

# **Novel modulators of non-selective and selective autophagy**

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## LIST OF PUBLICATIONS

1. Holland, P.\*, Knævesrud, H.\*, Sørensen, K., Mathai, B.J., Lystad, A. H., Pankiv, S., Bjørndal, G. T., Schultz, S. W., Lobert, V. H., Chan, R. B., Zhou, B., Liestøl, K., Carlsson, S. R., Melia, T. J., Di Paolo, G. & Simonsen, A., (2016), **HS1BP3 negatively regulates autophagy by modulation of phosphatidic acid levels**. Nature Communications, 7, 13889.
2. Abudu, Y.P.\*, Pankiv, S.\*, Mathai, B.J.\*, Lystad, A.H., Bindesbøll, C., Brenne, H.B., Thiede, B., Yamamoto, A., Nthiga, T.M., Lamark, T., Esguerra, C.V., Johansen, T & Simonsen, A. **NIPSNAP1 and NIPSNAP2 facilitate mitophagy to inhibit ROS production and neuronal death**. (Under revision, Developmental Cell)
3. Mathai, B.J., Pankiv, S., Esguerra, C.V & Simonsen, A. **Lack of Nipsnap1 causes gastrulation arrest in zebrafish**. (Manuscript in preparation)
4. Mathai, B.J., Meijer, A & Simonsen, A. (2017). **Studying autophagy in zebrafish**. Cells, 6(3), 21.

\* Contributed equally

## ABBREVIATIONS

AD	Alzheimer's disease
ALFY	Autophagy-linked FYVE
ALS	Amyotrophic Lateral Sclerosis
ANTH	AP180 N-terminal homology
APP	Amyloid precursor protein
ASE	Asymmetric enhancer element
ATG	Autophagy related
ATP	Adenosine triphosphate
ATP13A2	ATPase cation transporting 13A2
BATS	Barkor/Atg14(L) autophagosome targeting sequence
BCKDC	Branched-chain $\alpha$ -keto acid dehydrogenase enzyme complex
Bcl2-L-13	Bcl-2-like protein 13
BECLIN1	Coiled-Coil Myosin-Like BCL2-Interacting Protein 1
BMP	Bone morphogenetic protein
BNIP3	B-cell lymphoma 2 nineteen kilodalton interacting protein 3
CAG	Cytosine-Adenine-Guanine
CCCP	Carbonilcyanide m-cholorophenylhydrazone
CMA	Chaperone Mediated Autophagy
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9
DA	Dopaminergic neurons
DABB	Dimeric alpha-beta barrel
DAG	Diacylglycerol
DFCP1	Double FYVE domain containing protein 1
DUBs	Deubiquitinating enzymes
DV	Dorsal-Ventral

ENTH	Epsin N-terminal homology
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FCCP	Carbonilcyanide p-triflouromethoxyphenylhydrazone
FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
FGF	Fibroblast growth factor
FIP200	FAK Family Kinase-Interacting Protein of 200 KDa
FKBP	FK506-binding protein
FLIP	FLICE-like inhibitory protein
FUNDC1	FUN14 Domain Containing 1
FYCO1	FYVE and coiled-coil domain-containing 1
FYVE	F for Fab 1, Y for YOTB, V for Vac 1, and E for EEA1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GBAS	Glioblastoma Amplified Sequence
GDFs	Growth and differentiation factors
gp78	Glycoprotein 78
HD	Huntington's Disease
HS1BP3	HCLS1 binding protein 3
Htt	Huntington
IMM	Inner mitochondrial membrane
KO	Knock out
LBs	Lewy bodies
L-Dopa	Levodopa
LIR	LC3-interacting region
LLPD	Long lived protein degradation
LPA	Lysophosphatidic acid
LPAATS	Lysophosphatidic acid acyltransferases
LRRK2	Leucine rich repeat kinase 2

MAP1LC3B/LC3	Microtubule-associated proteins 1A/1B light chain 3B
MEFs	Mouse embryonic fibroblasts
MFN2	Mitofusin 2
MPP	Mitochondrial processing peptidases
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	mitochondrial DNA
MTMR3	Myotubularin related phosphatase 3
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex I
mTORC2	Mammalian target of rapamycin complex II
MTS	Mitochondrial targeting signal
NBR1	Neighbor of BRCA1
NDP52	Nuclear dot protein 52 kDa
NFL	Neurofilaments
NIPSNAP	4-nitrophenylphosphatase domain and non-neuronal synaptosomal associated protein 25 (SNAP25)-like protein
OA	Oligomycin and Antimycin
OMM	Outer mitochondrial membrane
OPTN	Optineurin
OXPHOS	Oxidative phosphorylation
p150	protein of 150 KDa
PA	Phosphatidic acid
PARL	Presenilin-associated rhomboid-like protease
PD	Parkinsons disease
PDH	Pyruvate dehydrogenase
PE/PtdEtn	Phosphatidylethanolamine
PH	Plekstrin Homology
PI	Phosphoinositide

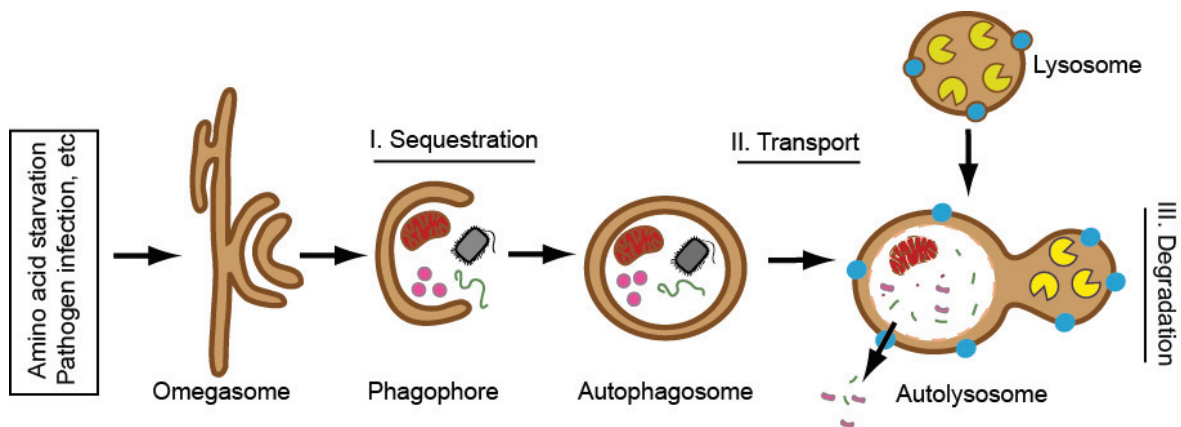
PI(3)K	Phosphatidylinositol 3-kinase
PI(3)P	Phosphatidylinositol-3-phosphate
PI(3,4,5)P3	Phosphatidylinositol-3,4,5-triphosphate
PI(3,5)P2	Phosphatidylinositol-3,5-biphosphate
PI(5)P	Phosphatidylinositol-5-phosphate
PIK3C3	Class III phosphatidylinositol 3-kinase
PINK1	PTEN-induced putative kinase 1
PKU	Phenylketonuria
PLD1	Phospholipase D1
PolyQ	Polyglutamine
PRKN	Parkin RBR E3 ubiquitin protein ligase
PROPPINs	$\beta$ -propellers that bind phosphoinositides
PRR	Pattern recognition receptors
PtdCho	Phosphatidylcholine
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
P-Ub	Phospho-ubiquitin
PX	Phox Homology
RE	Recycling endosome
ROS	Reactive oxygen species
SIAH1	Seven In Absentia Homolog 1
SLRs	SQSTM1-Like Receptors
SNCA	Synuclein alpha
SNpc	Substantia nigra pars compacta
SNX18	Sorting nexin 18
SQSTM1	Sequestosome1
TALENS	Transcription activator-like effector nucleases
TBK1	TANK binding kinase 1

TfR	Transferrin receptor
TGF- $\beta$	Transforming growth factor $\beta$
TH	Tyrosine hydroxylase
TIM	Translocase of inner membrane
TOM	Translocase of outer membrane
TRAF	Tumor Necrosis Factor (TNF) receptor-associated factors
ULK	UNC-51-like kinase
UPS	Ubiquitin-proteasome system
Vam7p	Vacuolar morphogenesis 7 protein
VPS34	Vacuolar Protein Sorting 34
WIPI	WD repeat domain phosphoinositide-interacting protein 1
Wnt	Wingless-type MMTV integration site family
WT	Wild type
ZFNs	Zinc finger nucleases

