondria and one for association with the OMM. In contrast, NIPSNAP3A and NIPSNAP3B contain an MTS but no signal for tethering to mitochondria (Figure S2F).

NIPSNAP1 Localizes to the Mitochondrial Surface upon Membrane Depolarization

As NIPSNAP1 and NIPSNAP2 bind autophagy proteins and contain signals for mitochondria surface localization, we assumed they might localize on the surface of mitochondria upon induction of mitophagy. Consistent with such a model, NIPSNAP1-EGFP accumulated on the OMM upon disruption of the mitochondrial membrane potential with Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Figure S3A), which induced mitophagy. As EGFP can be cleaved off from NIPSNAP1 (Figure S3B), making it difficult to distinguish surface-bound from matrix-localized NIPSNAP1 and free EGFP, we employed several imaging and biochemical approaches to investigate if surface localized NIPSNAP1 and NIPSNAP2 represent protein re-exported from the mitochondrial matrix and/or newly synthesized protein not yet imported. As expected, NIPSNAP1-3XFLAG expressed in U2OS cells co-localized extensively with Mitotracker Red and TIMM23 (Figures 3A and S3C) and was found to surrounded these mitochondrial markers in approx. 15% of untreated cells (Figures 3A and S3C, lower panel) and approx. 30% of CCCP-treated cells (Figure 3A). As an alternative approach, cells were transfected with NIPSNAP1-EGFP-CIB1 fusion protein and cytosolic CRY2low-tdTomato (Figure 3B), which, upon exposure to blue light, causes formation of transient (minutes) complexes between CIB1 and CRY2low (Duan et al., 2017). tdTomato labeling of NIPSNAP1 at mitochondria was seen, indicating a fraction of NIPSNAP1-EGFP-CIB1 is bound to the surface of mitochondria (Figure 3B). We further exploited the ability of globular proteins to block mitochondrial import, using HeLa cells stably expressing SUMOstar-NIPSNAP1-EGFP and a tet-regulated SUMOstar protease, allowing inducible cleavage of the SUMOstar tag from the fused protein by the SUMOstar protease (Liu et al., 2008) (Figure 3C). As expected, while these cells showed diffuse cytosolic EGFP-staining without tet-induction, mitochondrial EGFP staining and cleavage was observed upon induction of SUMOstar protease (Figures S3D and S3E). When cells were co-treated with tet and CCCP, NIPSNAP1-EGFP accumulated on the mitochondrial surface (Figure 3C).

Using anti-HA magnetic beads for rapid immunopurification from cells expressing 3xHA-tagged proteins (Chen et al., 2016), increased amounts of mitochondria were purified from cells expressing 3xHA-tagged NIPSNAP1 than from cells expressing 3xFLAG-tagged NIPSNAP1 or 3xHA-tagged PDH (Figure 3D). Moreover, more mitochondria were immunopurified from NIPSNAP1-EGFP-3xHA cells treated with CCCP (Figure 3D), confirming that the fraction of NIPSNAP1 on the mitochondrial surface increases upon disruption of the membrane potential.

Consistent with this, alkaline Na₂CO₃ extraction of isolated mitochondria showed that NIPSNAP1 was extracted into the supernatant in untreated cells, but was partly retained in the pellet in cells treated with a combination of Oligomycin and Antimycin A (OA) or CCCP (Figure 3E). NIPSNAP1 and NIPS-NAP2 found in the pellet upon Na₂CO₃ extraction of OA treated cells were highly sensitive to PK treatment, demonstrating their OMM association upon loss of membrane potential, in contrast to the inner mitochondrial membrane protein cvtochrome c oxidase subunit II (COXII) (Figure 3F). While in vitro-translated NIPSNAP1 was partially protected from PK when added to isolated untreated mitochondria, it was not protected from PK when added to mitochondria treated with Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), although there was no difference in NIPSNAP1 binding to isolated mitochondria (Figure 3G). Thus, a functional membrane potential is important for mitochondrial import of NIPSNAP1, but not for its binding to mitochondria.

⁽B) NIPSNAP1 is primarily an intra-mitochondrial protein. HeLa cell mitochondria isolated by differential centrifugation were digested with proteinase K (PK) in presence or absence of Triton X-100 and given osmotic shock followed by immunoblotting.

⁽C) Mitochondrial fractions from HeLa cells were incubated in mitochondrial-buffer alone or mitochondrial-buffer containing Na_2CO_3 (pH 11.5) and centrifuged at 16,000 x g for 15 min. The pellets (P) and supernatant (S) fractions were immunoblotted.

⁽D) N-terminal of NIPSNAP1 and NIPSNAP2 is necessary and sufficient for mitochondrial localization. HeLa cells were transfected with full-length, EGFP-tagged NIPSNAP1 and NIPSNAP2 or indicated deletion constructs. The NIPSNAP1 and NIPSNAP2 regions expressed are indicated in green (schematic figures below the images).

⁽E) The N-terminal 23 aas of NIPSNAP1 facilitate its mitochondrial import. *In vitro* translated ³⁵S-methionine-labeled full-length or deletion mutant (aa 24–284) of NIPSNAP1 were incubated with isolated mitochondria in mitochondrial import assay buffer for 45 min at 37°C, washed three times, treated with PK, and subjected to SDS-PAGE and autoradiography.

⁽F) NIPSNAP1 aa 24–64 function as a mitochondrial affinity signal. HeLa cells were transiently transfected with NIPSNAP1-EGFP deletion mutants (aa 24–284 or 24–64) for 24 h before confocal imaging.

⁽G) Full-length and NIPSNAP1 (24–284) localize to different mitochondrial compartments. HeLa cells transfected with indicated constructs imaged 24 h after. All scale bars are 10 µm. Region of insets are indicated. Results are representative of three independent experiments. See also Figure S2.



Figure 3. NIPSNAP1 Is Enriched on the OMM upon Depolarization

(A) NIPSNAP1-3XFLAG is present both inside (left image) and outside (right image) mitochondria. Representative images of the two phenotypes of subcellular distribution of transiently transfected NIPSNAP1-3xFLAG in U2OS cells and the frequency of both phenotypes, quantified in untreated and CCCP-treated cells. Values are mean \pm SD, *p < 0.05 (unpaired Student's t test).

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NIPSNAP1 and NIPSNAP2 Act as "Eat Me" Signals for Mitophagy

As NIPSNAP1 and NIPSNAP2 localize to the mitochondrial surface upon membrane depolarization, we speculated that they could function as an "eat me" signal for recognition of depolarized mitochondria by autophagy receptors. Indeed, interaction of NIPSNAP1 and NIPSNAP2 with p62 and NDP52 increased in CCCP-treated or hypoxic cells compared to untreated cells, as shown by immunoprecipitation (Figures 4A-4E and S3F) and proximity labeling (Figure S3G). Using a split-YFP bimolecular fluorescence complementation assay (Nyfeler et al., 2008), we show that a NIPSNAP1-GABARAP complex accumulates on the mitochondria surface upon CCCP treatment, with little or no mitochondrial YFP signal in untreated or control cells (Figures 4F and S3H). LC3B and ALFY were also recruited to mitochondria in a CCCP-dependent manner and detected on NIPSNAP1-positive structures (Figure 4G). Together, our data show that mitochondrial depolarization tethers NIPSNAP1 and NIPSNAP2 to the mitochondrial surface, where they recruit proteins involved in selective autophagy. Hence, NIPSNAP1 and NIPSNAP2 may act as "eat me" signals for mitophagy.

NIPSNAP1 and NIPSNAP2 Have Redundant Functions in Mitophagy

To investigate a possible role of NIPSNAP1 and NIPSNAP2 in mitophagy, HeLa cells stably expressing PARKIN were depleted of NIPSNAP1 and/or NIPSNAP2 using siRNA, followed by treatment with CCCP or OA for 12 h or 24 h. Depletion of ATG7 was used as a control (Figure S4A). Depletion of NIPSNAP1, either alone or together with NIPSNAP2, inhibited both CCCP- and OA-induced mitophagy, as analyzed by immunoblotting for COXII and TIMM23 (Figures S4B, S4C, and S4F-S4H) and immunostaining for TIMM23 (Figures S4D and S4E), while depletion of NIPSNAP2 alone had no effect. Using two independent siRNAs against NIPSNAP1, we noticed that its depletion also reduced NIPSNAP2 levels (Figure S4I). To further determine their individual contribution to PARKIN-dependent mitophagy, HeLa cells stably expressing mCherry-PARKIN with KO of NIPSNAP1 and/ or NIPSNAP2 were generated, with ATG7 KO as a control (Figures 5A and S4J). As expected, single NIPSNAP1 or NIPSNAP2 KO had no effect on OA-induced PARKIN-dependent mitophagy, as measured by COXII immunoblotting in two different clones of each, although mitophagy was strongly inhibited in ATG7 KO cells (Figures S4J-S4L). Similar to the NIPSNAP1 siRNA acting on both NIPSNAP1 and NIPSNAP2, double KO (DKO) of NIPSNAP1 and NIPSNAP2 (N1/N2 DKO) blocked both CCCPand OA-induced mitophagy, as evident by immunoblotting of COXII (Figures 5B, 5C, and S5A) and immunostaining of mitochondrial DNA nucleoids (Figures 5D and 5E). Moreover, using cells expressing the mCherry-EGFP-OMP25TM tandem tag mitophagy reporter, red only dots (representing mitochondria in lysosomes due to pH-sensitive quenching of GFP) were detected in wild-type (WT) cells, but not in N1/N2 DKO or ATG7 KO cells upon OA or CCCP treatment (Figures 5F and S5B). Importantly, re-expression of either NIPSNAP1 or NIPSNAP2 in DKO cells revealed that they are functionally redundant, as both were able to rescue CCCP- or OA-induced mitophagy (Figures 5G-5J and S5C). Over-expression of either NIPSNAP1 or NIPSNAP2 in HeLa PARKIN cells did not increase CCCP or OA-induced mitophagy (Figures S6A and S6B). Together, our data show that NIPSNAP1 and NIPSNAP2 are required for PARKIN-dependent mitophagy and have a redundant function.

Depletion of NIPSNAP1 and/or NIPSNAP2 did not inhibit PARKIN-independent mitophagy induced by the iron-chelator deferiprone (DFP) in U2OS cells expressing another tandem-tag mitophagy reporter (NIPSNAP (1-53)-GFP-mCherry), although siRNA-mediated depletion of ULK1 or addition of the lysosomal proton pump inhibitor Bafilomycin A1 (BafA1) efficiently blocked DFP-induced mitophagy (Figures S5D-S5F). Depletion of NIPSNAP1 or NIPSNAP2 had no effect on oxygen consumption rate of the mitochondria (Figure S5G) or on basal autophagy as measured by degradation of long-lived proteins upon starvation (Figure S5H) or mitochondria membrane depolarization (Figure S5I). Similarly, KO of both NIPSNAP1 and NIPSNAP2 did not affect degradation of p62 and NDP52 under basal or starved conditions (Figures S6C and S6D). This suggests a specific role for NIPSNAP1 and NIPSNAP2 in PARKIN-dependent mitophagy, supported by our finding of an interaction between NIPSNAP1 and PARKIN in U2OS cells (Figure S5J).

NIPSNAP1 and NIPSNAP2 Recruit Autophagy Receptors to Mediate Mitophagy

To determine if localization of NIPSNAP1 and NIPSNAP2 on the surface of mitochondria is responsible for their function in

(E) The mitochondrial fraction from HeLa mCherry-Parkin cells treated or not with OA or CCCP for 3 h, was incubated in mitochondrial-buffer alone or mitochondrial-buffer containing Na_2CO_3 (pH 11.5) and centrifuged at 16,000 × g for 15 min. The pellets (P) and supernatant (S) fractions were immunoblotted.

(F) Mitochondria isolated from HeLa mCherry-Parkin cells treated with OA for three hours were subjected to sodium carbonate (Na₂CO₃, pH 11.5) extraction in presence or absence of 15 µg/mL proteinase K. Indicated mitochondrial proteins were analyzed by immunoblotting.

(G) NIPSNAP1 mitochondrial import, but not mitochondrial binding, is dependent on intact mitochondrial membrane potential. *In-vitro* translated ³⁵S-methioninelabeled NIPSNAP1 was incubated with untreated or FCCP-treated mitochondria from HeLa or U2OS cells in mitochondrial import buffer for 45 min at 37°C, washed three times, treated with PK, and subjected to SDS-PAGE and autoradiography. See also Figure S3.

⁽B) NIPSNAP1-EGFP-CIB1 can localize to surface of mitochondria. HeLa cells transiently transfected with NIPSNAP1-EGFP-CIB1 and CRY2low-tdTomato. 24 h after transfection CRY2 was activated by 5 sec pulse of blue light (475 nm, 20 mW/cm²) and imaged on Zeiss AxioObserver Z1 fluorescent microscope. (C) HeLa cells stably transfected with SUMOstar-NIPSNAP1-EGFP under control of a constitutive CMV promoter and SUMOstar protease under control of tet-on

regulated CMV promoter were treated for 5 h with 1 μ g/ml tetracycline followed by 5 h treatment with 10 μ M CCCP and 1 μ g/mL tetracycline, followed by 15 min incubation with 50 nM Mitotracker Red. Scale bars in (A)–(C) are 10 μ m.