## INTRODUCTION

### Autophagy

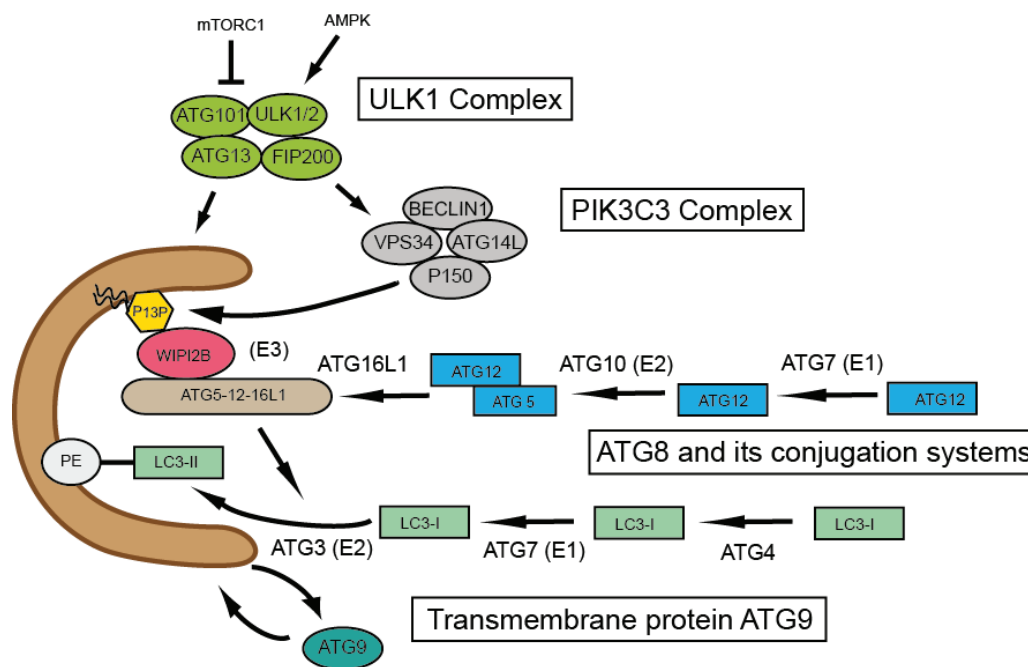
The Nobel Prize in Physiology or Medicine 2016 was awarded to Yoshinori Ohsumi "for his discoveries of mechanisms for autophagy", in particular the discovery of AuTophagy-related (Atg) genes in yeast (Tsukada & Ohsumi, 1993). The term 'autophagy' was however already coined in 1963 by Christian de Duve (derived from the ancient greek words "auto-phagin", which mean "self-eating") when he described single or double membraned vesicles that contained degraded cellular proteins and organelles. Autophagy is defined as degradation of intracellular materials in the lysosome. The resulting degradation products are recycled to be used as sources of energy or building blocks for the synthesis of new macromolecules, ultimately maintaining homeostasis and promoting survival at the cell, tissue and organism level. Three main types of autophagy have been described: chaperone-mediated autophagy (CMA), micro-autophagy and macro-autophagy (Boya et al., 2013). Macro-autophagy (hereafter referred to as autophagy) will be the main focus of this thesis. This pathway can be divided into three phases (Figure 1): first is the sequestration phase, where cellular constituents are sequestered into a double layered membranous structure (called the phagophore) that closes to form an autophagosome, the second phase involves transport of the autophagosome to the lysosome and the final phase is the maturation or degradation phase, which involves the fusion of the autophagosome with the lysosome, leading to degradation of the autophagosomal contents by lysosomal hydrolases to ultimately release the metabolites back into the cytosol through membrane permeases (Boya et al., 2013)



**Figure 1: The autophagy pathway.** The autophagosome formation is initiated after a strong stimulus such as amino acid starvation, through the formation of omegasome. The pathway includes sequestration of cargo into the phagophore (I), followed by closure of the phagophore - now termed as autophagosome - subsequent transport to the site of fusion with lysosome (II) and finally degradation in the lysosomal lumen by the proteases (III). (Modified from (Mathai et al., 2017))

### The core autophagy machinery and the origin of phagophore

In addition to the historical genetic screens performed by Yoshimori Ohsumi and co-workers (Tsukada & Ohsumi, 1993; Takeshige et al., 1992; Mizushima, 2017), other screens were conducted in parallel, leading to characterization of several autophagy mutants of *Saccharomyces cerevisiae* (Thumm et al., 1994; Harding et al., 1995). These were followed up by the identification of respective homologs in higher eukaryotes and resulted in the characterization of more than 30 ATG genes (Yang & Klionsky, 2009). The ATG proteins required for autophagosome formation are referred to as the ‘core’ autophagy machinery (Figure 2) (Xie & Klionsky, 2007) and include several highly conserved multimeric protein complexes, including in human (1) the UNC-51-like kinase (ULK) complex composed of ULK1 or ULK2, ATG13, ATG101 and FIP200; (2) the class III phosphatidylinositol 3-kinase (PI3K) complex (PIK3C3), consisting of the catalytic subunit VPS34, as well as BECLIN1, p150 and ATG14L; (3) the two ubiquitin-like conjugation systems that lead to the conjugation of ATG12 to ATG5 and the Atg8 homologs - microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B, hereafter referred to as LC3) / Gamma-aminobutyric acid receptor-associated protein (GABARAP) to phosphatidylethanolamine (PE/PtdEtn) in the phagophore membrane and finally (4) the transmembrane protein ATG9 (Figure 2) (Mizushima, Yoshimori, & Ohsumi, 2011; Yang & Klionsky, 2010).



**Figure 2: Core autophagic machinery involved in autophagosome biogenesis.** Upstream nutrient and energy-sensing kinases mTORC1 and AMPK regulates the ULK-complex (including ULK1/2, ATG101, ATG13 and FIP200) and induces autophagy by recruitment and activation of the PIK3C3-complex (consisting of catalytic subunit VPS34/PIK3C3, BECLIN1, ATG14L and p150). The resulting generation of PI(3)P in the ER recruits other core autophagic proteins to the site of phagophore formation. Recruitment of ATG12-5-16L1 complex to the membrane for lipidation of LC3/GABARAP proceeds binding of WIPI2 to PI(3)P in the phagophore. ATG12 conjugation to ATG5 is mediated by ATG7 and ATG10, whereas LC3/GABARAP is processed by ATG4, ATG7 and ATG3 before it is conjugated to PE in the membrane facilitated by ATG12-5-16L1 complex. Cycling of ATG9 to and from the phagophore site is crucial for autophagosome formation. (Modified from (Mathai et al., 2017))

Much before the identification of the core autophagy machinery, it was known that autophagy is responsive to fluctuations in amino acids (Mortimore & Schworer, 1977). It was later found that mammalian target of rapamycin or mechanistic TOR (mTOR) is the key downstream effector of amino acid mediated autophagy repression (Blommaert et al., 1995). mTOR, a serine/threonine kinase, integrates signals from various sources such as amino acids, energy levels, oxygen, growth factors and stress to maintain metabolic homeostasis and regulate cell growth (Laplante & Sabatini, 2012). mTORC1 and mTORC2 are the two functionally distinct complexes formed by mTOR in mammals, where the former gets activated in the presence of amino acids (Jewell, Russell, & Guan, 2013). Autophagy is induced by inhibiting mTORC1 even in the presence of

nutrients both in yeast and mammals, establishing mTORC1 as a conserved and critical repressor of autophagy (Noda & Ohsumi, 1998; Thoreen et al., 2009). The downstream mechanisms of repression by mTORC1 involves phosphorylation of core autophagy proteins (e.g. ULK1), but the details are beyond the scope of this thesis and elaborated elsewhere (Russell, Yuan, & Guan, 2014)

The regulation and execution of autophagy is tightly controlled by a large number of proteins and lipids, in addition to the core autophagic proteins. We are only beginning to understand how the different components interconnect and are regulated in time and space under various metabolic conditions and in different tissues. Perturbances in the interconnections or components of the pathway can lead to dysfunctional autophagy, which can result in tumorigenesis, immune disorders, neurodegeneration and aging (Plaza-Zabala, Sierra-Torre, & Sierra, 2017), infectious diseases (Deretic, Saitoh, & Akira, 2013) and diabetes (Laplante & Sabatini, 2012).

The origin of the phagophore membrane, and the mechanisms involved in its formation, have been under debate for a long time. There are evidences indicating that the endoplasmic reticulum (ER) (Axe et al., 2008), the mitochondria (Hailey et al., 2010), the ER-mitochondria contact sites (Hamasaki et al., 2013), the plasma membrane to recycling endosome trafficking process (Ravikumar et al., 2010; Bejarano et al., 2014; Puri et al., 2013) and the ER-Golgi intermediate compartment (ERGIC) (Ge et al., 2013; Ge, Zhang, & Schekman, 2014) can all act as membrane sources for autophagosome formation. There is no clear consensus on which endomembrane system provides membrane for the formation of autophagosome. Probably all of them - each under different conditions and in different ways.

## **Phospholipids and their binding proteins**

The autophagic pathway involves lipids as constituents, signaling molecules and as part of cargo in autophagosomes. Eukaryotic cells invest substantial resources and use ~5% of their genes in generating thousands of different lipids (Sud et al., 2007). Lipids serve three main functions in cells: energy storage, barrier function by being core constituents of membranes (structural lipids) and being second messengers in signal transduction. Glycerophospholipids are the major structural lipids in eukaryotic membranes and include phosphatidylcholine (PtdCho),

phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and phosphatidic acid (PA) (van Meer et al., 2008).

The bulk of the structural phospholipids and cholesterol are biosynthesized in the ER. The ER also harbors minor lipids that function as both pathway intermediates and pathway end products such as diacylglycerol (DAG), PA etc. (van Meer et al., 2008). Phosphoinositides (PI) are phosphorylated derivatives of PtdIns and function as key players in membrane dynamics and trafficking regulation (Schink et al., 2016). Phosphatidylinositol-3-phosphate (PI(3)P) is the major PI controlling autophagy. PI(3)P is produced from PtdIns by PIK3C3, one of the core components of the autophagy machinery (Suzuki et al., 2013).

PIK3C3 is targeted to contact sites between mitochondria and the ER by its autophagy-specific subunit ATG14L, for localized production of PI(3)P (Hamasaki et al., 2013) leading to formation of the phagophore. The PI(3)P binding effector proteins WIPI (WD repeat domain phosphoinositide-interacting proteins) (Grimmel et al., 2015) and DFCP1 (double FYVE domain containing protein 1) (Axe et al., 2008) are then recruited. DFCP1 has an ER-binding region and two FYVE domains that bind to PI(3)P containing subdomains on the ER. These subdomains are termed omegasomes as they have an  $\Omega$  shape, when observed through fluorescent microscope (Axe et al., 2008; Roberts & Ktistakis, 2013).