⁽D) NIPSNAP1 as an affinity tag for purification of mitochondria. HeLa cells stably transfected with NIPSNAP1-EGFP-3xHA, NIPSNAP1-EGFP-3xFLAG or another mitochondrial matrix protein, PDH-EGFP-3xHA, were treated with 20 μ M of CCCP for indicated periods of time or left untreated (NT), then lysed in KPBS under non-detergent conditions and subjected to immunoprecipitation with magnetic beads, conjugated to anti-HA antibody for 5 min. Immunoprecipitates and input lysates were immunoblotted with indicated antibodies.



Figure 4. Increased Interaction of NIPSNAP1 with LC3B, p62, NDP52, and ALFY after Induction of Mitochondrial Depolarization (A) HeLa PARKIN cells transiently transfected with 3xFLAG or 3xFLAG-p62 were treated with 10 μM CCCP or exposed to hypoxia (1% O₂) for 6 h. Cells were lysed and immunoprecipitated with FLAG resin. Protein levels in cell lysates (input) and immunoprecipitates were detected by immunoblotting.

mitophagy, NIPSNAP2 Δ 1-24, which binds to the mitochondrial surface but is not imported, was stably expressed in N1/N2 DKO cells. Indeed, both OA- and CCCP-induced PARKIN-dependent mitophagy was rescued comparably to the full-length protein (Figures 6A, 6B, and S6E), suggesting an important function of NIPSNAPs on the mitochondria surface in mitophagy.

After treatment with OA or CCCP, no mtDNA nucleoid aggregation or clustering occurred in N1/N2 DKO cells compared to WT or ATG7 KO cells (Figures 5D, 5F, and 5l), a role attributed to p62 (Okatsu et al., 2010). Thus, NIPSNAP1 and NIPSNAP2 may be required for recruitment of autophagy receptors to mitochondria following depolarization. To test this, WT and N1/N2 DKO HeLa mCherry-PARKIN cells were treated with CCCP in absence or presence of the proteasome inhibitor MG132, followed by fractionation of mitochondria and immunoblotting. While PARKIN recruitment and ubiquitination of mitochondria were similar in WT and N1/N2 DKO cells (Figure 6C), recruitment of NDP52, p62, OPTN, and TAX1BP1 to mitochondria in N1/N2 DKO cells was dramatically reduced compared to WT cells (Figure 6C), indicating that NIPSNAP1 and NIPSNAP2 are required for recruitment of autophagy receptors during mitophagy.

To test if PINK1 and/or PARKIN contribute to accumulation of NIPSNAP1 and NIPSNAP2 on the OMM upon depolarization, we first examined if NIPSNAP1 and NIPSNAP2 are phosphorylated upon depolarization. Using Phos-tag SDS-PAGE, we observed similar phosphorylation levels of NIPSNAP1 and NIPSNAP2 with and without CCCP treatment (Figure S6F). Consistently, no phosphorylation of NIPSNAP1 or NIPSNAP2 by PINK1 was detected, whereas PARKIN and ubiquitin were both phosphorylated by PINK1 *in vitro* (Figure S6G). Secondly, while Mitofusin-2 (MFN2) was ubiquitinated by PARKIN upon CCCP treatment, in agreement with Sarraf et al. (2013), no ubiquitination of endogenous or over-expressed NIPSNAP1 and NIPSNAP2 was observed (Figures S6H and S6I).

We found both p62 and NDP52 to bind the region encompassing aas 65–100 of NIPSNAP1 and NIPSNAP2 (Figures S6J and S6K). The C-terminal zinc-finger domain (aas 343–446) of NDP52, required for binding to ubiquitin, myosin VI, and galectin 8 (Thurston et al., 2012; Tumbarello et al., 2012) was sufficient for binding to both NIPSNAPs (Figure S6L). Of the two NDP52 zinc finger domains (ZF1 and ZF2), only the most C terminal domain is required for ubiquitin binding (Xie et al., 2015). We therefore asked whether interaction of NIPSNAP1 and NIPSNAP2 with NDP52 was ubiquitin dependent. Although binding of NDP52 to both NIPSNAP1, NIPSNAP2, and ubiquitin required the ZF2 domain, several mutations that abolished binding to ubiquitin did not affect binding to NIPSNAP1 or NIPSNAP2 (Figures 6D and 6E), suggesting that binding of NDP52 to NIPSNAP1 or NIPSNAP2 and ubiquitin can occur simultaneously. To examine how NDP52 is recruited to mitochondria, NDP52 KO cells were transfected with mCherry-PARKIN together with NDP52 WT, a ZF2 point mutant (L446A) that cannot bind ubiquitin but interacts with NIPSNAP1 and NIPSNAP2, or a deletion mutant (1-414) lacking the ZF2 domain and neither interacts with NIPSNAP1 and NIPSNAP2 nor ubiquitin. Both WT NDP52 and the L446A mutant were recruited to mitochondria, while NDP52 1-414 remained cytosolic after treatment with CCCP for 6 h (Figures 6F and S6M). The NDP52 L446A mutant was mostly recruited to fragmented mitochondria and not to perinuclear mitochondrial clusters. These results suggest that initial recruitment of NDP52 to damaged mitochondria is mediated by ubiquitination of OMM proteins. However, subsequent and sustained mitophagy-dependent recruitment of NDP52 is dependent on NIPSNAP1 and/or NIPSNAP2 (Figure 6C).

NIPSNAPs Are Evolutionary Conserved and Expressed during Zebrafish Embryogenesis

To elucidate a function for NIPSNAPs *in vivo* we used zebrafish as a model organism. Zebrafish Nipsnap1 and Nipsnap2 display high aa identity (\geq 75%) to the human and mouse proteins, indicating evolutionary conservation (Figures S7A and S7B). The temporal expression pattern of *nipsnap1* and *nipsnap2* during zebrafish embryogenesis, determined by quantitative PCR (qPCR), showed maternal expression from the 2 cell stage to 2 h post fertilization (hpf), which, for *nipsnap1*, gradually increased throughout gastrulation, peaking at 9 hpf, followed by a decrease down to 3 days post fertilization (dpf) and thereafter remained low. In contrast, *nipsnap2* expression was lower than *nipsnap1* during gastrulation and increased from 3 dpf (Figure S7C).

Whole-mount mRNA in situ hybridization (WISH) revealed nipsnap1 as ubiquitously expressed from early stages of embryogenesis, present in the endoderm by the 6-somites stage (11.5 hpf) with even stronger staining at 1 dpf (Figure 7A). nipsnap1 mRNA was detected in endoderm-derived organs such as liver, intestine, and pectoral fins and was predominantly expressed in the head from 1 to 4 dpf. Consistent with qPCR results, expression of nipsnap1 decreased considerably at 5 dpf. nipsnap2 was also expressed in the brain during development but was contrary to nipsnap1 expressed in the myotome at 1 dpf (Figure S7D). Western blot of different zebrafish adult tissues showed Nipsnap1 to be the predominant form expressed in brain, heart, muscle, liver, intestine, testis, and ovary and in low amounts in kidney, whereas Nipsnap2 was found in brain, heart, and testis and highly expressed in the ovary (Figure S7E). As these proteins have a redundant function in mitophagy, we focused our further investigation on Nipsnap1.

See also Figure S3.

⁽B) Densitometry of results shown in (A) from 3 independent experiments. Values are mean \pm SD ***p < 0.001; one-way ANOVA.

⁽C) HeLa cells stably expressing EGFP or NIPSNAP-EGFP were treated as in (A) followed by GFP-trap immunoprecipitation. Co-immunoprecipitation of p62 and NDP52 was detected by immunoblotting.

⁽D-E) Quantification of results shown in (C) based on 3 different experiments. Values are mean \pm SD ***p < 0.001, **p < 0.01; one-way ANOVA.

⁽F) U2OS cells were transiently transfected with NIPSNAP1 and GABARAP fused to split-YFP1 or split-YFP2, respectively, then treated 24 h after transfection with 10 μ M CCCP for 4 h, stained with 50 nM of Mitotracker Red for 20 min, and subjected to confocal microscopy. Scale bars are 10 μ m.

⁽G) U2OS cells were transiently transfected with NIPSNAP1-3xFLAG, treated or not with 10 µM CCCP overnight, stained with anti-FLAG together with anti-LC3B or anti-ALFY antibodies, and subjected to confocal imaging. Scale bars are 10 µm.



Figure 5. NIPSNAP1 and NIPSNAP2 Are Required for Mitophagy

(A) CRISPR-Cas9-mediated double knockout of NIPSNAP1 and NIPSNAP2 (N1/N2 DKO) or ATG7 in HeLa cells were confirmed by immunoblotting. The effect of ATG7 KO on autophagy was confirmed by immunoblotting against LC3B and GABARAP.

Mitophagy Is Reduced in Nipsnap1-Deficient Zebrafish Larvae

CRISPR-mediated genome editing of *nipsnap1* in zebrafish embryos achieved near complete depletion of Nipsnap1 protein (Figures 7G and S7F). Nipsnap1 KO larvae (*nipsnap1^{-/-}*) did not survive beyond day five and we also used a zebrafish line with the heterozygous *nipsnap1^{sa14357}* mutant allele (Kettleborough et al., 2013), having a single T > A base pair change in exon 6 (Figure S7F) resulting in a premature stop codon. Lysates from Nipsnap^{sa14357} embryos (*nipsnap* mutant) at 72 hpf had a significant reduction of the Nipsnap1 protein compared to WT, with no smaller molecular weight bands appearing, suggesting that the shorter transcripts undergo nonsense-mediated decay (Figure 7G).

To investigate if Nipsnap1-deficient larvae display reduced mitophagy, we generated stable transgenic zebrafish lines expressing a tandem-tagged mitochondria marker (cytochrome c oxidase subunit 8A (Cox8A)-GFP-mCherry) (Figure 7B) in the control (WT) or nipsnap1 mutant background. The ratio of red to yellow puncta was significantly reduced in the head region of Nipsnap1-deficient larvae at 3 dpf (Figures 7B and 7C), although large variation between different cell types could be detected. There was no difference in red dot formation in the muscle of WT and Nipsnap1-deficient larvae (data not shown). The reduced level of mitophagy in the head of nipsnap1 mutants could be partially rescued by injection of zebrafish WT nipsnap1 mRNA into one-cell nipsnap1 mutant embryos (Figures 7B, 7C, and S7G). Thus, Nipsnap1 is required for efficient mitophagy in the head but not in muscles of zebrafish larvae. As Nipsnap2 is abundantly expressed in muscles (Figure S7D), we speculate it could facilitate mitophagy in this tissue.

Nipsnap1-Deficient Zebrafish Larvae Display Parkinsonism

PD is characterized by death of dopaminergic (DA) neurons in the substantia nigra, which can be linked to dysfunctional mitophagy and increased level of reactive oxygen species (ROS) (Pickrell and Youle, 2015). As Nipsnap1 was mainly detected in the brain of zebrafish larvae (Figure 7A) and mouse (Figure 1C), consistent with its reported high expression in DA neurons in the midbrain and noradrenergic neurons in the brainstem of mice (Nautiyal et al., 2010), we asked if Nipsnap1deficient larvae displayed parkinsonian phenotypes. Indeed, a significant increased level of ROS was seen in the nipsnap1 mutant and nipsnap1^{-/-} embryos compared to WT controls (Figures 7D and S7H). Zebrafish have two orthologs of tyrosine hydroxylase (Th1 and Th2), catalyzing conversion of L-tyrosine to L-DOPA, the precursor for dopamine, where the level of Th1 can be used to infer DA neuron health (Holzschuh et al., 2001). Interestingly, both *nipsnap1* mutant and nipsnap1^{-/-} embryos showed a dramatic reduction in th1 staining compared to controls as analyzed by WISH (Figures 7E and 7F) and immunoblotting for Th1 (Figures 7G and S7I). TUNEL staining showed increased cell death in the nipsnap1 mutants relative to WT controls (Figures 7H and 7I). Interestingly, the locomotor activity of nipsnap1 mutant larvae at 7 dpf was dramatically reduced compared to WT larvae, as analyzed by guantification of swimming activity in the light and dark over a time course using the Zebrabox (Figure 7J). The swimming defect of nipsnap1 mutants was rescued with exogenous addition of 5 mM L-DOPA (Figures 7J and S7J), indicating that the locomotion defect of nipsnap1 mutants is due to a reduced number of DA neurons and possibly lower levels of dopamine in the mutants (Figures 7E-7G).