The PI(3)P level in the autophagic membrane is regulated by PI(3)P phosphatases, including Jumpy and myotubularin related phosphatase 3 (MTMR3), which dephosphorylate PI(3)P and thus negatively regulate autophagy (Vergne et al., 2009; Taguchi-Atarashi et al., 2010). PI(3)P dephosphorylation by Jumpy prevents recruitment of WIPI1 and other early autophagy proteins to the phagophore membrane (Vergne et al., 2009) and thus inhibits autophagy. There is also evidence for PIK3C3-independent autophagy, whereby PI(3)P may be produced by an alternate source, especially by the class II PI3K (Xue et al., 2003) and also good evidence that PI(5)P, generated by the PtdIns 5-kinase PIKfyve, promotes autophagy induced by glucose starvation (Vicinanza et al., 2015). Other phosphoinositides such as PI(3,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> have been implicated in autophagosome maturation (Rusten et al., 2007; Martin et al., 2013) and found to regulate mTOR activity, respectively (Dall'Armi, Devereaux, & Di Paolo, 2013).

Other phospholipids such as PE/PtdEtn and PA have been shown to be important for autophagy. PE/PtdEtn is mainly synthesized in the ER (Gibellini & Smith, 2010; Vance, 2015), but can also be produced from PtdSer in the mitochondria. PE/PtdEtn is shuttled from mitochondria to ER, through mitochondria–ER contact sites, and can be delivered efficiently from there to other organelles (Rowland & Voeltz, 2012). PE/PtdEtn is the target of LC3 and GABARAP conjugation. Being cone shaped in structure, PE/PtdEtn might further contribute to the membrane curvature and possibly to the regulation of autophagosome size, as insertion of PE/PtdEtn into membranes is known to induce local curvature stress (Marsh, 2007), which may facilitate formation of vesicular structures such as autophagosomes (Carlsson & Simonsen, 2015).

PA is also a cone-shaped membrane lipid thought to help membrane curvature if it sits on the inside of a curving membrane (Barr & Shorter, 2000). The role of PA in autophagy is however not clear. PA has been shown to activate the mTOR signaling by interacting on the same domain in mTOR where rapamycin interacts (Fang et al., 2001). The Phox homology (PX) domain (explained later) containing enzyme Phospholipase D1 (PLD1), which makes PA from PtdCho, was found to translocate to lysosomes in a VPS34 and PI(3)P dependent manner by amino acids known to stimulate mTORC1, suggesting their involvement in stimulating mTORC1 activity (Yoon et al., 2011). In contrast, PLD1 was shown to induce autophagy on starvation (Dall'Armi et al., 2010). Ablation of PLD1 inhibited autophagosome formation, indicating that PLD1 and its enzymatic product PA is implicated in autophagy. There are further studies confirming the role and importance of PLD1 in autophagy. Pharmacological inhibition of PLD1 results in enhanced levels of p62 and tau aggregates in organotypic brain slices, suggesting the role of PLD1 in starvation independent autophagy and its requirement for aggregate clearance (Dall'Armi et al., 2010). It was also seen that PLD enzymes are a critical source of DAG to initiate the host cell's antibacterial autophagy of *Salmonella typhimurium* (Shahnazari et al., 2010). Furthermore, PA can be generated from lysophosphatidic acid (LPA) by LPA acyltransferases (LPAATS) (Leung, 2001). Not much is known about LPAATs in the autophagic process, though it has been demonstrated that LPAATs are necessary for mTOR signaling (Blaskovich et al., 2013).

## **Autophagic phosphatidylinositol binding proteins**

Pleckstrin Homology (PH) domain from phospholipase C delta was the first identified lipid-binding domain (Garcia et al., 1995; Lemmon et al., 1995). Later, other PtdIns-binding domains were identified, including FYVE, FERM, PX, ENTH/ANTH, PROPPINS, TRAF and BATS (Hammond & Balla, 2015; Lystad & Simonsen, 2016). Only the ones seemingly having a role in autophagy will be discussed.

The FYVE domain is a zinc domain, that binds to PI(3)P and is found in several proteins implicated in autophagy. As mentioned earlier, DFCP1 has two FYVE domains and an ER binding domain that helps it bind to PI(3)P-enriched subdomains on the ER, called the omegasomes (Axe et al., 2008). The FYVE and coiled-coil domain-containing 1 (FYCO1) is another PI(3)P binding protein that facilitates autophagosome transport via binding to RAB7 and ATG8/LC3 on the autophagosome membrane (Pankiv et al., 2010; Olsvik et al., 2015). The autophagy-linked FYVE protein (ALFY, also called WDFY3, described in detail later) binds to PI(3)P through a C-terminal FYVE domain (Simonsen et al., 2004) and has been found to be required for degradation of protein aggregates by autophagy (Filimonenko et al., 2010). ALFY binds to autophagic membranes through GABARAP subfamily of the ATG8 family proteins (Lystad et al., 2014) and is known to interact with the ubiquitin binding autophagy receptor p62 (SQSTM1) (Clausen et al., 2010) and the ATG12-5-16L1 complex (Filimonenko et al., 2010).

The PX domain is found to interact mainly with PI(3)P but can also interact with PtdIns(4,5)P<sub>2</sub> or PtdIns(3,4)P<sub>2</sub> (Lystad & Simonsen, 2016). PLD1 (Dall'Armi et al., 2010), Sorting nexin 18 (SNX18) (Knævelsrud et al., 2013), HCLS1 binding protein 3 (HS1BP3) (Holland et al., 2016), Vacuolar morphogenesis 7 protein (Vam7p) (Cheever et al., 2001), Atg24 and Atg20 (Nice et al., 2002; Zhao et al., 2016) and Kip98a are some of the PX domain containing proteins implicated in autophagy (Lystad & Simonsen, 2016).

β-propellers that bind phosphoinositides (PROPPINS) display an essential PI(3)P effector function in autophagy conserved from yeast to human. The WIPI proteins (WIPI1-4 in humans) have affinity for both PI(3)P and PtdIns(3,5)P<sub>2</sub> via the two individual phosphoinositide-binding sites in the WD40 domain (Proikas-Cezanne et al., 2015). WIPI1 and WIPI2 are the best studied of the WIPI proteins, both being recruited to PI(3)P containing phagophores upon starvation (Proikas-Cezanne et al., 2004). WIPI2B and WIPI2D are the two functional splice variants of

WIPI2 that localize to autophagic structures upon induction of autophagy (Proikas-Cezanne et al., 2004). WIPI2B also binds specifically to ATG16L1 and recruits the ATG12-5-16L1 complex to autophagic membranes in a PI(3)P-dependent manner (Dooley et al., 2014).

### **Non-selective and Selective autophagy**

Autophagic degradation of intracellular components can be a non-selective bulk process (and was thought as the only mode of autophagy earlier) induced upon cellular stress like starvation to restore nutrient supply and ensure cell survival (Yang & Klionsky, 2010). It has lately become evident that autophagy is also a highly selective quality control mechanism to facilitate selective removal of toxic, superfluous or surplus structures (Reggiori et al., 2012). Based on the type of cargo, selective autophagy is subdivided into several sub-types, including lipophagy (autophagy of lipid droplets), ferritinophagy (autophagy of iron bound ferritin), lysophagy (autophagy of lysosomes), reticulophagy (autophagy of ER), ribophagy (autophagy of ribosomes), xenophagy (autophagy of pathogens), aggrephagy (autophagy of protein aggregates) and mitophagy (autophagy of damaged mitochondria) (Anding & Baehrecke, 2017)

Selective autophagy is characterized by specific recognition of the cargo, tethering of the cargo to a nascent phagophore and exclusion of non-cargo from the autophagosome (Zaffagnini & Martens, 2016). The recognition and tethering of the cargo to the phagophore is established via cargo receptor proteins, which confers selectivity by simultaneously binding the cargo and ATG8-family proteins on the phagophore membrane. The binding of the receptor proteins to the cargo may be direct or indirect. Indirect binding involves recognition of poly-ubiquitin chains attached to the surface of the cargo by specific ubiquitin-binding domains in the receptor proteins (Khaminets, Behl, & Dikic, 2016). Receptor proteins bind ATG8 family proteins through a short LIR (LC3-interacting region) motif. The LIR motif is a degenerate sequence with a common (W/F/Y)XX(L/I/V) (X = any amino acid) sequence (Svenning & Johansen, 2013)

### **Autophagy receptor proteins**

As mentioned above, autophagy receptor proteins link the cargo to be degraded to the autophagy membrane through their binding to cargo and membrane-conjugated ATG8-family proteins. The function of autophagy receptors can be regulated by protein phosphorylation, ubiquitination and oligomerization (Deng et al., 2017). Different types of autophagy receptors are known, including

p62/ Sequestosome1 (SQSTM1) and SQSTM1-Like Receptors (SLRs) that comprises optineurin (OPTN), NBR1 (neighbor of BRCA1), and NDP52 (nuclear dot protein 52 kDa); mitophagy receptors including FUN14 Domain Containing 1 (FUND C1), NIX, Atg32, Bnip3 and VCP/p97; specialized receptors as Cbl and Stbd1 and Cvt receptors including Atg19 and Atg34 (Svenning & Johansen, 2013; Wild, McEwan, & Dikic, 2014; Deng et al., 2017). Several review articles have elaborated further on the characterization and function of various autophagy receptors, and their importance in health and disease (Johansen & Lamark, 2011; Svenning & Johansen, 2013; Stolz, Ernst, & Dikic, 2014; Zaffagnini & Martens, 2016; Deng et al., 2017) and in this thesis I will limit the discussion to autophagy receptors involved in mitophagy (described in more detail in the “Mitophagy” section).

### **Autophagic adaptor proteins**

Autophagy adaptor proteins also interact with ATG8 family proteins through a LIR motif, but in contrast to autophagy receptors they do not bind cargo and are not themselves degraded by autophagy. Autophagy adaptors typically serve as scaffolds for the autophagy machinery and may modulate autophagy receptors in a way that can alter their affinity for LC3 and cargo (Deng et al., 2017; Stolz et al., 2014). Some of the known adaptor proteins are members of the ULK complex, ATG4B, FYCO1 and ALFY.

FYCO1 binds to PI(3)P via its FYVE domain in autophagic membranes in a LC3-dependent manner. It is the only reported LIR containing protein which interacts solely with LC3 of the ATG8 family proteins. It functions as an adaptor between autophagosomes and tubulin-coupled molecular motors (Pankiv et al., 2010). ULK1, ULK2, ATG13 and FIP200, proteins of the ULK complex, were shown to contain LIR motifs (Alemu et al., 2012). The LIR motif in ULK1 is conserved in the yeast Atg1 (ULK1 homolog in yeast) and its interaction with Atg8 is important for phagophore expansion (Kraft et al., 2012; Nakatogawa et al., 2012). Out of the four human homologs of yeast Atg4, ATG4B is the sole enzyme reported to efficiently cleave LC3 precursors and LC3-PE (Kabeya et al., 2004). It interact with LC3/GABARAPs via a LIR in its N-terminus (Satoo et al., 2009).

The importance of LIR motifs to establish interactions to ATG8 family protein is unambiguous and the list of LIR-containing proteins is constantly growing. It still remains to understand how these adaptor proteins prevent being degraded. Likely, they either avoid association within the

luminal side of the forming autophagosome or dissociate before the vesicle is formed. How this is regulated is not known, but one can speculate that post translational modifications of critical residues are involved.

### **The autophagy adaptor protein ALFY**

The autophagy-linked FYVE protein is a large (400kDa) scaffolding, multi-domain protein that was initially found to bind to PI(3)P and colocalize with autophagic markers (Simonsen et al., 2004). ALFY has three functional domains, including a PH-BEACH domain assembly, five WD40 repeats and a PI(3)P-binding FYVE domain (Isakson, Holland, & Simonsen, 2013). p62 binds to the PH-BEACH domain, while the WD40 repeats are important for interaction of ALFY with ATG5 (Clausen et al., 2010; Filimonenko et al., 2010). ALFY interacts with the GABARAP subfamily of ATG8 family proteins via a LIR motif in the WD40 domain (Lystad et al., 2014). ALFY was shown to be recruited to ubiquitin-positive protein inclusions under stress conditions (Simonsen et al., 2004) and deletion of the *Drosophila* ALFY homolog blue cheese (bchs), resulted in accelerated accumulation of ubiquitin-positive inclusions, neuronal degeneration and shorter life span (Finley et al., 2003). In line with this, ALFY was found to be involved in autophagic clearance of aggregated proteins (Filimonenko et al., 2010). Furthermore, ALFY was found indispensable for the turnover of midbody remnants (Pauline Isakson et al., 2013). Recently, it was established that ALFY is important for the proper development of the central nervous system in mice and its depletion resulted in major forebrain commissures throwing light on the importance of selective autophagy during development (Dragich et al., 2016). ALFY has also been implicated in determining brain size by attenuating the canonical wingless-type MMTV integration site family (Wnt) signaling pathway (Kadir et al., 2016).

### **Mitophagy**

Mitochondria are the powerhouses of eukaryotic cells, but also have key functions in cellular processes such as intermediary metabolism, calcium signaling and apoptosis. Although a vast majority of the mitochondrial proteins are encoded by the nuclear genome (almost 900) and imported to the mitochondria, several proteins required for their respiratory function are encoded by the mitochondria genome. The intricate mitochondrial network is dynamically maintained in order to remain healthy and meet changing demands for adenosine triphosphate (ATP). Any

disturbance in the mitochondrial homeostasis can lead to dysfunctional mitochondria, culminating in diseases such as ischaemia, diabetes and neurodegeneration (Chan, 2006). Dysfunctional mitochondria can lead to the over-production of reactive oxygen species (ROS) and the release of pro-apoptotic proteins into the cytoplasm, often with dire consequences, including deleterious mitochondrial DNA (mtDNA) mutations and protein damage (Youle & Narendra, 2011). Therefore, it is of utmost importance for the cell to get rid of superfluous or dysfunctional mitochondria and thereby uphold homeostasis and restore steady production of energy. The process by which damaged or superfluous mitochondria are targeted for degradation via autophagy is called mitophagy (Lemasters, 2005). Though the outer mitochondrial membrane (OMM) proteins can be targeted for degradation by the proteosomal machinery (Yoshii et al., 2011), mitophagy is the only known pathway by which the whole mitochondria can be selectively eliminated.

The degradation of mitochondria by autophagy was already reported in the late 1950s when Clark and Novikoff observed mitochondria within membrane-bound compartments called “dense-bodies”, which were later shown to contain lysosomal enzymes (Clark, 1957; Novikoff, 1959). The mechanism of mitophagy was however first characterized in yeast in 2004 (Kiššová et al., 2004). Mitophagy in yeast is a concerted effort of various players in response to a number of conditions including nitrogen starvation and rapamycin treatment (Bhatia-Kiššová & Camougrand, 2010). The outer mitochondrial membrane (OMM) protein Atg32 is an autophagy receptor important for mitophagy in yeast (Kiššová et al., 2004; Kanki et al., 2009; Okamoto et al., 2009). Atg32 interacts with Atg8 and the selective autophagy adaptor protein Atg11 (Kanki & Klionsky, 2008). The Atg32-Atg11 interaction is stabilized by the phosphorylation of serine residues (Ser<sup>114</sup>) on Atg32, leading to mitophagy in yeast (Aoki et al., 2011).

Atg32 and Atg11 do not have corresponding mammalian orthologues, but have functional homologues. The mammalian OMM protein NIX acts both like Atg32 and Atg11 (Matic et al., 2017). NIX is essential for the mitophagy-mediated elimination of mitochondria from the red blood cells during erythropoiesis in mammalian systems (Kundu et al., 2008; Zhang et al., 2009; Zhang & Ney, 2010) and its depletion was found to cause defective erythroid maturation and anemia in mice (Sandoval et al., 2008; Schweers et al., 2007). Mitophagy in reticulocytes is the best example of how healthy but redundant mitochondria are removed.

The process of mitophagy follows a general pattern that involves a receptor mediated mechanism, where the receptors physically connect the dysfunctional mitochondria to LC3 (via their LIR), leading to mitochondrial degradation. Receptors involved in mammalian mitophagy can be divided into two classes: mitochondrial membrane receptors and non-mitochondrial receptors.

### **Mitochondrial membrane receptors**

OMM proteins such as B-cell lymphoma 2 nineteen kilodalton interacting protein 3 (BNIP3), Nix, Bcl-2-like protein 13 (Bcl2-L-13) and FUNDC1 can act as mitophagy receptors under certain situations in certain cell types. All of them have a LIR that connects to either LC3 or GABARAP. BNIP3 and its analog Nix (described earlier) are pro-apoptotic proteins belonging to the Bcl-2 family. They were initially classified as proteins involved in programmed cell death, but later was shown also to act as mitophagy receptors (Zhang & Ney, 2009). Both BNIP3 and NIX can be regulated by hypoxia, where the transcriptional factor, hypoxia-inducible factor-1 (HIF-1) activates BNIP3 and NIX expression in response to low oxygen levels (Quinsay et al., 2010; Zhang & Ney, 2009). Studies have shown that serine residues adjacent to the LIR motif in BNIP3 have to be phosphorylated to activate mitophagy (Zhu et al., 2013). Another Bcl-2 family protein, Bcl-2-L13 was identified as a mitophagy receptor and homolog to the yeast mitophagy receptor Atg32. Bcl-2-L13 can mediate both mitochondrial clearance by mitophagy and also mitochondrial fragmentation (Murakawa et al., 2015).

Another OMM protein that gets activated and regulates mitophagy in response to hypoxia is FUNDC1. As BNIP3, FUNDC1 is also regulated by several reversible phosphorylations mediated by different kinases and phosphatases, including ULK1 (Wu et al., 2014). FUNDC1 shows no obvious effect on starvation induced bulk autophagy (Liu et al., 2012; Wu et al., 2016). AMBRA1 was also described to act as a mitophagy receptor. It regulates mitophagy by partially localizing to mitochondria and binding to LC3 through its LIR (Strappazzon et al., 2015). In a very recent report, the IMM membrane protein Prohibitin2 was found to mediate PINK1/Parkin mediated mitophagy in PARKIN-expressing HeLa cells. On mitochondrial insult, the OMM undergoes proteasome induced rupture that exposes Prohibitin2 to interact with LC3 (via a LIR) and mediate mitophagy (Wei et al., 2017). Cardiolipin, an IMM phospholipid, conducts mitophagy by translocating to OMM, on depolarization cues, where it acts as a mitophagy

receptor. Despite being a phospholipid, it also contains an LC3-binding motif and can directly bind the N-terminal domain of LC3-II (Chu et al., 2013; Maguire et al., 2017).

### **Non-mitochondrial receptors**

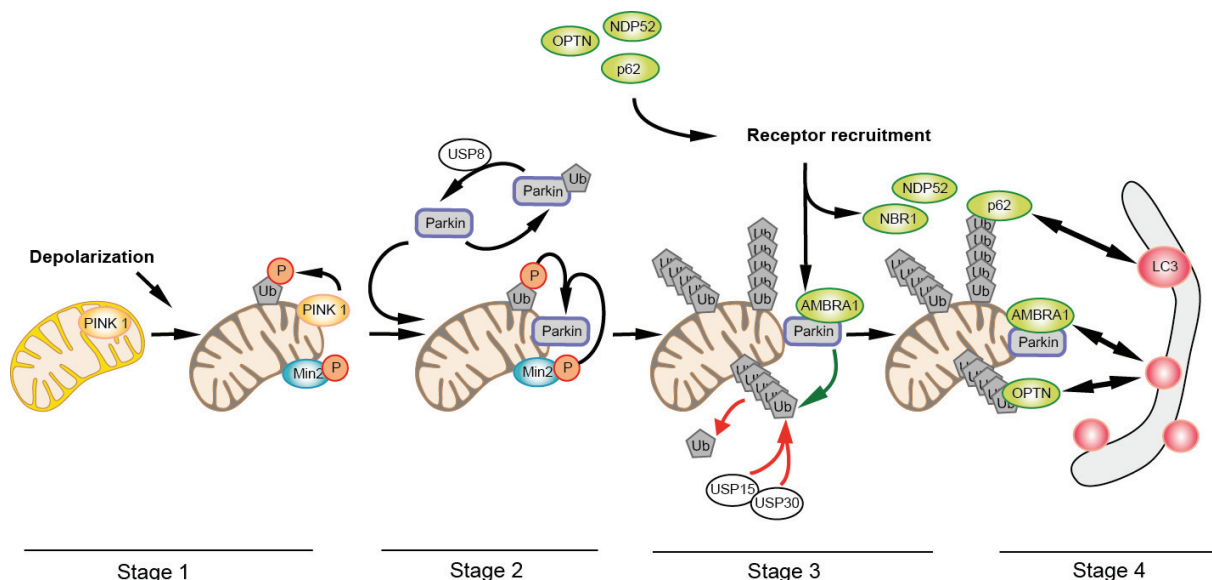
Mitophagy can also be regulated by cytosolic autophagy receptors proteins that recognize both the target mitochondria and the autophagosome via binding to poly-ubiquitinated OMM proteins and to LC3 (via their LIR), respectively (Wild et al., 2014). These receptors are recruited to the mitochondrial surface upon membrane induction of mitophagy. As the mitophagy process described in this thesis is dependent of PINK1/Parkin, I will describe this process and the receptors involved in more detail in the next few sections. To date, five different autophagy receptors (p62, NBR1, OPTN, NDP52 and TAX1BP1) have been implicated in PINK1/Parkin mediated mitophagy.