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DISCUSSION

The Mitochondrial Matrix Proteins NIPSNAP1 and NIPSNAP2 Have a Redundant Function as "Eat Me" Signals in PARKIN-Mediated Mitophagy

Much effort has been put into deciphering mechanisms of recognition of damaged mitochondria. Here we identified NIPSNAP1 and NIPSNAP2 as binding partners of p62 and ALFY, both involved in selective autophagy (Rogov et al., 2014; Stolz et al., 2014). NIPSNAP1 and NIPSNAP2 are predominantly mitochondrial matrix proteins. However, they also localize to the OMM upon CCCP- or OA-induced mitochondrial depolarization to recruit autophagy receptors and ATG8 homologs and effectively act as "eat me" signals for PARKIN-dependent mitophagy.

A mitochondrial membrane potential is required for the import of most mitochondrial proteins (Kulawiak et al., 2013; Truscott et al., 2003). The imported proteins generally do not decorate the OMM upon treatment with CCCP. In contrast, cytosolic

⁽B) WT, N1/N2 DKO, and ATG7 KO HeLa cells with or without mCherry-PARKIN expression were treated with OA for 12 or 24 h and extracts immunoblotted as indicated.

⁽C) Densitometry of COXII protein levels relative to WT in (B). Values are mean ± SD ***p < 0.001, one-way ANOVA.

⁽D) Representative images of mCherry-Parkin expressing WT, N1/N2 DKO and ATG7 KO cells treated with OA for 6 or 24 h, immunostained for mtDNA nucleoids (green) and DAPI (blue). Scale bars are 20 μ m.

⁽E) Quantification of mtDNA nucleoid staining shown in (D) from three independent experiments. More than 100 cells were quantified per sample. Values are mean ± SD ***p < 0.001, one-way ANOVA.

⁽F) WT, N1/N2 DKO, and ATG7 KO HeLa cells stably expressing the mCherry-EGFP-OMP25TM mitophagy reporter were left untreated or treated with OA for 6 h and analyzed by confocal microscopy. Scale bars are 10 μ m.

⁽G) mCherry-PARKIN expressing WT, and N1/N2 DKO HeLa cells were rescued or not with untagged NIPSNAP1 or NIPSNAP2, followed by treatment with OA for 12 or 24 h and immunoblotting.

⁽H) Quantification of COXII levels relative to WT from data shown in (F). Values are mean ± SD ***p < 0.001, **p < 0.005, one-way ANOVA.

⁽I) Representative images of mCherry-PARKIN expressing N1/N2 DKO cells rescued or not with NIPSNAP1 or NIPSNAP2 treated with OA for 6 or 24 h, then immunostained for mtDNA nucleoids (green) and DAPI (blue). Scale bars are 20 µm.

⁽J) Quantification of mtDNA staining of data shown in (I). More than 100 cells were quantified per sample. Similar results were obtained in three independent experiments. Values are mean \pm SD **p < 0.005, one-way ANOVA. See also Figures S4 and S5.



Figure 6. NIPSNAP1 and NIPSNAP2 Have Redundant Functions in Mitophagy

(A) WT cells, N1/N2 DKO cells, and N1/N2 DKO cells rescued with untagged NIPSNAP2 or NIPSNAP2 Δ1-24 and stably expressing mCherry-PARKIN were treated with OA for 12 or 24 h and immunoblotted as indicated.

NIPSNAP1 was readily detected on the surface of depolarized mitochondria and on the mitochondrial surface in non-treated cells. The possibility that intra-mitochondrial NIPSNAP1 and NIPSNAP2 become stabilized at the surface cannot be completely excluded.

Mitophagy depends on autophagy receptors, but it is not clear why only some autophagy receptors are crucial for mitophagy in certain cell lines. Only NDP52, TAX1BP1, and OPTN are required for PARKIN-dependent mitophagy in HeLa cells (Lazarou et al., 2015), while p62 is sufficient for PARKINdependent mitophagy in macrophages (Zhong et al., 2016). NDP52, NBR1, OPTN, p62, and TAX1BP1 all have ubiquitinbinding domains (Birgisdottir et al., 2013). p62 and OPTN bind to damaged mitochondria in a PARKIN- and ubiquitin-dependent manner (Okatsu et al., 2010). PARKIN-mediated ubiquitination of OMM proteins can however not fully account for these differences. Recently, the inner mitochondrial membrane protein prohibitin 2 (PHB2) was found to bind LC3 upon mitochondrial depolarization and function as a receptor for PARKINdependent mitophagy (Wei et al., 2017). Here, we show that recruitment of autophagy receptors to depolarized mitochondrial is mediated by NIPSNAP1 and NIPSNAP2, which both interact with NDP52, p62, NBR1, TAX1BP1, and the autophagy adaptor ALFY. ALFY interacts with p62 and facilitates recruitment of the phagophore for selective autophagy by binding to PtdIns3P and GABARAP (Clausen et al., 2010; Filimonenko et al., 2010; Lystad et al., 2014). We find that the NDP52 L446A mutant, which binds NIPSNAP1 and NIPSNAP2 but not ubiquitin, is recruited to mitochondria after 6 h of CCCP treatment. Thus, PARKIN-dependent ubiquitination of OMM proteins leading to their proteasomal degradation upon mitochondrial damage may "prime" mitochondria for lysosomal degradation (Chan et al., 2011; Geisler et al., 2010; Glauser et al., 2011; Shlevkov et al., 2016; Tanaka et al., 2010; Yoshii et al., 2011), while OMM-localized NIPSNAP1 and NIPSNAP2 are required to sustain recruitment of autophagy receptors required for mitophagy. We confirmed the interaction of NIPS-NAP1 and NIPSNAP2 with ATG8 proteins (Behrends et al., 2010) and showed GABARAPs as preferred interacting partners. Thus, efficient targeting of dysfunctional mitochondria for mitophagy involves several layers of specific interactions between mitochondrial proteins and the autophagy machinery.

The function of NIPSNAP1 and NIPSNAP2 in mitophagy is likely specific to PARKIN-dependent mitophagy. NIPSNAP1 was immunoprecipitated by PARKIN, and we did not see a difference between control and NIPSNAP1- and NIPSNAP2-depleted cells when inducing mitophagy by iron depletion, shown to be PARKIN independent (Allen et al., 2013). Depletion of NIPSNAPs also did not affect starvation-induced autophagy.

Ablation of Nipsnap1 in Zebrafish Causes Parkinsonism

PD is characterized by death of DA neurons in the substantia nigra, linked to dysfunctional turnover of mitochondria (Pickrell and Youle, 2015). NIPSNAP1 was most significantly downregulated in a study of genome-wide gene expression data of PD samples compared to controls (Fu and Fu, 2015). Nipsnap1 was also downregulated in a proteomics study of neural SH-SY5Y cells responding to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment, known to lead to degeneration of DA neurons (Choi et al., 2014). Mouse NIPSNAP1 is highly expressed in midbrain DA neurons and brainstem noradrenergic neurons (Nautiyal et al., 2010). Consistently, zebrafish depleted of Nipsnap1 had reduced mitophagy in the brain. Because of their post-mitotic state and metabolic requirements, neurons are particularly vulnerable to mitochondrial dysfunction. Zebrafish lacking Nipsnap1 showed significant loss of Th1-positive DA neurons in the diencephalospinal tract, and the aberrant locomotion phenotype of nipsnap1 mutants was rescued completely by exogenous addition of L-DOPA. Nipsnap1-deficient larvae also displayed increased ROS production compared to WT larvae. Hence, we propose that reduced mitophagy in Nipsnap1-deficient larvae leads to increased ROS, resulting in death of DA neurons and a locomotion defect.

Our results are similar to other studies that have modeled PDrelated genes in zebrafish. *parkin, pink1*, and *Irrk2* knockdown using antisense morpholinos resulted in decreased levels of DA neurons. *pink1* morphants had elevated ROS level and *Irrk2* morphants displayed motor defects (Anichtchik et al., 2008; Flinn et al., 2009; Sheng et al., 2010). A *pink1* TALEN-mediated KO zebrafish line showed 30–40% DA neuronal loss (Zhang et al., 2017). Autophagy was found to protect DA neurons in an MPTP-induced PD model in zebrafish (Hu et al., 2017). *atg5* downregulation caused a pathological locomotor behavior, DA neuron loss, and accumulation of α -Synuclein aggregates, which was reversed by Atg5 overexpression.

We show that zebrafish lacking a functional Nipsnap1 display parkinsonism, including reduced Th1-positive DA neurons and dysfunctional neuronal motor activity rescued by exogenous addition of L-DOPA. Zebrafish Nipsnap1 and Nipsnap2 show 75% aa identity with the corresponding human and mouse orthologs. The expression pattern of *nipsnap1* and *nipsnap2* in zebrafish, as in mouse and humans, is largely tissue specific. It is clearly conceivable that our data in zebrafish are characteristic for the function of mouse and human NIPSNAPs.

⁽B) Densitometry of COXII protein levels relative to WT from data shown in (A). Values are mean ± SD ***p < 0.001, **p < 0.005, one-way ANOVA.

⁽C) Whole cell lysate and mitochondria fraction from WT and N1/N2 DKO cells stably expressing mCherry-PARKIN were treated with CCCP with or without MG132, subjected to immunoblotting and Ponceau S staining of proteins used as loading control.

⁽D) Domain structure of NDP52 indicating binding sites for myosin VI, galectin 8, and ubiquitin. aa indicated in red are point mutations (mutated to alanines) that affect ubiquitin binding to the zinc finger (ZF2) domain. The LIR required for interaction with hATG8 proteins is annotated in green.

⁽E) MYC-tagged NDP52 wild-type and indicated mutants were in vitro translated and used in GST-pulldown assay with GST-tagged NIPSNAP1, NIPSNAP2, ubiquitin, 4x-ubiquitin, or galectin 8.

⁽F) HeLa NDP52 KO cells were co-transfected with mCherry-Parkin and EGFP or EGFP- NDP52 WT or mutants (indicated in D) and treated with CCCP for 6 h. Recruitment of EGFP-tagged proteins to mitochondria was analyzed by staining with an antibody against TIMM23. Regions of insert are indicated. Scale bars are 10 μ m.



Figure 7. Nipsnap1-Deficient Zebrafish Larvae Display Parkinsonism

(A) Spatial expression pattern of *nipsnap1* across different development stages of zebrafish as demonstrated by whole-mount *in situ* hybridizations. All embryos are in lateral view. Scale bar is 200 µm.

STAR***METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 O Cell Culture
 - Mouse and Zebrafish Husbandry
- METHODS DETAILS
 - Antibodies and Reagents
 - Generation of Human Knockout Cell Lines Using CRISPR-Cas9 System
 - Generation of Stable Cell Lines and Reconstitution of KO Cell Lines
 - CRISPR/Cas9 Genome Editing in Zebrafish
 - Genotyping of nipsnap1 Mutants
 - RT-PCR/qPCR Analysis
 - Whole-Mount In Situ Hybridization
 - Tandem-Tag Transgenic Mitofish Generation and Imaging
 - Zebrafish Rescue Experiments
 - Immunoblotting, Immunoprecipitation and Mass Spectrometry
 - Proximity Biotinylation Assay
 - O Phos-tag SDS-PAGE Analysis
 - Live Cell and Confocal Immunofluorescence Microscopy
 - Long-Lived Protein Degradation
 - Recombinant Protein Expression, *In-Vitro* Translation and GST-Pulldown Assay
 - Subcellular Fractionation, Proteinase K/trypsin Treatment and Sodium Carbonate Extraction
 - Mitophagy Assay
 - In Vivo Ubiquitination Assay
 - *In Vitro* Kinase Assay
 - Mitochondrial Import Assay
 - Mitochondrial Oxygen Consumption Rate

- ROS Analysis
- Zebrafish Locomotor Assay
- Visualizing Apoptosis by TUNEL Staining
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j. devcel.2019.03.013.

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AUTHOR CONTRIBUTIONS

Y.P.A., S.P., B.J.M., A.H.L., C.B., T.M.N., and M.Y.W.N. designed and performed the experimental research, analyzed data, drafted the article, and made figures. H.B.B. helped with some experiments, T.L. assisted in analyses of data and experimental design. B.T. and T.M.N. did the MS analysis. A.Y. and C.V.E. provided essential reagents and knowhow, and C.V.E. aided with phenotypic analysis for zebrafish experiments. T.J. and A.S. designed the project, analyzed the data, and wrote the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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WEB RESOURCES

GTEx Portal, gtexportal.org/.

(C) Quantification of ratio of red puncta to yellow puncta (representative of mitophagy) per cell. Average of 15 cells from each of 25 larvae for control (WT), nipsnap1 mutant, and nipsnap1 mutant + rescue; tandem-tagged mitofish larvae from three independent experiments were used for quantification.

(D) Control (WT), nipsnap1^{-/-} and nipsnap1 mutant embryos at 3 dpf incubated with CellROX and ROS levels quantified from six different experiments. Error bars represent ± SEM.

(E) Spatial expression pattern of *tyrosine hydroxylase 1 (th1*) gene in control (WT), nipsnap1^{-/-} and *nipsnap1* mutant embryos at 3 dpf determined by whole-mount *in situ* hybridization. Orientation dorsal. Scale bar is 200 µm.

(F) Quantification of the number of Th1 positive neurons from images in (D). 10 control (WT), 20 mutant and 20 knockout larvae were used for quantification. Values were normalized to control (WT) values.

(G) Representative immunoblots of Nipsnap1, Th1, and β -tubulin on whole embryo lysates of WT, nipsnap1^{-/-} and *nipsnap1* mutant embryos at 3 dpf. β -tubulin served as loading control.

(H) Representative images of TUNEL assay on control (WT), *nipsnap1* mutant and DNase-treated WT larvae (positive control) at 3dpf. Orientation lateral. Scale bar is 200 µm.

(J) Motility analysis of WT and *nipsnap1* mutant embryos at 7 dpf using the "Zebrabox" automated videotracker (Viewpoint, Lyon). Assay was carried out during daytime, one cycle of 20 min exposure to light (white horizontal bar) followed by 20 min of darkness (black horizontal bar). Vertical bars indicate average distance moved during 5 min intervals. Controls and *nipsnap1* mutants were treated or not with 5 mM of L-DOPA for 1 h, after which they were analyzed for motility. Each group consisted of 20–24 larvae.

All error bars indicate SEM. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0005, unpaired Student's t test. See also Figure S7.

⁽B) Representative images of control (WT), *nipsnap1* mutant, and rescue (*nipsnap1* mutant + zebrafish *nipsnap1* mRNA) transgenic tandem-tagged mitofish larvae at 3 dpf. Images are from head region of zebrafish larvae. Scale bars are 20 μ m.

⁽I) Quantification of mean fluorescent intensity from demarcated region from images in (H). 10 control (WT), 15 *nipsnap1* mutant, and 10 positive control larvae were used for quantification, respectively. Values were normalized to control (WT) values.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies	· · · · · · · · · · · · · · · · · · ·	
Rabbit monoclonal anti-NIPSNAP1	Cell Signaling	Cat#D1Y6S
Rabbit polyclonal anti-NIPSNAP1	Abcam	Cat#ab67302; RRID:AB_2151304
Rabbit polyclonal anti-NIPSNAP1	Abcam	Cat#ab133840
Mouse monoclonal anti-NIPSNAP2	LSBio	Cat#LS-B13280
Rabbit polyclonal anti-NIPSNAP2	Abgent	Cat#AP6752c; RRID:AB_10667243
Rabbit polyclonal anti-NIPSNAP2	Abcam	Cat#ab153833
Rabbit polyclonal anti-ALFY	Simonsen et al., 2004	N/A
Rabbit polyclonal anti-GFP	Abcam	Cat#ab290; RRID:AB_303395
Rabbit polyclonal anti-GFP	Santa Cruz	Cat#sc-8334; RRID:AB_641123
Mouse monoclonal anti-GFP	Clontech	Cat#632381; RRID:AB_2313808
Mouse monoclonal anti-MYC tag	Cell Signaling	Cat#2276; RRID:AB_331783
Mouse monoclonal anti-HA tag	Roche	Cat#11583816001; RRID:AB_514505
Mouse monoclonal anti-p62	BD Biosciences	Cat#610833; RRID:AB_398152
Guinea pig polyclonal anti-p62	Progen	Cat#GP62-C; RRID:AB_2687531
Rabbit polyclonal anti-CALCOCO2	Sigma-Aldrich	Cat#HPA023195; RRID:AB_1845916
Mouse monoclonal anti-NBR1	Santa Cruz	Cat#sc-130380; RRID:AB_2149402
Rabbit polyclonal anti-TAX1BP1	Sigma-Aldrich	Cat#HPA024432; RRID:AB_1857783
Rabbit polyclonal anti-Optineurin	Sigma-Aldrich	Cat#HPA003360; RRID:AB_1079528
Mouse monoclonal anti-MTOC2	Abcam	Cat#ab110258; RRID:AB_10887758
Mouse monoclonal anti-DNA	Progen	Cat#61014; RRID:AB_2750935
Rabbit polyclonal anti-SOD-2	Santa Cruz	Cat#sc-30080; RRID:AB_661470
Mouse monoclonal anti-MFN2	Santa Cruz	Cat#sc-100560; RRID:AB_2235195
Mouse monoclonal anti-Ubiquitin	Enzo	Cat#BML-PW8810; RRID:AB_10541840
Rabbit monoclonal anti-ATG7	Cell Signaling	Cat#D12B11; RRID:AB_10831194
Rabbit polyclonal anti-Actin	Sigma-Aldrich	Cat#A2066; RRID:AB_476693
Rabbit polyclonal anti-PDH	Cell Signaling	Cat#2784S
Mouse monoclonal anti-Cytochrome C	Abcam	Cat#ab110325; RRID:AB_10864775
Rabbit polyclonal anti-Parkin	Cell Signaling	Cat#2132; RRID:AB_10693040
Rabbit polyclonal anti-LC3B	Novusbio	Cat#NB100-2220; RRID:AB_10003146
Mouse monoclonal anti-GABARAP	MBL	Cat#M135-3; RRID:AB_10364779
Rabbit polyclonal anti-LC3B	Sigma-Aldrich	Cat#L7543; RRID:AB_796155
Mouse monoclonal anti-TOMM20	Santa Cruz	Cat#sc-17764; RRID:AB_628381
Rabbit polyclonal anti-TOMM20	Santa Cruz	Cat#sc-11415; RRID:AB_2207533
Mouse monoclonal anti-TOMM40	Santa Cruz	Cat#sc-365467; RRID:AB_10847086
Rabbit polyclonal anti-IKKa	Cell Signaling	Cat#2682; RRID:AB_331626
Rabbit polyclonal anti-histone H3	Abcam	Cat#ab1791; RRID:AB_302613
Mouse monoclonal anti-TIMM23	BD Biosciences	Cat#611223; RRID:AB_398755
Mouse monoclonal anti-FLAG tag	Sigma-Aldrich	Cat#F1804; RRID:AB_262044
Rabbit monoclonal anti-FLAG tag	Cell Signaling	Cat#14793
Mouse monoclonal anti-Tyrosine Hydroxylase	ImmunoStar	Cat#22941; RRID:AB_572268
Mouse monoclonal anti-a-Tubulin	Sigma-Aldrich	Cat#T5168; RRID:AB_477579
Bacterial and Virus Strains		
SoluBL21 Competent Escherichia coli	Genlantis	Cat#C700200