### **PINK1/Parkin-mediated mitophagy (Figure 3)**

The importance of PTEN-induced putative kinase 1 (PINK1) and Parkin in mitophagy came to light when seminal work by Richard Youle's group postulated that neuronal loss in Parkinson's disease (PD) may be due to the accumulation of dysfunctional mitochondria triggered by the loss of Parkin (Narendra et al., 2008). The different stages of PINK1/Parkin-mediated mitophagy is described in detail below.

#### **Stage I: PINK1 as a mitochondrial stress sensor (Figure 3)**

Under basal conditions, PINK1 is imported from the cytosol to the inner mitochondria membrane (IMM), in a process that involves TOM and TIM (translocase complexes) in a mitochondrial transmembrane-potential dependent manner (Kato et al., 2013; Ashrafi & Schwarz, 2013). PINK1 is then cleaved by MPP (mitochondrial processing peptidases) and PARL (Presenilin-associated rhomboid-like protease), both of which are mitochondrial matrix proteases.



**Figure 3: PINK1/Parkin-mediated mitophagy.** The whole process can be divided into 4 stages. Upon mitochondrial depolarization, PINK1 is stabilized at the OMM (Stage1). This results in the phosphorylation of ubiquitin and Mitofusin 2 and consequent recruitment of the E3 ligase Parkin (Stage2). De-ubiquitylation of Parkin by USP8 in the cytosol preps Parkin's translocation to the mitochondria. At the mitochondria, Parkin ubiquitylates OMM proteins, resulting in recruitment of ubiquitin-binding autophagy receptors such as p62, OPTN, and NBR1 which then can attach to autophagosomes via their LIR motifs (Stage3), resulting in the expansion and elongation of the autophagic membrane around the damaged and tagged mitochondria, to be delivered to lysosome for degradation (Stage 4). AMBRA1 also acts as a mitophagy receptor tethering to LC3 via its LIR. Deubiquitinating enzymes like USP15, USP30 etc helps remove the poly-ubiquitin chain and thus can modulate the whole process of mitophagy.

The resulting cleaved protein is degraded by ubiquitin-proteasome system (UPS) after being externalized back to the cytosol (Jin et al., 2010; Deas et al., 2011; Greene et al., 2012; Meissner et al., 2015). When damaged mitochondria lose their membrane potential, PINK1 accumulates in the OMM in association with the TOM complex, owing to incomplete processing of PINK1 (Jin et al., 2010). Activated PINK1 in the OMM triggers recruitment of Parkin and mitophagy of dysfunctional mitochondria (Figure).

#### Stage II: Recruitment of Parkin (Figure 3)

Before the translocation of PARKIN to mitochondria, its deubiquitylation by USP8 is required, in the cytosol (Durcan et al., 2014). PINK1-mediated translocation of Parkin to mitochondria involves two mechanisms. First, PINK1 at the OMM phosphorylates Mitofusin2 (MFN2) at serine-442 and threonine-111, and phosphorylated MFN2 can act as a receptor to recruit Parkin (Chen & Dorn, 2013). Second, PINK1 phosphorylates ubiquitin at serine-65, leading to

recruitment of Parkin owing to its high affinity to phosphorylated ubiquitin chains (P-UB chains) (Narendra et al., 2008). Once at the mitochondrial surface, Parkin too is phosphorylated at its serine-65 by PINK1, causing activation of Parkin (Sha, Chin, & Li, 2009; Shiba-Fukushima et al., 2012; Shiba-Fukushima et al., 2014; Kazlauskaitė et al., 2014). Active Parkin serves as an E3 ubiquitin ligase, promoting the ubiquitination of several mitochondrial surface proteins, that subsequently become phosphorylated by PINK1, generating a positive feedback loop mediated by PINK1 and Parkin (Riley & Olzmann, 2015; Matsuda & Tanaka, 2015). All this culminates to the damaged mitochondria being coated by P-UB chains that act as an “eat-me” signal for the mitophagy receptors. Parkin is however dispensable for the poly-ubiquitination of OMM proteins as other E3 UB ligases such as Seven In Absentia Homolog 1 (SIAH1) and gp78 (glycoprotein 78) can catalyze poly-ubiquitination in the absence of Parkin (Szargel et al., 2015; Fu et al., 2013).

#### Stage III: Autophagy receptors join the party (Figure 3)

Poly-ubiquitinated OMM proteins are recognized by different LIR-containing autophagy receptors, including p62/SQSTM1 (Geisler et al., 2010), Optineurin (Wong & Holzbaur, 2014), NBR1 (Hollville et al., 2014), NDP52 (Heo et al., 2015) and TAX1BP1 (Moore & Holzbaur, 2016b). p62 has been known to target diverse substrates for autophagy (Pankiv et al., 2007; Kim et al., 2008; Babu et al., 2005) and is known to cluster mitochondria during mitophagy (Narendra et al., 2010; Okatsu et al., 2010). There are reports stating p62 to be required (Geisler et al., 2010) or dispensable for mitophagy (Wong & Holzbaur, 2014; Narendra et al., 2010). The role of p62 in PINK1/Parkin-mediated mitophagy seems to be cell type dependent and context-specific. p62 is essential for PARKIN-dependent mitophagy in macrophages treated with inflammasome NLRP3 agonists (Z. Zhong et al., 2016). In a recent study, it was shown that OPTN and NDP52 are the main receptors required for PINK1/Parkin mediated mitophagy in HeLa cells expressing PARKIN and that others are dispensable (Lazarou et al., 2015; Baumann, 2015). However, the nature of the ubiquitinated OMM proteins recognized by these autophagy receptors is not clear. Thus depending upon situations, type of stress, cell type, organ type and organism type, there might be different players required for mitochondrial turn-over.

#### Stage IV: Stabilization by TBK1 and recruitment of autophagic machinery (Figure 3)

Soon after the recruitment of the autophagy receptors and their binding to the polyubiquitin

chains, the TANK binding kinase 1 (TBK1) is activated by mitochondrial depolarization and recruited by the autophagic receptors to the mitochondria, where it phosphorylates both OPTN and NDP52, strengthening their binding to ubiquitin chains (Heo et al., 2015; Richter et al., 2016). TBK1 has also been shown to positively regulate p62-ubiquitin binding by phosphorylating it at serine-403 (Matsumoto et al., 2011). The autophagy receptors via their LIR motif, recruit LC3 together with other autophagy machinery members, resulting in the expansion and elongation of the autophagic membrane around the damaged mitochondria to be delivered to lysosome for degradation. Deubiquitinating enzymes (DUBs) that can remove the P-UB chains require a special mention here as modulation of their activity can modulate the whole process of mitophagy. Different identified DUBs linked to mitophagy are USP30, USP35, USP8 and USP15 (Bingol et al., 2014; Cornelissen et al., 2014; Durcan & Fon, 2015; Wang et al., 2015)

### **Parkin-independent mitophagy**

The discovery that autosomal recessive mutations in PINK1 and PARKIN leads to loss of function and possible causality of PD led to a flurry of studies modelling PINK1 and Parkin *in vivo*. In *Drosophila*, it was shown that Parkin is important for mitochondrial maintenance (Greene et al., 2003) and that PINK1/Parkin deficiency triggered the accumulation of enlarged and damaged mitochondria in sperm, flight muscle and dopaminergic neurons, suggesting that this pathway influences mitochondrial integrity in energetically demanding tissues (Greene et al., 2003; Clark et al., 2006; Park et al., 2006). Interestingly, *PINK1/parkin* knockout mice show no apparent lack of gross physiological, neurological or behavioral phenotypes, questioning the importance of PINK1/Parkin-mediated mitophagy. However, post-natal conditional *parkin* knockout in mice did result in loss of nigral dopaminergic neurons (Shin et al., 2011; Stevens et al., 2015), indicating a possible compensatory mechanism in germline *parkin* knockouts during development. One obvious compensatory mechanism is Parkin-independent mitophagy.

Most *in vitro* Parkin dependent mitophagy studies have been performed using non-neuronal cells with over-expression of Parkin, where mitophagy is triggered by an uncoupler (e.g. carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP) or carbonilcyanide m-chlorophenylhydrazone (CCCP)), inducing a massive reduction of mitochondrial membrane potential. Such conditions are somewhat artificial and do not reflect the process of mitophagy in basal conditions, where a cell would want to turnover surplus mitochondria. The recruitment of

endogenous Parkin to mitochondria upon depolarization cannot be reproduced in a neuronal cell line, unless until Parkin is overexpressed and also this process is slower than in non-neuronal cell lines (Cai et al., 2012; Rakovic et al., 2013). This implies an alternate mechanism to maintain a healthy mitochondrial population in neurons, which might or might not involve PINK1 and Parkin.