Cell²ress

BRAGENCY OF RESOURCESOURCEIDENTIFIERChemicals, Peptides, and Recombinant ProteinsDEI RNA Labeling MixRochaCal#119272073910Anti-Digoxigenin AP Fab fragmentsRochaCal#119272073910Porleinage KCal#119272073910Cal#C10422L-DopaSigma-AdrichCal#S119822001CeilRoxThermoFisherCal#C10422L-DopaSigma-AdrichCal#S4117Torula Yeast RNASigma-AdrichCal#R025Collegenase PSigma-AdrichCal#M0265CaleGenase PSigma-AdrichCal#M0265CaleGraymeSigma-AdrichCal#M0265CaleGraymeSigma-AdrichCal#M0265CaleGraymeSigma-AdrichCal#M0265CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichCal#M0266CaleGraymeSigma-AdrichC	Continued		
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Anti-Digoxigenin AP Fab fragments Roche Cat#1108224001 Proteinase K Cat#3115828001 CollRox ThermoFisher Cat#S115828001 L-Opa Sigma-Aldrich Cat#335786 Formanide Sigma-Aldrich Cat#3827806 Formanide Sigma-Aldrich Cat#B826 Hepain Sodium Salt Sigma-Aldrich Cat#18626 Collagenase P Sigma-Aldrich Cat#104202001 Oligomycin Sigma-Aldrich Cat#04876 Antimycin A Sigma-Aldrich Cat#04876 Carbonyl cyanide <i>m</i> -chlorophenyl hydrazine Sigma-Aldrich Cat#04876 Carbonyl cyanide <i>m</i> -chlorophenyl hydrazine Sigma-Aldrich Cat#04876 Carbonyl cyanide <i>s</i> oblution Sigma-Aldrich Cat#04876 Carbonyl cyanide <i>s</i> oblution Sigma-Aldrich Cat#04876 Carbonyl cyanide <i>m</i> -chlorophenyl hydrazine Sigma-Aldrich Cat#04762 Carbonyl cyanide <i>m</i> -chlorophenyl hydrazine Sigma-Aldrich Cat#0472211 Ponceau Sigma-Aldrich Cat#0472211 Ponceau Sigma-Aldrich Cat#0472211 Ponceau Sigma-Aldrich Cat#042211 Ponceau Sigma-Aldrich Cat#042211 Ponceau Sigma-Aldrich Cat#042211	DIG RNA Labelling Mix	Roche	Cat#11277073910
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Collagenase PSigma-AldrichCat#11249002001OligomycinSigma-AldrichCat#04876Antimycin ASigma-AldrichCat#04876Carbonyl cyanide m-chlorophenyl hydrazoneSigma-AldrichCat#02759Carbonyl cyanide 4.thffucornebnyl hydrazoneSigma-AldrichCat#02290Hanks Balanced Salt SolutionSigma-AldrichCat#18264Bafliomycin A1Sigma-AldrichCat#18264Bafliomycin A1Sigma-AldrichCat#18264Bafliomycin A1Sigma-AldrichCat#1973MG132 (Z-Lou-Leu-Leu-a)Sigma-AldrichCat#19268Vectafectere PoSigma-AldrichCat#19268Nectafectere PoSigma-AldrichCat#1020N-ethylmaleimideSigma-AldrichCat#211Cornassie Brillant Blue R-250 DyeThernoFisherCat#20278Phos-Tag AcrylamideWakoCat#101974-088Biotin TyramideIIS BiotechCat#104974-088Biotin TyramideIIS BiotechCat#104974-088Biotin TyramideNethonCat#Al1340MCat#Al14610SigenCat#Al1401QProteome mitochondria isolation kitOigenCat#Al1414QProteome mitochondria isolation kitGigenCat#Al14140MLiAPA SYBRE FAST QPCR KitsKAPA BiosystemsCat#Al14340MCat#Al14340MCat#Al14340MCat#Al14340MLiAPAS SyBRE FAST QPCR KitsAplientCat#Al14340MLiAMACS GPI Isolation KitAglientCat#Al1430MLiAMACS GPI Isolation KitAplientCat#Al1430M <tr< td=""><td>Heparin Sodium Salt</td><td>Sigma-Aldrich</td><td>Cat#H4784</td></tr<>	Heparin Sodium Salt	Sigma-Aldrich	Cat#H4784
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Seahorse XF Cell Mito Stress Test KitAgilentCat#103015-100Genelute mammalian genomic DNA miniprep kitSigma-AldrichCat#G1N350Hank's Balanced Salt Solution (HBSS)ThermoFisherCat#14025-092Poly(A) Tailing KitThermoFisherCat#AM1350Click-iT Plus TUNEL assayThermoFisherCat#C10619DNase I, RNase-freeThermoFisherCat#EN0521Experimental Models: Cell LinesATCCCCL-2HELaATCCCRL-1573HeLa NIPSNAP1 ^{KO} This paperN/AHeLa NIPSNAP2 ^{KO} This paperN/AHeLa N1/N2 ^{DKO} This paperN/AHeLa NDF52 ^{KO} This paperN/A	μMACS GFP Isolation Kit	Miltenyi Biotec	Cat#130-091-125
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HeLa NIPSNAP1 ^{KO} This paper N/A HeLa NIPSNAP2 ^{KO} This paper N/A HeLa N1/N2 ^{DKO} This paper N/A HeLa NDP52 ^{KO} This paper N/A	HEK-293	ATCC	CRL-1573
HeLa NIPSNAP2 ^{KO} This paper N/A HeLa N1/N2 ^{DKO} This paper N/A HeLa NDP52 ^{KO} This paper N/A	HeLa NIPSNAP1 ^{KO}	This paper	N/A
HeLa N1/N2 ^{DKO} This paper N/A HeLa NDP52 ^{KO} This paper N/A	Hel a NIPSNAP2 ^{KO}	This paper	N/A
HeLa NDP52 ^{KO} This paper N/A	Hel a N1/N2 ^{DKO}	This paper	N/A
······ papa,	HeLa NDP52 ^{KO}	This paper	N/A
HeLa ATG7 ^{KO} This paper N/A	HeLa ATG7 ^{KO}	This paper	N/A
U2OS ATCC HTB-96	U2OS	ATCC	HTB-96
MEF Alfy ^{WT} Dragich et al., 2016 N/A	MEF Alfy ^{WT}	Dragich et al., 2016	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MEF Alfy ^{KO}	Dragich et al., 2016	N/A
MEF p62 ^{WT}	Komatsu et al., 2007	N/A
MEF p62 ^{KO}	Komatsu et al., 2007	N/A
Experimental Models: Organisms/Strains		
Male Mouse C57BL/6J wild-type	This paper	N/A
Zebrafish wild-type	ZIRC, Oregon	N/A
Zebrafish Nipsnap1 ^{KO}	This paper	N/A
Zebrafish Nipsnap1 ^{sa14357}	Kettleborough et al., 2013	N/A
Oligonucleotides		
siRNA targeting sequence: NIPSNAP1 #1: CCAGGA ACCAUGAUCGAGU	Dharmacon	Cat# J-011489-19
siRNA targeting sequence: NIPSNAP1 #2: CGUAAC AGGAACUCGGAAG	Dharmacon	Cat# J-011489-20
siRNA targeting sequence: NIPSNAP2: GCCAAAGA UUCACGAAGAU	Dharmacon	Cat# J-011282-11
siRNA targeting sequence: ATG7: CAGUGGAUCUA AAUCUCAAACUGAU	Høyer-Hansen et al., (2007)	N/A
NIPSNAP1 CRISPR sgRNA #1: GCGGCTCCAACAT GGCTCCG	Custom order (ThermoFisher)	N/A
NIPSNAP1 CRISPR sgRNA #2: GCAGCATCTCTGT GACGGCG	Custom order (ThermoFisher)	N/A
NIPSNAP2 CRISPR sgRNA #1: CGAGGCGCCGAG CAAGATGG	Custom order (ThermoFisher)	N/A
NIPSNAP2 CRISPR sgRNA #2: GTCTTCTCGAGAT CTGTTGC	Custom order (ThermoFisher)	N/A
NDP52 CRISPR sgRNA: CCTCGTCGAAAGGATTGGAT	Custom order (ThermoFisher)	N/A
ATG7 CRISPR sgRNA: AGAAGAAGCTGAACGAGTAT	Custom order (ThermoFisher)	N/A
Zebrafish Nipsnap1 CRISPR sgRNA #1: attaatacgactc actataGGAAATGCTGCTGTGTGTTGgttttagagctagaaatagc	Custom order (ThermoFisher)	N/A
Zebrafish Nipsnap1 CRISPR sgRNA #2: aattaatacgac tcactataGGAAGCTGGAACACATGGTAgttttagagctagaaatagc	Custom order (ThermoFisher)	N/A
Zebrafish Nipsnap1 CRISPR sgRNA #3: aattaatacga ctcactataGGCGGATTCTTCACACAGATgttttagagctagaaatagc	Custom order (ThermoFisher)	N/A
Nipsnap1 genotyping forward primer: TGCATCTGT GGAGATACTCTGGAGG	Custom order (ThermoFisher)	N/A
Nipsnap1 genotyping reverse primer: CCCATAAA TGATGCACTACATAC	Custom order (ThermoFisher)	N/A
NIPSNAP1 RT-qPCR forward primer: TCCCTGTG AAGTTGTTGGAAGCTG	Custom order (ThermoFisher)	N/A
NIPSNAP1 RT-qPCR reverse primer: TGCACTGC CTGATCCTGTTCAC	Custom order (ThermoFisher)	N/A
NIPSNAP2 RT-qPCR forward primer: TGCACTTGT GGAGGTACAGAGG	Custom order (ThermoFisher)	N/A
NIPSNAP2 RT-qPCR reverse primer: TGCGGTACT CCAGAAACTCCTTG	Custom order (ThermoFisher)	N/A
β-actin RT-qPCR forward primer: CGAACGACCAA CCTAAACCTCTCG	Custom order (ThermoFisher)	N/A
β-actin RT-qPCR reverse primer: ATGCGCCATACA GAGCAGAAGC3	Custom order (ThermoFisher)	N/A
Nipsnap1 in situ hybridization 5'UTR probe forward primer: CGGAATCAACAGACAAGGCC	Custom order (ThermoFisher)	N/A
Nipsnap1 in situ hybridization 5'UTR probe reverse primer: TACTCAGGCTTGACATTGTG	Custom order (ThermoFisher)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nipsnap1 in situ hybridization internal probe forward primer: ACTCCAATCTGCTCTCCAAG	Custom order (ThermoFisher)	N/A
Nipsnap1 in situ hybridization internal probe reverse primer: TCTCTTCTCTGGACTGCAGG	Custom order (ThermoFisher)	N/A
Nipsnap1 in situ hybridization 3'UTR probe forward primer: CAGATCATATCAGCTACTGC	Custom order (ThermoFisher)	N/A
Nipsnap1 in situ hybridization 3'UTR probe reverse primer: ACATGCTGTATAGCTCAAGC	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII MLS forward primer: cATGTCTGGACTTCTGAGGGGACTAGCTCGCGTCC GCGCCGCTCCGGTTCTGCGGGGATCCACGATCA CCCAGCGAGCCAACCTCGTTACGCGAgc	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII MLS reverse primer: catggcTCGCGTAACGAGGTTGGCTCGCTGGGTG ATCGTGGATCCCCGCAGAACCGGAGCGGCGCG GACGCGAGCTAGTCCCCTCAGAAGTCCAGA	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII CMV forward primers: ctgatgcccagtttaatttaaatagatctggccatCGATGTACG GGCCAGATATAC	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII CMV reverse primers: cctcagaagtccagacatCCTATAGTGAGTCGTATTAATTTCG	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII MLSGFP forward primers: aatacgactcactataggATGTCTGGACTTCTGAGGG	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII MLSGFP reverse primers: ctcctcgcccttgctcacCCTTGAATTCCCAGATCTTC	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII mCherry forward primers: agatctgggaattcaaggGTGAGCAAGGGCGAGGAG	Custom order (ThermoFisher)	N/A
Zebrafish Tandem-tag CoxVIII mCherry reverse primers: aactagagattcttgtttaagcttgatatccatggACGCCTTAAGAT ACATTGATGAGTTTG	Custom order (ThermoFisher)	N/A
Wild-type zebrafish nipsnap1 forward primer: ATGATGG CTACCGCACGACCTCTGC	Custom order (ThermoFisher)	N/A
Wild-type zebrafish nipsnap1 reverse primer: TTACTGC AGAGGTGAATGTACCATG	Custom order (ThermoFisher)	N/A
Recombinant DNA		
pEGFP-N3	Clontech	Cat# 6080-1
pGEX-5X3	GE Healthcare	Cat# 27-4586-01
pEGFP-N3-NIPSNAP1	This paper	N/A
pEGFP-N3-NIPSNAP1 ⁽¹⁻²⁰⁾	This paper	N/A
pEGFP-N3-NIPSNAP1 ⁽²⁴⁻²⁸⁴⁾	This paper	N/A
pEGFP-N3-NIPSNAP1 ⁽⁵⁹⁻²⁸⁴⁾	This paper	N/A
pEGFP-N3-NIPSNAP1 ⁽²⁴⁻⁶⁴⁾	This paper	N/A
pmCherry-N3-NIPSNAP1	This paper	N/A
p-3xFLAG-N3-NIPSNAP1	This paper	N/A
pTH1-NIPSNAP1	This paper	N/A
pNIPSNAP1	This paper	N/A
pDestEGFP-LC3A	Alemu et al., 2012	N/A
pDestEGFP-LC3B	Alemu et al., 2012	N/A
pDestEGFP-GABARAP	Alemu et al., 2012	N/A
pDestEGFP-GABARAPL1	Alemu et al., 2012	N/A
pDestEGFP-GABARAPL2	Alemu et al., 2012	N/A
pDest15-LC3A	Pankiv et al., 2007	N/A
pDest15-LC3B	Pankiv et al., 2007	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pDest15-LC3C	This paper	N/A
pDest15-GABARAP	Pankiv et al., 2007	N/A
pDest15-GABARAPL1	Pankiv et al., 2007	N/A
pDest15-GABARAPL2	Pankiv et al., 2007	N/A
pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene	Cat#62988
pCS2-nls-zCas9-nls	Addgene	Cat#47929
pCRII-TH1	Holzschuh et al., 2001	N/A
ZeroBlunt® TOPO PCR Vector	ThermoFisher	Cat#450245
pMXs-puro	Cell Biolabs	Cat#RTV-012
pMXs-neo	Cell Biolabs	Cat#RTV-011
pMXs-puro mCherry-Parkin	This paper	N/A
pMXs-neo MYC-Parkin	This paper	N/A
pMXs-neo NIPSNAP1	This paper	N/A
pMXs-neo NIPSNAP2	This paper	N/A
pMXs-neo NIPSNAP2 ^{del1-24}	This paper	N/A
pMXs-neo NIPSNAP1-EGFP	This paper	N/A
pMXs-neo NIPSNAP2-EGFP	This paper	N/A
pMXs-neo NIPSNAP1-MYC	This paper	N/A
pMXs-neo NIPSNAP2-MYC	This paper	N/A
pMXs-puro mCherry-EGFP-OMP25TM	This paper	N/A
pDest15-NIPSNAP1	This paper	N/A
pDest15-NIPSNAP2	This paper	N/A
pDest15-NIPSNAP2 ¹⁻¹⁸⁰	This paper	N/A
pDest15-NIPSNAP2 ¹⁸¹⁻²⁸⁶	This paper	N/A
pDest15-NIPSNAP2 ¹⁻¹⁵⁰	This paper	N/A
pDest15-NIPSNAP2 ¹⁵¹⁻²⁸⁶	This paper	N/A
, pDest15-NIPSNAP2 ¹⁻²²⁰	This paper	N/A
pDest15-NIPSNAP2 ¹⁰¹⁻²⁸⁶	This paper	N/A
pDest15-NDP52	This paper	N/A
pDestEGFP-NDP52	This paper	N/A
pDestEGFP-NDP52 ¹⁻⁴¹⁴	This paper	N/A
DestEGFP-NDP52 L446A	This paper	N/A
pDest15-p62	Jain et al., 2010	N/A
pDest15-Galectin8	This paper	N/A
pDestMYC-NDP52	This paper	N/A
pDestMYC-NDP52 ¹⁻⁴¹⁴	This paper	N/A
pDestMYC-NDP52 ¹⁻³⁸²	This paper	N/A
pDestMYC-NDP52 ¹⁻³⁶⁴	This paper	N/A
pDestMYC-NDP52 L446A	This paper	N/A
pDestMYC-NDP52 ^{C425A}	This paper	N/A
pDestMYC-NDP52 C422A	This paper	N/A
pDestMYC-NDP52 ^{D439A}	This paper	N/A
pDestMYC-NDP52 ^{F442A}	This paper	N/A
pDestMYC-NDP52 ^{134LVV/AAA}	This paper	Ν/Δ
nDestMVC-n62	Lamark et al. 2003	N/Δ
nDestMVC-TAX1RP1	This paper	N/A
nDeetMVC_NRR1	Lamark et al. 2003	N/A
	This paper	N/A
puestivit o-IVIF SIVAF I	mis paper	

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pDestMYC-NIPSNAP2	This paper	N/A
PDestEYFPN1-NIPSNAP1	This paper	N/A
PDestEYFPN1-NIPSNAP2	This paper	N/A
pDest3xFLAG-p62	Jain et al., 2010	N/A
pcDNA3-sYFP1	Nyfeler et al., 2008	N/A
pcDNA3-sYFP2	Nyfeler et al., 2008	N/A
pDestFlpIn-sYFP1-GABARAP	This paper	N/A
pDestFlpIn-sYFP2-GABARAP	This paper	N/A
pcDNA5/FRT/TO-NIPSNAP1-sYFP1	This paper	N/A
pcDNA5/FRT/TO-NIPSNAP1-sYFP2	This paper	N/A
pNIPSNAP1-EGFP-CIB1	This paper	N/A
CRY2low-tdTomato	Duan et al., 2017	N/A
pcDNA5-FRT-SUMOStar-NIPSNAP1-EGFP	This paper	N/A
pLVX-SUMOstar protease	This paper	N/A
pEGFP-N3-NIPSNAP2	This paper	N/A
pEGFP-N3-NIPSNAP2 ¹⁻¹⁹	This paper	N/A
pEGFP-N3-NIPSNAP2 ²²⁻²⁸⁶	This paper	N/A
pEGFP-N3-NIPSNAP ²⁵⁹⁻²⁸⁶	This paper	N/A
pEGFP-N3-NIPSNAP3A ²⁵⁻²⁴⁷	This paper	N/A
pEGFP-N3-NIPSNAP3B	This paper	N/A
pEGFP-N3-NIPSNAP3B ²⁵⁻²⁴⁷	This paper	N/A
pLVX-NIPNSPA1-EGFP-3xHA	This paper	N/A
pLVX-NIPNSPA1-EGFP-3xFlag	This paper	N/A
pLVX-PDHA1-EGFP-3xHA	This paper	N/A
pLVX-NIPSNAP1-APEX2	This paper	N/A
Software and Algorithms		
DNASTAR v14	DNASTAR	N/A
ApE (A Plasmid Editor v.2.0.47)	University of Utah	N/A
ZEN Software	Carl Zeiss	N/A
Scaffold (version Scaffold_4.3.4)	Proteome Software Inc.	N/A
ScienceLab ImageGuage	Fujifilm	N/A
Prism 7	GraphPad	N/A
Viewpoint software (version 3,10,0,42)	Viewpoint Life Sciences	N/A
Leica Application Suite X (LAS X)	Leica Microsystems	N/A
Other		
PGEM vector	Promega	Cat##A3600
GFP-TRAP	Chromotek	Cat## gta-20
MYC-TRAP	Chromotek	Cat## yta-20
Anti-FLAG M2 affinity gel	Sigma-Aldrich	Cat##A2220
Anti-HA magnetic beads	ThermoFisher	Cat#88837
Glutathione Sepharose 4 Fast Flow beads	GE Healthcare	Cat##17-5132-01
cOmplete Mini EDTA-free protease inhibitor cocktail tablets	Roche	Cat#11836170001
phosphatase inhibitor cocktail	Merck Millipore	Cat#524625

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead Contact, Anne Simonsen (anne. simonsen@medisin.uio.no).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

Human cells (U2OS, HeLa and HEK293) were all from ATCC. Mouse embryonic fibroblasts (MEFs) were kindly provided by Masaaki Komatsu (p62 KO MEFs) and Ai Yamamoto (ALFY KO MEFs) (Dragich et al., 2016; Komatsu et al., 2007). All cells were maintained in DMEM supplemented with 1X L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin.

Mouse and Zebrafish Husbandry

Male C57BL/6J wild-type (WT) mice were housed in a temperature-controlled (22°C) facility with a strict 12 h light/dark cycle and free access to water and food at all times. The mice were euthanized by cervical dislocation and tissues were snap-frozen in liquid nitrogen and stored at -80°C. Proteins were extracted from mouse tissues with a precipitation buffer (50 mM Tris/HCl pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA). Equal amount of protein was resolved by SDS/PAGE and immunoblotted with indicated antibodies. Fish (WT strains and the *nipsnap1* mutant lines) were held at the zebrafish facility at the Centre for Molecular Medicine Norway (AVD.172) using standard practices. Embryos were incubated in egg water (0.06 g/L salt (Red Sea)) or E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, equilibrated to pH 7.0). From 12 hpf, 0.003% (w/v) 1-phenyl-2-thiourea (Sigma-Aldrich) was used to inhibit pigmentation. Embryos were held at 28 °C in an incubator following collection. All use of animals was approved and registered by the Norwegian Animal Research authority. **Experimental procedures followed the recommendations of the** Norwegian Regulation on Animal Experimentation ("Forskrift om forsøk med dyr" fra 15.jan.1996). All experiments conducted on larvae at 7 dpf were approved by Mattilsynet (FDF Saksnr. 16/153907).

METHODS DETAILS

Antibodies and Reagents

The following antibodies were used for human cells: rabbit monoclonal anti-NIPSNAP1 antibody (Cell Signaling, #D1Y6S), rabbit polyclonal anti-NIPSNAP1 antibody (Abcam, #ab67302 and #ab133840), mouse monoclonal anti-NIPSNAP2 antibody (LSBio, #LS-B13280), rabbit polyclonal anti-NIPSNAP2 antibody (ABGENT, #AP6752c), rabbit polyclonal anti-NIPSNAP2 antibody (Abcam, #ab153833), rabbit polyclonal anti-ALFY antibody (Simonsen et al., 2004), rabbit polyclonal anti-GFP antibody (Abcam, #ab290) and (Santa-Cruz; #sc-8334), mouse monoclonal anti-GFP antibody (Clontech, #632381), mouse monoclonal anti-MYC tag antibody (Cell Signaling, #2276), mouse monoclonal anti-HA tag antibody (Roche, #11583816001), anti-p62 mouse monoclonal (BD Biosciences, #610833) and guinea pig polyclonal (Progen, #GP62-C) antibodies, rabbit polyclonal anti-CALCOCO2 Antibody (Sigma, #HPA023195), mouse monoclonal anti-NBR1 antibody (Santa Cruz, #sc-130380), rabbit polyclonal Anti-TAX1BP1 antibody (Sigma, #HPA024432), rabbit polyclonal Anti-Optineurin antibody (Sigma, #HPA003360), mouse monoclonal anti-MTCO2 antibody (Abcam, #ab110258), mouse monoclonal anti-DNA antibody (Progen, #61014), rabbit polyclonal anti-SOD-2 antibody (Santa Cruz, #sc-30080), mouse monoclonal anti-MFN2 antibody (Santa Cruz, #sc-100560), mouse monoclonal anti-Ubiquitin (FK2) antibody (Enzo, #BML-PW8810), rabbit monoclonal anti-ATG7 antibody (Cell Signaling, #D12B11), rabbit polyclonal anti-Actin antibody (Sigma, #A2066), rabbit polyclonal anti-PDH antibody (Cell Signaling, #2784S), mouse monoclonal anti-Cytochrome-C antibody (Abcam, #ab110325), rabbit polyclonal anti-Parkin antibody (Cell Signaling, #2132), rabbit polyclonal anti-LC3B antibody (Novusbio, #NB100-2220), mouse monoclonal anti-GABARAP antibody (MBL, #M135-3), rabbit polyclonal anti-LC3B antibody (Sigma, #L7543), mouse monoclonal anti-TOMM20 antibody (Santa Cruz, #sc-17764), rabbit polyclonal anti-TOMM20 antibody (Santa Cruz, #sc-11415), mouse monoclonal anti-TOMM40 antibody (Santa Cruz, #sc-365467), rabbit polyclonal anti-IKKα antibody (Cell Signaling, #2682), rabbit polyclonal anti-histone H3 antibody (Abcam, #ab1791), mouse monoclonal anti-TIMM23 antibody (BD Biosciences, #611223), mouse monoclonal anti-FLAG epitope M2 antibody (Sigma, #F1804), rabbit monoclonal anti-DYKDDDDK-tag (FLAGtag) antibody (Cell Signaling, #14793S). The following antibodies were used for zebrafish: rabbit polyclonal anti-Nipsnap1 (Abcam, #ab133840) and Nipsnap2 (Abcam, #ab153833), mouse monoclonal anti-tyrosine hydroxylase (TH) (ImmunoStar, #22941) and mouse monoclonal anti-α-Tubulin (Sigma, #T5168). The following kits and reagents were used: KAPA SYBR® FAST qPCR Kits (KAPA Biosystems #KK4601), DIG RNA Labelling Mix (Roche #11277073910), Anti-Digoxigenin AP fragments (Roche #11093274910), Proteinase K (PK) (Roche #3115828001), QProteome mitochondria isolation kit (Qiagen, #37612), TnT T7 coupled reticulocyte lysate system (Promega, #L4610), CellRox (ThermoFisher, #C10422), Propidium Iodide (ThermoFisher, #P1304MP), FCCP, L-Dopa (Sigma, #333786), Formamide (Sigma, #S4117), Torula Yeast RNA (Sigma, #R6625), Heparin Sodium Salt (Sigma, #H4784), Collagenase P (Sigma, #11249002001), SP6/T7 mMessage mMachine (Ambion #AM1340M/#AM1344), 10 μM Oligomycin (Sigma, #495455 or #04876) and 4 µM Antimycin A (Sigma, #A8674), 10 µM Carbonyl cyanide m-chlorophenyl hydrazine CCCP (Sigma, #C2759), Hanks Balanced Salt Solution (HBSS) (Sigma, #H8264), Bafilomycin A1 (Sigma, #B1793), MG132 (Z-Leu-Leu-Leu-al) (Sigma, #C2211) and Ponceau S (Sigma, #P3504). All siRNAs were purchased from Dharmacon Inc. The target sequences include NIPSNAP1 (CCAGGAACCAUGAUCGAGU, CGUAACAGGAACUCGGAAG), NIPSNAP2 (GCCAAAGAUUCAC GAAGAU) and ATG7 (CAGUGGAUCUAAAUCUCAAACUGAU) (Høyer-Hansen et al., 2007).

Generation of Human Knockout Cell Lines Using CRISPR-Cas9 System

Human NIPSNAP1 and NIPSNAP2 knockout cell lines were generated with the CRISPR-Cas9 system as described previously (Ran et al., 2013). Two guide RNAs designed for both NIPSNAP1 and NIPSNAP2 where annealed and ligated into a BbsI linearized vector

(Addgene #62988 or #48138) carrying both the Cas9 and puromycin-resistance or EGFP gene, respectively. HeLa cells were transfected with the gRNA-containing Cas9 vector using Mectafectene Pro (Biontex #T020). For vector with the puromycin resistance gene, HeLa cells were treated with 1 µg/mL of puromycin 24 h post transfection for 36 h. Single cells were then sorted and plated into 96-well plates. For vector with EGFP gene, EGFP-positive cells were sorted by FACS and plated into 96-well plates 48 h post transfection. Single colonies were then expanded and screened by immunoblotting. Once knockout were confirmed by immunoblotting, genomic DNA were extracted using the GenElute mammalian genomic DNA miniprep kit (Sigma #G1N350) and the area of interest amplified by PCR. The amplified region was ligated into the PGEM vector (Promega #A3600) and sequenced to identify indels. To generate double knockouts, the parental cell lines were transfected sequentially with gRNAs for each protein at a time. Guide RNAs include NIPSNAP1 (GCGGCTCCAACATGGCTCCG, GCAGCATCTCTGTGACGGCG), NIPSNAP2 (CGAGGCGC CGAGCAAGATGG, GTCTTCTCGAGATCTGTTGC), NDP52 (CCTCGTCGAAAGGATTGGAT) and ATG7 (AGAAGAAGCTGAACGA GTAT).

Generation of Stable Cell Lines and Reconstitution of KO Cell Lines

Stable cell lines and reconstituted KO cell lines were generated using the pMXs vector with Puromycin or Neomycin (G418) resistance gene. NIPSNAP1, NIPSNAP2, NIPSNAP1del1-24, NIPSNAP1-MYC, NIPSNAP2-MYC, NIPSNAP1-EGFP, NIPSNAP2-EGFPi and mCherry-EGFP-OMP25TM were PCR amplified and ligated into the pMXs vector using *Bam*H1 and *Not*1 sites. These retroviral vectors were packaged in HEK293 cells and resulting viral particles were used to transduce both HeLa WT and N1/N2 DKO cells three times for 24h each in combination with 8 µg/ml Hexadimethrine bromide (Sigma, #H9268). Protein expression was optimized by selection in appropriate antibiotics.

CRISPR/Cas9 Genome Editing in Zebrafish

Zebrafish *nipsnap1* KO embryos were generated using CRISPR/Cas9 technology as described (Jao et al., 2013). Briefly, the web tool "CHOPCHOP" was used to design a set of three sgRNA molecules (designated G1-G3), targeting exon 1, 4 and 7 of the zebrafish *nipsnap1* gene, respectively. A plasmid encoding zebrafish codon-optimized Cas9 (pCS2-nls-zCas9-nls) was procured from Addgene (Plasmid ID 47929). sgRNA and Cas9 mRNA were generated essentially as described (Jao et al., 2013). (Guide#1-Exon1attaatacgactcactataGGAAATGCTGCTGTGTGTGTGTGTGttttagagctagaaatagc, Guide#2-Exon4aattaatacgactcactataGGAAATGCTGCTGTGTGTGTGttttagagctagaaatagc, Guide#2-Exon4aattaatacgactcactataGGAAGCTGGAACACATGGTAgttttagagctagaaatagc, Guide#3-Exon7aattaatacgactcactataGGCGGATTCTTCACACAGATgttttagagcta gaaatagc). Synthesized sgRNA integrity was checked on 1% TBE gel. Individual sgRNAs (50–200 ng/µL) were mixed with capped and poly-adenylated Cas9 mRNA (300 pg/µL) before microinjection into the 1 cell stage. Nipsnap1 depletion was validated by immunoblotting.

Genotyping of nipsnap1 Mutants

The zebrafish line carrying the heterozygous *nipsnap*1^{sa14357} mutant allele (Zebrafish Mutation Project (ZMP)) (Kettleborough et al., 2013) was verified by PCR and sequencing of genomic DNA from adult fish fin-clips. DNA was extracted from single embryos or fin clips from adult fish using the HotSHOT protocol (Meeker et al., 2007). Purified PCR or gel extracted PCR products (Zymoclean Gel DNA Recovery Kit, Zymo Research) were then cloned into Blunt End TOPO Vector. Colonies were picked and grown in liquid broth with the appropriate antibiotics. Plasmid DNA was isolated, purified and sent for sequencing. 25 μ L PCR reactions consisted of 0.5 μ L Phusion High Fidelity DNA Polymerase (Thermo scientific), 5 μ L 5X Phusion HF Buffer, 2 μ L dNTP (2.5 mM), 0.5 μ L forward primer (10 μ M) (5'TGCATCTGTGGAGATACTCTGGAGG3'), 0.5 μ L reverse primer (10 μ M) (5' CCCATAAATGATGCACTACATAC3'), 5 μ L genomic DNA and 11.5 μ L of nuclease free water. The Bio-Rad S1000 thermal cycler with the following program was used for amplification: 90 sec at 95 °C, 30 cycles of: 30 sec at 95 °C, 30 sec at 63.1 °C and 30 sec at 72 °C, followed by 72 °C for 5 min. Sequencing was performed using the reverse M13 primer. Sequencing traces were analyzed using DNASTAR (Version 14) and ApE (A Plasmid Editor v.2.0.47). Mutations were identified manually by comparing mutant and WT traces.