Several studies have shown that mitophagy can happen independently of Parkin. Treatment of cells with iron-chelators was shown to induce mitophagy under glycolytic conditions, independent of PINK1 stabilization or Parkin activation in primary human fibroblasts, as well as in cells isolated from a PD patient with Parkin mutations (Allen et al., 2013). Another study found that the mitochondrial fission protein Drp1 and Parkin synergistically maintain the integrity of mitochondrial structure and function in mouse heart and brain, as simultaneous loss of Drp1 and Parkin worsened cardiac defects (Kageyama et al., 2014). Mitochondrial ubiquitination was independent of Parkin in Drp1KO hearts. In another study it was found that Synphilin-1 interacted with PINK1 and was recruited to the mitochondria, where it activates a PINK1-dependent mitophagy by recruiting LC3 and Lamp1 to the mitochondria. This occurred in the absence of Parkin, but relied on synphilin-1-mediated recruitment of SIAH-1 to the mitochondria where it promoted mitochondrial protein ubiquitination and subsequent mitophagy (Szargel et al., 2015). Recently, it was demonstrated that the OMM protein FKBP8, a member of the FK506-binding protein (FKBP) family, mediates Parkin-independent mitophagy. FKBP8 interacts with LC3A via a LIR motif in its N-terminal region, leading to efficient recruitment of LC3A to damaged mitochondria (Bhujabal et al., 2017; Lim & Lim, 2017).

## **Mitophagy and neurodegeneration**

Neurons are specialized post-mitotic non-proliferating cells with very high energy demands, requiring a healthy and highly functional mitochondrial population (Bélanger, Allaman, & Magistretti, 2011; Magistretti & Allaman, 2015). Their organelles are prone to accumulate oxidative damage and have to cope with high levels of  $\text{Ca}^{2+}$ . It is therefore of utmost necessity that the neurons maintain protein and organelle homeostasis, not just for their functionality but also for their viability. To this end, neurons rely on the UPS system and the autophagy pathway. Any tweak in these systems, can result in the accumulation of misfolded and/or aggregated proteins and dysfunctional mitochondria, which form the two main hallmarks of

neurodegeneration. I will describe in brief some of the common neurodegenerative disorders and later on describe in detail the role of mitophagy in the progression of PD.

### **Alzheimer's disease (AD)**

AD is the most common neurodegenerative disorder, resulting in severe memory loss and cognitive dysfunction. The neuropathological hallmarks of AD include “positive” lesions such as amyloid plaques and cerebral amyloid angiopathy, neurofibrillary tangles mostly constituted by hyperphosphorylated Tau proteins, glial responses, and “negative” lesions such as neuronal and synaptic loss (Serrano-Pozo et al., 2011). There is considerable evidence showing that alterations in mitochondrial dynamics and activity together with oxidative stress is associated with AD conditions (Nunomura et al., 2001; Cai & Tammineni, 2016) and ample evidences implicating dysfunctional autophagy in the etiology of AD (Nixon, Cataldo, & Mathews, 2000; Nixon et al., 2005; Ihara, Morishima-Kawashima, & Nixon, 2012). There is not much known about the role of mitophagy in AD (Khandelwal et al., 2011; Wang et al., 2016) and this could be worth looking into since mitochondrial dysfunction is a hallmark of AD and since AD neurons often show aberrant autophagy.

### **Amyotrophic Lateral Sclerosis (ALS)**

ALS is characterized by the selective degeneration of motor neurons. A hallmark of this disease is the aggregation of ubiquitinated proteins within the affected neurons (Blokhuys et al., 2013). Almost 90% of the cases are sporadic. Several genes linked to ALS have been identified in the familial cases through exome sequencing (Cirulli et al., 2015). Among the identified genes are the mitophagy receptors OPTN and p62, as well as TBK1, stabilizing binding of mitophagy receptors like OPTN and NDP52 to ubiquitin-chains via their phosphorylation. There are studies that confirm the role of these mitophagy receptors in ALS progression (Wong & Holzbaur, 2014; Moore & Holzbaur, 2016a) and it is highly likely that the inefficient turnover of damaged mitochondria and aggregates may contribute to neurodegeneration in ALS.

### **Huntington's Disease (HD)**

HD is an autosomal-dominant neurodegenerative disorder associated with cell loss within a specific subset of neurons in the basal ganglia and cerebral cortex. It is caused by an expansion of a cytosine-adenine-guanine (CAG) trinucleotide repeat encoding polyglutamine (PolyQ) tract

in the amino-terminal region of the Huntington (Htt) protein. HD is characterized by mobility dysfunction, cognitive deterioration and psychiatric disturbances caused by atrophy of GABAergic medium spiny neurons (Rubinsztein, 2003). Dysfunctional autophagic and endosomal systems, mitochondrial damage and proteostasis defects are linked to the pathogenesis of HD (Ross & Tabrizi, 2011)

### **Parkinson's disease (PD)**

PD is the second most common neurodegenerative disease affecting 1% of the population over the age of 60 and the most common movement disorder (Martinez-Vicente, 2017). PD is manifested by the cardinal motor symptoms characterized by bradykinesia, resting tremor, rigidity and postural instability (Szeto et al., 2015). The motor symptoms of PD become progressively worse as the disease advances. It can also be termed as a heterogeneous disease due to the lack of consistent symptoms overall, some may exhibit rigidity and bradykinesia whereas tremor is predominant in others (Xia & Mao, 2012)

At the molecular level, these symptoms are caused by the progressive degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and a resulting decrease of dopamine levels in the striatum (Gautier, Corti, & Brice, 2014). PD is also characterized by the presence of cytoplasmic protein inclusions called Lewy bodies (LBs) within the affected neurons, having  $\alpha$ -synuclein protein as their main component (Dauer & Przedborski, 2003). There is no cure for PD as of now despite great progression in biomedical research.

Although most cases of PD are sporadic, cases of familial PD have facilitated the identification of different autosomal recessive and dominant genes linked to PD, as well as single nucleotide polymorphisms (SNPs) identified as PD risk factors (Klein & Westenberger, 2016). The different monogenic forms of PD include genes linked to autosomal dominant forms, such as SNCA (synuclein alpha) and LRRK2 (leucine rich repeat kinase 2), as well as autosomal recessive forms such as PRKN (parkin RBR E3 ubiquitin protein ligase), PINK1, PARK7 (DJ-1) and ATP13A2 (ATPase cation transporting 13A2) (Klein & Westenberger, 2016). The ones that link PD to mitophagy are PRKN and PINK1 – main players in PINK1/Parkin mediated mitophagy, leading researchers to speculate that impaired mitochondrial turnover might be one of the major contributors to PD pathogenesis.

As mentioned earlier, Parkin and PINK1 knock out mouse models do not recapitulate the cardinal hallmarks of PD. Though they show an age-dependent moderate reduction of dopamine levels, they do not exhibit major abnormalities in the dopaminergic neurons (DA) neurons or striatal dopamine levels nor show the presence of LBs or show any drastic abnormalities in motor behavior (Gautier, Kitada, & Shen, 2008; Gispert et al., 2009; Goldberg et al., 2003; Von Coelln et al., 2004; Blesa & Przedborski, 2014). In contrast, Parkin and PINK1 mutant fly and zebrafish models have a more clear PD phenotype, including mitochondrial dysfunction, oxidative stress (ROS formation), dopaminergic neuronal loss, significant motor disabilities and reduced longevity (Greene et al., 2003; Pesah et al., 2004; Clark et al., 2006; Yang et al., 2006; Burman et al., 2012; Anichtchik et al., 2008; Xi et al., 2010; Flinn et al., 2009)

### **The mitochondrial protein – Nipsnap1**

The 4-nitrophenylphosphatase domain and non-neuronal synaptosomal associated protein 25 (SNAP25)-like protein homolog (NIPSNAP) family includes four proteins, NIPSNAP1-4 in humans (Seroussi et al., 1998; Brittain et al., 2012; Buechler et al., 2004). The name comes from the presence of the *C. elegans* Nipsnap gene in an operon harboring proteins with homology to 4-nitrosphenylphosphate (NIP) and synaptosomal associated protein 25 (SNAP) domains (Seroussi et al., 1998). The Nipsnaps all have a predicted mitochondrial targeting signal (MTS) in their N-terminus and one or two dimeric alpha-beta barrel (DABB) domains, also called Nipsnap domains, towards the C terminus. These domains exhibit an alpha-beta sandwich fold with an antiparallel beta sheet that forms a closed barrel and can be found in of many different protein families, especially mono-oxygenases including bacterial actinorhodin biosynthesis monooxygenase (ActVA-Orf6) (Sciara et al., 2003). Sequence alignment confirms high sequence homology between NIPSNAP1 and NIPSNAP2 and between NIPSNAP3 and NIPSNAP4, suggesting that NIPSNAP1-NIPSNAP2 and NIPSNAP3-NIPSNAP4 may have redundant functions. NIPSNAP1 has been shown to be highly expressed in brain, liver and kidney (Seroussi et al., 1998; Satoh et al., 2002; Schoeber et al., 2008; Tummala et al., 2010; Nautiyal et al., 2010), while NIPSNAP2 (also called GBAS (Glioblastoma Amplified Sequence)) is predominantly expressed in heart and brain (Wang et al., 1998; Martherus et al., 2010) and NIPSNAP3 and -4 expressed in brain, muscle and testis (Buechler et al., 2004)

Since Nipsnap in *C. elegans* was found in an operon coding for SNAP25, a protein involved in

synaptic vesicle fusion and docking, it was assumed that Nipsnaps would have a function in vesicular transport. To this end, it was found that a Nipsnap homologue (termed as TassC) is a host cell factor that determines vesicular trafficking in macrophages and is inactivated by Salmonella virulence factor SpiC. SpiC was found to interfere with vesicular trafficking via inhibition of the phagosome-lysosome maturation (Uchiya et al., 1999). The exact function of Nipsnap1 has however not yet been elucidated, though it has been linked with various diseases and has shown to be differentially expressed under different conditions.