RT-PCR/qPCR Analysis

Total RNA was extracted from approximately 50 zebrafish embryos at the indicated developmental stages with Trizol (Invitrogen, Inc., USA). The RNA quality was checked by 260/280 nm absorption using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA) and gel analysis. First-strand cDNA was prepared using the SuperScript First-Strand Synthesis System according to manufacturer's instructions (Thermo Fisher). Amplification was performed with KAPA SYBR FAST qPCR Kit and the CFx96 real-time PCR system (Bio-Rad). In brief, reactions were done in 10 μ L volumes containing 400 nM of each primer, 2.5 μ L cDNA (3 ng), 5 μ L 2 × KAPA SYBR Green Master Mix Reagent and the rest nuclease free water. Reactions were run using the manufacturer's recommended cycling parameters of 50°C for 2 min, 95°C for 5 min, 40 cycles of 94°C for 15 s, and 60°C for 30 s. All reactions were performed in triplicate. Relative expression levels were calculated after correction for the expression of β -actin as an endogenous reference using the 2^{- $\Delta\Delta$ C(t)} method. Amplification specificity/quality was assessed by analyzing the melting curve. The primer sequences used for Nipsnap1 were forward primer (5'TGCACTTGTGGAAGTTGTTGGAAGCTG3') and reverse primer (5'TGCACTGCCTGATCCCAGAAA CTCCTTG3') and for β -actin were forward primer (5' CGAACGACCAACCTAAACCTCTCG3') and reverse primer (5' ATGCGC CATACAGAGCG3').

Whole-Mount In Situ Hybridization

Whole-mount *in situ* hybridizations for *nipsnap1* and *tyrosine hydroxylase 1* (TH1) were performed as previously described (Thisse and Thisse, 2008) using digoxigenin-labelled riboprobes. Primer sequences for *nipsnap1* sense and antisense probes were; 5'UTR probe: forward primer (5'CGGAATCAACAGACAAGGCC3'), reverse primer (5'TACTCAGGCTTGACATTGTG3'), internal probe: forward primer (5'ACTCCAATCTGCTCTCCAAG3'), reverse primer (5'TCTCTTCTCGGACTGCAGG3'), 3'UTR probe: forward primer (5'CAGATCATACAGCTACTGC3'), reverse primer (5'ACATGCTGTACAGCAGG3'), 3'UTR probe: forward primer (5'CAGATCATATCAGCTACTGC3'), reverse primer (5'ACATGCTGTATAGCTCAAGC3'). The TH1 plasmid was a kind gift from Wolfgang Driever (Department of Biology I, University of Freiburg).

Tandem-Tag Transgenic Mitofish Generation and Imaging

The pME-EGFP no stop vector from the Tol2 kit was cut with NCol restriction enzyme and then dephosphorylated with calf intestinal phosphatase. This was later phosphorylated with T4 polynucleotide kinase and annealed with mitochondrial localization signal (MLS) of zebrafish COXVIII (Kim et al., 2008). The oligo sequences used for annealing were forward primer (5'cATGTCTGGACTTCT GAGGGGACTAGCTCGCGTCCGCGCCGCTCCGGTTCTGCGGGGGATCCACCCAGCGAGCCAACCTCGTTACGCGAgc3') and reverse primer (5'catggcTCGCGTAACGAGGTTGGCTCGCTGGGTGATCGTGGATCCCCGCAGAACCGGAGCGGCGCGGA CGCGAGCTAGTCCCCTCAGAAGTCCAGA3'). Gibson assembly was used to generate pTol2-CMV-MLS-EGFP-Cherry with linearized pTol2mini and PCR products from the following primers: CMVFw (5'ctgatgcccagtttaatttaaatagatctggccatCGATGTACGGGC CAGATATAC3'), CMVRev (5'cctcagaagtccagacatCCTATAGTGAGTCGTATTAATTTCG3'), MLSGFPFw (5'aatacgactcactataggA TGTCTGGACTTCTGAGGG3'), MLSGFPRev (5'ctcctcgcccttgctcacCCTTGAATTCCCAGATCTTC3'), mCherryFw (5'agatctgggaattc aaggGTGAGCAAGGGCGAGGAG3') and mCherryRev (5'aactagagattcttgtttaagcttgatatccatggACGCCTTAAGATACATTGATGAG TTTG3'). Templates for PCR were used from Tol2 Kit. The MLS-EGFP-mCherry was subcloned into iTol2 vector using XhoI and Agel. 35 pg (final concentration) of iTol2 MLS-EGFP-mCherry vector and 50 pg (final concentration) of in-vitro transcribed transposase mRNA (in-vitro transcribed from linearized pCS2FA-transposase vector from the Tol2 kit) was injected into the 1 cell stage of control (WT) and Nipsnap1 mutants. Injected embryos were raised to adulthood (F0) and out-crossed to wild-type fish to identify transgenic founders. Control (WT) tandem-tagged mitofish transgenic founders and Nipsnap1 mutant tandem-tagged mitofish transgenic founders were incrossed respectively. Resulting respective larvae (F1) were fixed in 4% PFA (pH 7.2) overnight at 3dpf and co-stained with 50ug Hoechst reagent for 3-4 h at room temperature. Each larva was mounted on depression slides using low melting point agarose. Confocal images were obtained using an Apochromat 40x/1.2 WC or 60x/1.2 oil DIC objective on an LSM 780 microscope (Zeiss). Red and yellow dots were counted manually for each cell and the ratio of red to yellow dots (per cell) were interpreted as mitophagy.

Zebrafish Rescue Experiments

Full length wildtype zebrafish nipsnap1 was amplified using the oligos: FP – ATGATGGCTACCGCACGACCTCTGC and RP – TTA CTGCAGAGGTGAATGTACCATG. Amplified product was cloned into zero-blunt end TOPO vector (ThermoFisher). Capped full-length zebrafish wildtype nipsnap1 mRNA was transcribed from linearized zero-blunt TOPO vector using mMessage mMachine (Ambion) and later poly(A) tailed using the poly(A) tailing kit (ThermoFisher). 75pg of the transcribed mRNA was injected into 1 cell stage of Nipsnap1 mutant tandem-tagged transgenic larvae. Larvae at 3dpf were fixed in 4% PFA (pH 7.2) overnight at 3dpf and co-stained with 50ug Hoechst reagent for 3-4 h at room temperature. Each larva was mounted on depression slides using low melting point agarose. Confocal images were obtained using an Apochromat 40x/1.2 WC or 60x/1.2 oil DIC objective on an LSM 780 microscope (Zeiss). Red and yellow dots were counted manually for each cell and the ratio of red to yellow dots (per cell) were interpreted as mitophagy.

Immunoblotting, Immunoprecipitation and Mass Spectrometry

HeLa cells seeded in either 6-well plates or 6 cm plates were treated as indicated. Cells were lysed in 1xSDS (50 mM Tris pH 7.4, 2% SDS, 10% Glycerol) supplemented with 200 mM dithiothreitol (DTT, Sigma, #D0632) and heated to 99°C for 8-10 min. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermofischer Scientific, #23227). 10-40 µg protein per sample were separated by SDS-PAGE, transferred to nitrocellulose membrane, stained with Ponceau S (Sigma, #P3504) and immunoblotted by the indicated antibodies. For immunoprecipitation, HeLa cells stably expressing EGFP or EGFP tagged proteins were immunoprecipitated by GFP-TRAP (Chromotek, # gta-20) while those expressing MYC-tagged proteins were immunoprecipitated using MYC-TRAP (Chromotek, # yta-20). HeLa cells transiently transfected with 3xFLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma, #A2220). Cells were lyses in modified RIPA buffer (50 mM Tris-Cl pH 7.4, 120 mM NaCl, 1 mM EDTA pH 8.0, 1% NP-40, 0,25% Triton X-100) supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail tablets (Roche AppliedScience, #11836170001) on ice for 30 min, followed by centrifugation at 10.000 x g for 10 min. Supernatants were then incubated with either GFP-TRAP or anti-FLAG M2 affinity gel for 2 h and washed five times with RIPA buffer. FLAG-tagged protein were eluted by flag-peptide in RIPA buffer before boiling in 2x SDS gel loading buffer, while GFP-tagged protein were eluted by boiling in 2X SDS gel loading buffer. GFP-tagged proteins were also immunoprecipitated using the µMACS GFP Isolation Kit (Miltenyi Biotec) according to the instruction manual. For immunoprecipitation of endogenous ALFY, ALFY^{+/+} or ALFY^{-/-} MEFs were incubated with lysis buffer (150 mM NaCl, 20 mM Hepes pH 7.4, 1% NP40, protease inhibitors and phosphatase inhibitors cocktail (Roche)) for 20 min, 4°C, then centrifuged for 10 min, 18.000 x g, and supernatant containing 5 mg of protein was incubated with 20 µL of anti-ALFY antibody for 2 h, 4°C, followed by 1 h incubation with 20 µL of protein G Dynabeads (ThermoFisher). After

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incubation, beads were washed four times in washing buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 0,1% NP40) and bound proteins were eluted by boiling with 0.25% SDS in washing buffer. For immunoblotting of proteins from zebrafish, embryos were de-yolked and homogenized in lysis buffer (50 mM Tris-HCI (pH 8), 150 mM NaCI, 5 mM EDTA, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail and (Roche)) 3 d post injections. Protein lysates were separated on Criterion TGX Gels (Bio-Rad), transferred to PVDF membrane (Millipore), and incubated overnight at 4°C with the indicated primary antibodies, followed by 1 h incubation with far-red/green fluorophore-conjugated secondary antibodies (LI-COR) and analysis on a LI-COR Odyssey Imaging Systems Application. Approximately 107 HeLa cells stably transfected with NIPSNA1-EGFP-3xHA, NIPSNAP1-EGFP-3xFLAG or PDHA1-EGFP-3xHA were washed 2 times with ice-cold PBS, then scraped in 1ml of ice-cold KPBS (136mM KCI, 10mM KH₂PO₄, pH 7.25) and centrifuged at 1000g, 4°C for 2 min. The pellet was resuspended in 1ml of KPBS and lysed by 25 plunger strokes in homogenizer vessel (VWR, cat. no. 89026-386/89026-398). Lysate was centrifuged at 1000g, 4°C for 2 min and supernatant was incubated for 5min at 4°C with 100µl of anti-HA magnetic beads (Thermo Fisher Scientific, #88837), then washed 3 times with 1ml KPBS and subjected to SDS PAGE and immunoblotting. For protein analysis by liquid chromatography-mass spectrometry (LC-MS), the SDS-PAGE was cut in 12 bands, each band digested with 0.1 µg trypsin (Promega, Madison, WI, USA) for 16 h at 37°C, the generated peptides were purified using a ZipTip μ-C18 (Millipore, Billerica, MA, USA), and dried using a Speed Vac concentrator (Concentrator Plus, Eppendorf, Hamburg, Germany). The tryptic peptides were dissolved in 10 µL 0.1% formic acid/2% acetonitrile and 5 µL analyzed using an Ultimate 3000 nano-HPLC system connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano electrospray ion source. For liquid chromatography separation, an Acclaim PepMap 100 column (C18, 3 µm beads, 100 Å, 75 µm inner diameter, 50 cm length) (Dionex, Sunnyvale CA, USA) was used with a flow rate of 300 nL/min and a solvent gradient of 4-35% B in 47 min, to 50% B in 10 min and then to 80% B in 1 min. Solvent A was 0.1% formic acid and solvent B was 0.1% formic acid/ 90% acetonitrile. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 2,000) were acquired with the resolution R = 60,000 at m/z 400 after accumulation to a target of 1e6. The maximum allowed ion accumulation times were 60 ms. The method used allowed sequential isolation of up to the seven most intense ions, depending on signal intensity (intensity threshold 1.7e4), for fragmentation using collision induced dissociation (CID) at a target value of 10,000 charges and NCE 35 in the linear ion trap. Target ions already selected for MS/MS were dynamically excluded for 60 sec. For accurate mass measurements, the lock mass option was enabled in MS mode. Data were acquired using Xcalibur v2.5.5 and raw files were processed to generate peak list in Mascot generic format (*.mgf) using ProteoWizard release version 3.0.331. Database searches were performed using Mascot in-house version 2.4.0 to search the SwissProt database (Mouse, 16,460 proteins) assuming the digestion enzyme trypsin, at maximum one missed cleavage site, fragment ion mass tolerance of 0.6 Da, parent ion tolerance of 10 ppm, propionamidylation of cysteines, oxidation of methionines, and acetylation of the protein N-terminus as variable modifications. Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.

Proximity Biotinylation Assay

Approximately $5x10^{6}$ HeLa cells stably transfected with NIPSNAP1-APEX2 were incubated for 30 min with 500μ M of biothin tyramide in complete medium, followed by treatment for 1min with 1mM H₂O₂ and washing 3 times with 5 ml of quenching solution (10mM sodium azide, 10mM sodium ascorbate, 5mM trolox in PBS). Cells then were scraped in 1ml of quenching solution, centrifuged at 3000g for 10min. 4°C, and pellet was lysed in 2xSDS gel loading buffer and subjected to SDS PAGE and immunoblotting with indicated antibodies.

Phos-tag SDS-PAGE Analysis

WT or NIPSNAP1/2 KO cells were plated in 6-well plate and treated 24h after transfection with 20µM CCCP for 0.5, 1, 2 and 4h. After treatment cells were lysed in 20mM Tris, pH7,5, 150mM NaCl, 1% Triton and either left untreated or were treated with lambda protein phosphatase (400U phosphatase, 1xPMP buffer, 1mM MnCl₂, 30°C, 30min). Samples were resolved on 8% SDS PAGE containing 50µM Phos-tag and 100µM MnCl₂, transferred to nitrocellulose membrane after washing gel 3x10min in 10mM EDTA and immuno-stained with NIPSNAP1/2 or actin antibodies.

Live Cell and Confocal Immunofluorescence Microscopy

HeLa cells were seeded in 8-well Lab-tek chamber coverglasses (Thermofischer Scientific, # 155409 &155411) or on coverslips (VWR, #631-0150) and treated as indicated. Cells were either examined directly or fixed. Cells were fixed for 10 min at 37 °C in preheated (37 °C) 4% PFA, followed by permeabilization in 0.1% Triton X-100 for 5 min and blocking with 3% goat serum for 30 min. Cells were incubated with primary antibodies diluted in PBS for 1 h at room temperature and washed five times with PBS, followed by incubation in Alexa Fluor 488-, Fluor 555-, or Fluor 647-conjugated secondary antibodies diluted in PBS for 30 min at room temperature and washed five times with PBS. During this final wash step, cells were incubated with 10 μ g/mL DAPI diluted in PBS for 10 min. Confocal images were obtained using an Apochromat 40x/1.2 WC or 60x/1.2 oil DIC objective on an LSM 780 microscope (Zeiss) or Leica TCS SP5 confocal microscope, 63×1.2W-objective.

Long-Lived Protein Degradation

To measure the degradation of long-lived proteins by autophagy, cellular proteins were first labelled with 0.25 mCi/m L-14C-valine (Perkin Elmer) for 24 h in GIBCO-RPMI 1640 medium (Invitrogen) containing 10% FBS. The cells were washed and then chased for 16 h in nonradioactive Dulbecco's modified Eagle's medium (Invitrogen) containing 10% FBS and 10 mM valine (Sigma), to allow degradation of short-lived proteins. The cells were washed twice with EBSS (Invitrogen), and starved or not for 4 h in the presence or absence of 10 mM 3-methyladenine (Sigma). The medium was then collected and added to 50% Trichloroacetic acid, followed by 2 h incubation at 4 °C and centrifugation to pellet any contaminants. 0.2 M potassium hydroxide solution was added to the cells for 2 h before the lysate was collected. Ultima Gold LSC cocktail (Perkin Elmer) was added to the medium and cell samples and protein degradation was determined by measuring the ratio of radioactivity in the medium relative to the total radioactivity detected by a liquid scintillation analyser (Tri-Carb 3100TR, Perkin Elmer), counting 3 min per sample.

Recombinant Protein Expression, In-Vitro Translation and GST-Pulldown Assay

GST and GST-fusion proteins were expressed in SoluBL21 Competent *Escherichia coli* (Genlantis, #C700200) and purified by immobilization on Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, #17-5132-01). MYC-tagged proteins were *in-vitro* translated in the presence of radioactive ³⁵S-methionine using the TNT T7 Reticulocyte Lysate System (Promega, #l4610). For GST-pulldown assay, $10 \,\mu$ L of *in-vitro* translated protein was pre-cleared with $10 \,\mu$ L of empty Glutathione sepharose beads in $100 \,\mu$ L of NETN buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail tablets for 30 min at 4 °C to remove unspecific binding. The precleared mixture was then incubated with the immobilized GST-fusion protein and incubated for 1-2 h at 4 °C. The beads were washed five times with NETN buffer (500 μ L NETN buffer followed by centrifugation at 2500 x *g* for 1 min). After the last wash, 2xSDS gel-loading buffer (100 mM Tris pH 7.4, 4% SDS, 20% Glycerol, 0.2% Bromophenol blue and 200 mM dithiothreitol DTT (Sigma, # D0632) was added and boiled for 10 min followed by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 Dye (ThermoFisher Scientific, #20278) for 30 min to visualized the fusion proteins, vacuumed-dried (in Saskia HochVakuum combined with BIO-RAD Gel dryer model 583, #1651746) for 30 min. Radioactive signals were detected by Fujifilm bioimaging analyzer BAS-5000 (Fujifilm) and quantified with ScienceLab ImageGuage software (Fujifilm).

Subcellular Fractionation, Proteinase K/trypsin Treatment and Sodium Carbonate Extraction

Subcellular fractionation was performed with a QProteome mitochondria isolation kit (Quiagen) according to the instruction manual. In brief, 10^7 HeLa cells were resuspended in 1 mL of lysis buffer, incubated for 10 min at 4°C and centrifuged at 1000 x *g* for 10 min. The supernatant was transferred into a separate tube as cytosolic fraction, while the pellet was resuspended in 1.5 mL of ice-cold disruption buffer, rapidly passed through 21 *g* needle 10 times to disrupt cells and centrifuged at 1000 x *g* for 10 min, 4°C. The pellet was saved as nuclear fraction, while the supernatant was re-centrifuged at 6000 x *g* for 10 min, 4°C. The pellet obtained after centrifugation comprised the mitochondrial fraction, while the supernatant contained the microsomal fraction. For PK digestion, mitochondria were resuspended in Mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5) with 50 or 100 µg/mL of PK and incubated 30 min at RT. For trypsin digestion, mitochondria were re-suspended in trypsin digestion buffer (10 mM sucrose, 0.1 mM EGTA/Tris and 10 mM Tris/HCl, pH 7.4) with 200 µg/mL of trypsin. Both reactions were stopped by addition of 5 mM phenylmethylsulfonyl fluoride. For the analysis of integral membrane proteins, the mitochondrial fraction was resuspended in MB buffer or MB buffer containing freshly prepared 0.1 M Na₂CO₃ (pH 11.5) and incubated on ice for 30 min. The insoluble membrane fraction was centrifuged at 16.000 x *g* for 15 min.

Mitophagy Assay

Hela cells seeded in 6 cm dishes (or 24 well plates for confocal microscopy) were either treated with 10 μ M Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) or a combination of 10 μ M Oligomycin and 4 μ M Antimycin A for indicated times. Mitophagy was analyzed by measuring the degradation of cytochrome C oxidase subunit II (COXII), a mtDNA encoded inner membrane protein, and TIMM23, a nuclear encoded mitochondria inner membrane protein. For confocal miscroscopic analyses of mitophagy, we immunostained for mtDNA nucleoids and TIMM23. In addition, we also used a tandem tagged mCherry-EGFP-OMP25TM mitophagy reporter for visualizing acidified mCherry dots in the lysosome.

In Vivo Ubiquitination Assay

HeLa WT cells stably expressing mCherry-PARKIN were transfected with NIPSNAP1-MYC, NIPSNAP2-MYC, MFN2-MYC and HA-UBIQUITIN. 24h after transfection cells were treated with CCCP and MG132 for 3h before harvesting. Cells were collected in lysis buffer (2% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, #11836170001) and N-ethylmaleimide (Sigma,#E1271)) and heated at 90 °C for 10 min to denature proteins. Lysates were diluted 1:10 in dilution buffer (1% TritonX-100, 150 mM NaCl, 50 mM Tris, pH 8.0) and myc-tagged proteins immunoprecipitated with MYC-TRAP. Ubiquitination was detected by immunoblotting with HA antibody.

In Vitro Kinase Assay

In vitro kinase assays with PINK1 were performed with 50 ng of recombinant active PINK1 kinase (Ubiquigent, #66-0043-050), 1-2 μ g of GST-tagged proteins, 60 μ M ATP and 2 μ Ci [γ -³²P] ATP (PerkinElmer, #NEG002A250UC) in 30 μ l of kinase buffer (35.5 mMTris

pH7.5, 10 mM MgCl₂, 0.5 mM EGTA pH 8.0, 0.1 mM CaCl₂ supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail tablets (Roche AppliedScience, #11836170001) and phosphatase inhibitor cocktail (Merck Millipore, #524625)). The reaction was incubated at 30 °C for 20 min and terminated by addition of 6x SDS-loading buffer. The reaction were then analyzed by SDS-PAGE and autoradiography.

Mitochondrial Import Assay

Mitochondria were isolated from HeLa or U2OS cells. Approximately 10^7 cells were pelleted, washed in PBS and resuspended in 2 mL of ice-cold isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES, 0.5% BSA). Cells were sheared with 6 passes through Cell Homogenizer (Isobiotech) with 16 µm clearance and centrifuged at 800 x g for 5 min at 4°C. The supernatant was transferred to a new tube and re-centrifuged at 10.000 x g for 10 min to pellet the mitochondrial fraction. The pellet was washed two times in washing buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 5 mM MgOAc, 80 mM KOAc, 1 mM DTT) and pretreated or not with 10 µM FCCP in mitochondrial import buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 5 mM MgOAc, 80 mM KOAc, 80 mM KOAc, 10 mM sodium succinate, 2 mM ATP, 0.4 mM ADP, 1 mM DTT). S³⁵-labeled NIPSNAP1 was co-transcribed/translated in vitro using TNT T7 coupled reticulocyte lysate system (Promega) and its product from 100 ng of pNIPSNAP1 plasmid was added to isolated mitochondria from 2x10⁶ cells in 30 µL of mitochondrial import buffer and reaction was incubated at 37°C for 45 min. Mitochondria were then washed three times with washing buffer and treated or not with PK, 25 µg/mL at room temperature for 10 min followed by addition of 1 mM PMSF.

Mitochondrial Oxygen Consumption Rate

Mitochondrial oxygen consumption rate was measured with the Seahorse XF CellMito Stress Test Kit (Agilent Technologies) according to the manufacturer's instruction.

ROS Analysis

The analysis of reactive oxygen species (ROS) levels in zebrafish was performed as described (Mugoni et al., 2014). Briefly, after dissociation into single cells of the respective larvae, cells were treated with 10 μ M of CellRox (Thermo Fisher Scientific) in Hanks Balanced Salt Solution (HBSS) for 30 min at 28°C in the dark. Cells were then centrifuged for 5 min at 250 x g at 4°C, the supernatant discarded and the cells washed with HBSS. FACS estimations were done on a BD FACS Calibur under standard settings. The voltage for the FL1 channel was optimized. 1 μ M of propidium iodide (PI) (Sigma) was added at room temperature for 5 min in the dark prior to FACS.

Zebrafish Locomotor Assay

Larval motility was monitored using the ZebraBox and Viewpoint software (version 3,10,0,42; Viewpoint Life Sciences, Inc.; Montreal, Quebec, Canada) under infrared light. At 6 days post fertilization (dpf), larvae were singly placed in 96-well plates with 300 µL of fish water per well, followed by incubation at 28.5°C on a normal light cycle overnight. All experiments were completed in a quiet room at 7 dpf between 10 AM and 2 PM. Larvae were allowed to acclimate in the ZebraBox measurement apparatus for 2 h before recording. Larvae were then exposed to alternating cycles of light and dark, invisible to the camera, every 20 min as described (Emran et al., 2008). Each light transition took approximately 1 ms. Larval locomotion was tracked with the ViewPoint software. Motility was defined as tracks moving less than 10 cm/s, but more than 0.1 cm/s.

Visualizing Apoptosis by TUNEL Staining

Control (WT) and *nipsnap1* mutant zebrafish larvae were fixed in 4% PFA overnight at 3dpf. After a couple of washes with PBST, they were dehydrated in methanol and then rehydrated gradually. The respective larvae were permeabilized with 25 µg/ml proteinase K for 30 minutes at 37°C followed by 20 minutes of 4% PFA fixation at room temperature. The larvae were then subjected to TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; Thermo Fisher Scientific) according to the manufacturer's instructions. As a positive control, fixed and permeabilized WT larvae were incubated with 1 unit of DNase I diluted into 1X DNase I Reaction Buffer (20 mM Tris-HCl, pH 8.4, 2 mM MgCl2, 50 mM KCl) for 1 hour at room temperature, followed by a rinse with deionized water prior to the TUNEL assay.

QUANTIFICATION AND STATISTICAL ANALYSIS

Descriptive and analytical statistics were generated in Prism 7.0 (GraphPad Software). Statistical significance was analyzed by oneway ANOVA or unpaired Student's t test and data represented as mean \pm SD or SEM from three or more independent experiments. All zebrafish experiments were done at least 3-6 times with 20-24 larvae in each experiment. p < 0.01 was taken to indicate a significant difference.