NIPSNAP1 was found to be highly expressed in kainite treated mice that induced acute epilepsy (Sato et al., 2002) and lowly expressed in a cognitively impaired mice having the condition Phenylketonuria (PKU) – a genetic metabolic disorder (Surendran, Tying, & Matalon, 2005). NIPSNAP1 was found to localize to the mitochondria in catecholaminergic neurons where it was shown to colocalize with tyrosine hydroxylase (TH), a marker for dopaminergic neurons of SNpc and noradrenergic neurons in locus coeruleus (Nautiyal et al., 2010). Interestingly, NIPSNAP1 was shown to interact with dihydrolipoyltransacylase and -transacetylase components of the branched-chain  $\alpha$ -keto acid dehydrogenase enzyme complex (BCKDC) and pyruvate dehydrogenase (PDH) complexes *in vitro*, but a function for NIPSNAP1 in mitochondria metabolism was not shown (Nautiyal et al., 2010). In a study linking NIPSNAP1 and calcium, it was demonstrated that Nipsnap1 could modulate TRPV6-mediated  $\text{Ca}^{2+}$  entry (Schoeber et al., 2008), but it had no effect on L-type  $\text{Ca}^{2+}$  channels, which were more interactive with NIPSNAP2/GBAS (Brittain et al., 2012). NIPSNAP1 has also been linked to AD, as it was found that amyloid precursor protein (APP) forms a stable complex with Nipsnap1 in mouse brain and *in vitro* (Tummala et al., 2010). Interestingly, human AD brains were found to have aggregates of non-glycosylated APP in the protein import channels of mitochondria (Devi et al., 2006), but whether this is linked to NIPSNAP1 is not clear.

NIPSNAP1 has also been linked to modulation of inflammatory pain (Okuda-Ashitaka et al., 2012; Okamoto et al., 2016; Okuda-Ashitaka & Ito, 2015; Avenali et al., 2017). In a very recent study, it was shown that Nipsnap1 and -2 are stabilized by binding to mitochondrial HSP60, an essential chaperone. This study also found an interaction between NIPSNAP1 and the autophagy receptor p62/SQSTM1 (Yamamoto et al., 2017). Moreover, it was reported that NIPSNAP1 and -2 regulate proinflammatory cytokine and chemokine production induced by pattern recognition

receptors (PRR), linking NIPSNAP1 to innate immunity (Yamamoto et al., 2017). NIPSNAP1 has also been suggested to function as a tumor suppressor protein, as it was upregulated when a lung cancer cell line was treated with umbelliprenin, a natural coumarin known to have anti-tumor properties (Khaghanzadeh et al., 2016) and because of its total absence in the prostate cancer cell line WPE1-NB26 (Malhotra et al., 2013). In another study pertaining to the nervous system, it was found that NIPSNAP1 was strongly upregulated in a mental disorder condition characterized by a behavioral phenotype called prepulse inhibition, induced by the knockout of *Xbp1* in mice (Takata et al., 2010).

NIPSNAP1 has also been shown to play a role during development. In a study to identify human liver proteins associated with different stages of liver development, NIPSNAP1 was found to be highly upregulated in 16 week old liver sample, but steadily decreasing thereafter, suggesting its importance in early development (Brizard et al., 2009). *nipsnap1* was also identified as one of the most highly induced genes (upregulated 7.9 folds) in a microarray approach to identify novel direct bone morphogenetic pathway (BMP) target genes involved in early embryonic development of *Xenopus*. This was confirmed by in situ hybridization as *nipsnap1* expression partially resembled that of BMP4 (known BMP marker) (Peiffer et al., 2005). It was also demonstrated that *nipsnap1* is very highly upregulated during the trans-differentiation from cornea to lens, and in situ hybridization showed the stable expression of *nipsnap1* in eye structures in *Xenopus* larvae (Day & Beck, 2011).

Taken together, it is clear that NIPSNAP1 is a very important protein, having pleiotropic functions both during the early life stages and later on. It seems to play a key role in the nervous system, but the molecular mechanisms underlying the functions of NIPSNAP1 still remain poorly characterized.

## **Autophagy in zebrafish**

Our understanding of how autophagy is regulated under different physiological and pathological conditions is largely based on research performed in different tractable animal model systems such as the fruit fly *Drosophila melanogaster* (Rearick Shoup, 1966; Juhász et al., 2003) nematode *Caenorhabditis elegans* (Meléndez et al., 2003), the mouse *Mus musculus* (Mizushima et al., 2001; Mizushima et al., 2003), *Crassostrea gigas* (Pacific oysters) (Moreau et al., 2015)

and *Dictyostelium discoideum* (Calvo-Garrido et al., 2010). This has provided insights into the role of autophagy in the pathophysiologies of different diseases, including tumorigenesis, immune disorders, neurodegeneration and aging (Plaza-Zabala et al., 2017), infectious diseases (Deretic et al., 2013) and diabetes (Laplane & Sabatini, 2012; Saxton & Sabatini, 2017)

There has been a steep rise in zebrafish research owing to the various advantages that it offers, including its small size, high fecundity, external fertilization, transparent embryos, rapid development and genetic tractability (Basu & Sachidanandan, 2013). Research on autophagy using zebrafish as a model organism got a jump start when Klionsky and colleagues generated GFP-LC3 and GFP-Gabarap transgenic zebrafish lines (He et al., 2009). Several existing studies of autophagy in zebrafish have presented invaluable insight into the role of autophagy in development, disease progression and drug discovery. We have recently published a review on “Studying autophagy in zebrafish” where we review the current literature and the methods used to study autophagy in zebrafish, including DNA, RNA and protein-based methods. We also discuss different types of selective autophagy, with emphasis on mitophagy, xenophagy and aggrephagy and how these can be studied in zebrafish and provide detailed information about different antibodies, chemical reagents and reporter lines that have been used to analyze autophagy in zebrafish and further discuss how current methods could be improved to better understand autophagy in zebrafish (Mathai et al., 2017). The review is included in this thesis as paper IV.

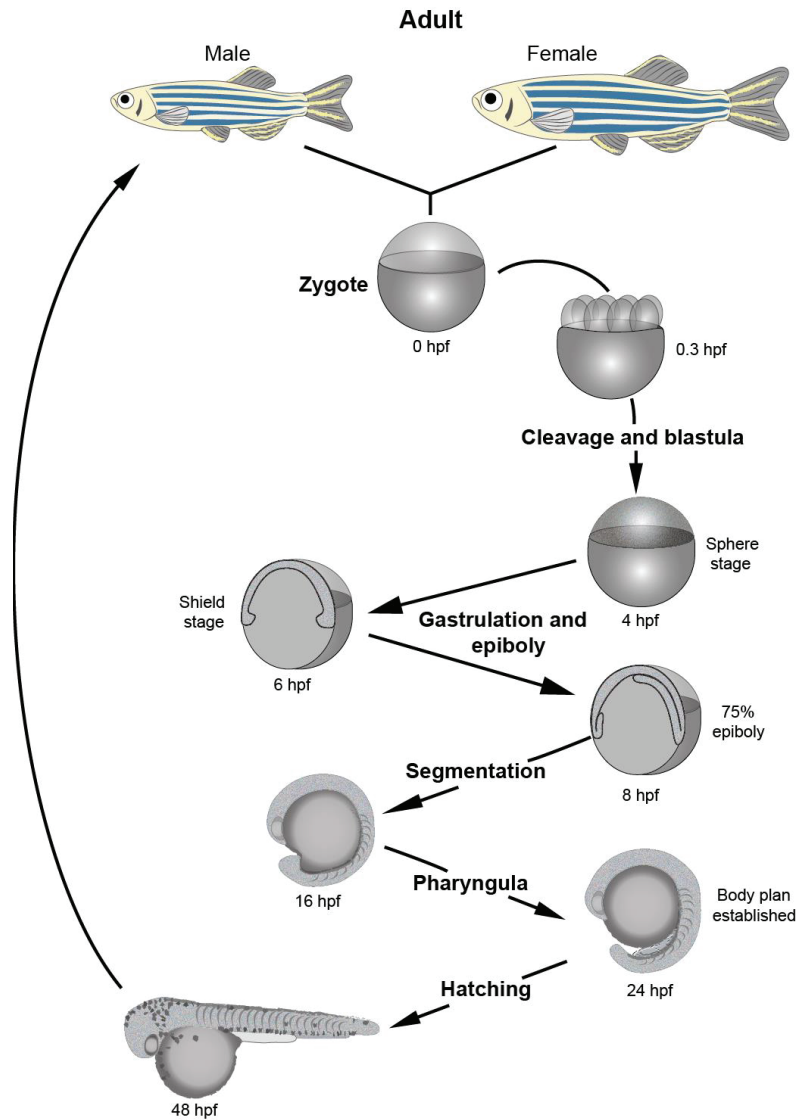
## **Zebrafish as a model organism and its development**

Zebrafish is a small slender fish characterized by white and neon blue stripes, native to paddy fields of East India and Myanmar. It was first recognized by George Streisinger – better known as the father of zebrafish genetics – as a potential model organism for scientific research and he introduced it in his lab in the late 1960’s. But it was only in the 1980’s that fruits of his decade long hard work came to bore. He inspired a generation of core zebrafish biologists like Charles Kimmel who is now recognized as a pioneer in zebrafish developmental genetics. Christiane Nüsslein-Volhard and her ex-student Wolfgang Driever pioneered the largest screen ever in zebrafish biology in the 1990’s, which culminated in 37 papers in 1996 in a special zebrafish edition of ‘Development’ putting forward over 4000 mutations (Nüsslein-Volhard, 2012; Driever et al., 1996). This opened the doors to further “forward genetic” screens. The past decade or so

has seen an exponential rise in the use of zebrafish to probe for answers, owing to the enormous advantages that it offers. Several “reverse-genetic” tools have been evolving at an unprecedented rate. Morpholino oligonucleotides were considered a landmark advance; zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) soon took over, but the introduction of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR Associated Protein 9 (Cas9) technology is currently driving a revolution in biomedical research. The ease at which these “reverse-genetic” tools can be employed in zebrafish makes it highly desirable to work with.

Zebrafish development (Figure 4) begins with the maturation (the process of oogenesis) and then the fertilization of the oocyte. This is followed by transition of the newly fertilized egg through cellular cleavage, to gastrulation, and then patterning of its pluripotent embryonic cells into a fully formed organism. These events are tightly regulated and involve the precise coordination and regulation of multiple signaling pathways and morphogenetic movements to establish the body plan. Prior to gastrulation the primary axes of the vertebrate embryo are established, which provide the foundation for its body plan. This requires dynamic molecular cues that help differentiate between the animal pole, marked by the blastodisc and the vegetal pole where the yolk sac resides (Langdon & Mullins, 2011). The primary axes formation and subsequent patterning of multiple tissues around the axes is mediated by maternal and zygotic factors acting through Wnt, BMP, Nodal, and fibroblast growth factor (FGF) signaling pathways (Langdon & Mullins, 2011). Nodal and BMP are part of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of growth factors that contain over 30 members including TGF- $\beta$ s, growth and differentiation factors (GDFs) and Activins (Feng & Derynck, 2005). They play key roles in embryonic stem cell self-renewal, gastrulation, differentiation, organ morphogenesis, and adult tissue homeostasis (Feng & Derynck, 2005; Weiss & Attisano, 2013). These are vital for development and homeostasis of most organisms.

The signal transduction pathways utilized by TGF- $\beta$  growth factors involve binding of ligand dimers to heteromeric complexes of type I and type II transmembrane receptors, leading to their activation. Activated receptors then phosphorylate the intracellular mediators (Smads), which form complexes with each other and other proteins to modulate transcription of target genes in



**Figure 4: Snapshot of stages of development in zebrafish.** The zebrafish development can be classified into 7 broad periods starting from the formation of zygote stage where the newly fertilized egg preps itself for the first cleavage. This is followed by the cleavage stage, that results in the formation of 16 celled blastomeres. Next is the blastula stage, where flattening of the cellular materials occur leading to the formation of blastula, when the blastodisc begins to look ball-like, at the 128-cell stage. Next is the gastrulation stage, starting from the morphogenetic cell movement of involution and ending with the formation of tailbud. This is followed by the segmentation stage which involves the development of somites and rudiments of the primary organ become visible and also results in the elongation of the embryo. Pharyngula stage marks the presence of a well-developed notochord with newly-completed set of somites, with a 5-lobed sculpted brain. The final stage is the hatching stage where the embryo rupture the egg membrane or the chorion to break free (Kimmel et al., 1995)

the nucleus (Wu & Hill, 2009). Thus, TGF- $\beta$  signaling pathway components (Figure 5) includes ligands, extracellular ligand binding proteins, the heteromeric serine/threonine kinase, cell

surface-associated co-receptors, the Smads and non-Smad signaling (Weiss & Attisano, 2013). Early vertebrate division and signaling events prior to gastrulation is beyond the scope of this thesis and is elaborated elsewhere (Kimmel et al., 1995). The TGF- $\beta$  pathway and two of its effectors – BMP and the Nodal pathway, are described in more detail below.

### **The Nodal signaling pathway during zebrafish development**

The Nodal signaling pathway is initiated around the gastrulation stage of the zebrafish embryogenesis. It primarily establishes two axes of the vertebrate body plan. First, it specifies and patterns mesendodermal tissues around the animal-vegetal axis (as called in xenopus and zebrafish) (Feldman et al., 1998; Shen, 2007) and second, Nodal functions to break the symmetry of the embryo around the left-right axis of the embryo, shortly proceeding the gastrulation (Rebagliati et al., 1998; Lin et al., 2017). The initial nodal expression is triggered by dorsally localized  $\beta$ -catenin (Feldman et al., 1998). A regulatory element known as the asymmetric enhancer element (ASE), deeply conserved within the first intron of Nodal, helps activating the expression of nodal itself. ASE contains the binding site for Smad2 cofactor FoxH1 (Fan & Dougan, 2007). During gastrulation, Nodal patterns the formation of germ layers by triggering the involution and ingression of Nodal specified mesendodermal cells (Feldman et al., 2000). Loss-of-function nodal pathway zebrafish mutants fail to specify the mesendoderm and the cells fail to ingress (Gritsman et al., 1999; Feldman et al., 2000)

Ligands: The three nodal genes in zebrafish are *ndr1* (*squint*), *ndr2* (*cyclops*) and *ndr3* (*southpaw*). Elimination of two of the three nodal genes, namely *ndr1* (*squint*) and *ndr2* (*cyclops*), is required to completely eliminate mesendoderm specification (Feldman et al., 1998; Rodaway et al., 1999). *ndr3* (*southpaw*) is not expressed during gastrulation and is indispensable later for LR patterning (Long, Ahmad, & Rebagliati, 2003).

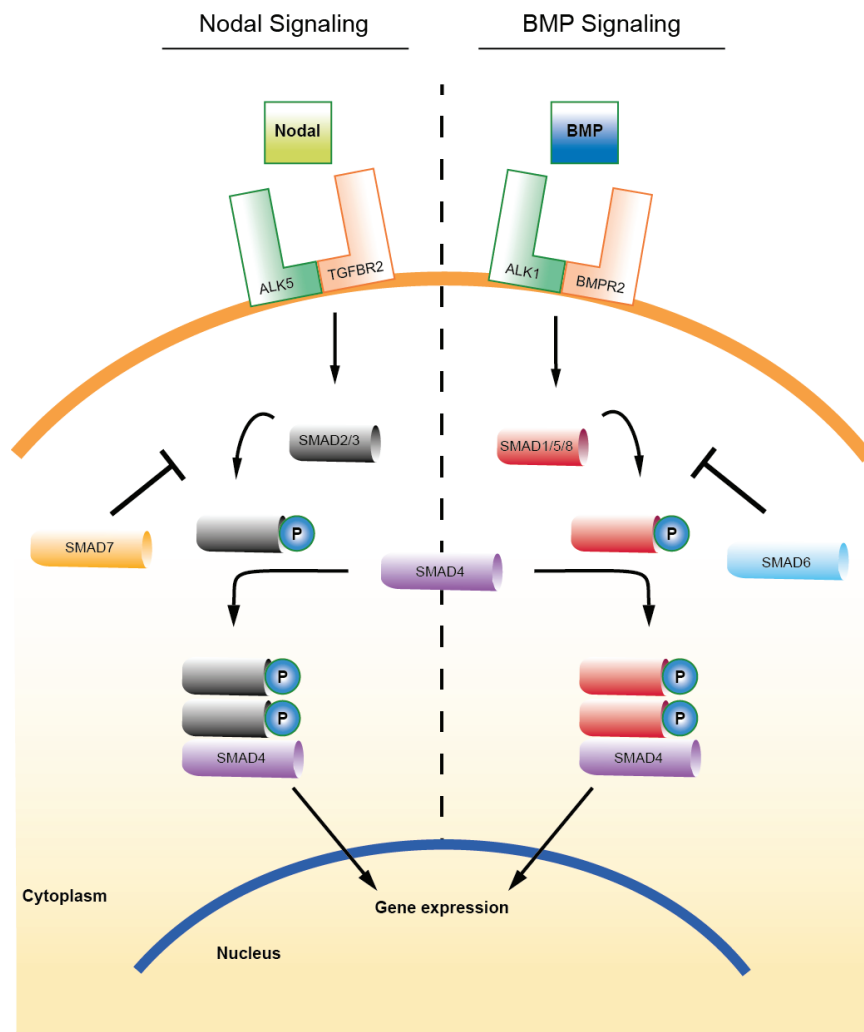
Intracellular effectors and co-factors: On binding of the Nodal on its receptor complex, the type I receptor phosphorylates the primary effectors Smad2 and Smad3 (Attisano & Wrana, 2002). Loss of *smad2* function in zebrafish abolishes mesendodermal specification (Dubrulle et al., 2015). As mentioned before, Smad2 does not bind to DNA directly and hence it requires a co-factor that associate to DNA to regulate transcription. During mesendodermal patterning, the important co-factor is FoxH1 (Fan & Dougan, 2007), whose ablation results in truncation of body axis, loss of anterior mesoderm and impaired formation of craniofacial structures (Slagle,

Aoki, & Burdine, 2011). In FoxH1's absence, the Nodal still patterns mesendodermal tissues through other co-factors like eomesodermin and Mixl1 (Slagle et al., 2011). Also the E3 ubiquitin ligase Siah2 enhances Nodal signaling activity (Kang et al., 2014)

**Inhibitors:** Nodal activates the expression of many of its own inhibitors, including the extracellular antagonists Lefty and Cerberus as well as the intracellular inhibitor Smad7. Negative feedback is as important as the positive ones - both of which are invoked by the Nodal signaling - for proper mesendoderm patterning and LR axis formation. Lefty genes include *lefty1* and *lefty2* in zebrafish. Lefty proteins have been shown to function by blocking EGF-CFC (Oep in zebrafish) co-receptors, therefore prevention interaction with receptor complex and hence Nodal signaling (Sakuma et al., 2002; Zinski, Tajer, & Mullins, 2017)

### **Bone Morphogenetic Protein (BMP) signaling pathway during zebrafish development**

The BMP pathway runs parallel to the Nodal signaling pathway and patterns tissues along an axis perpendicular to the animal-vegetal axis, called the dorsal-ventral (DV) axis of the blastula and gastrula embryo (Little & Mullins, 2006). High and intermediate levels of BMP signaling induce ventral tissue fates such as epidermis, blood and lateral tissue such as neural crest respectively whereas they should be completely blocked for dorsal tissue development into notochord, brain and prechordal plate tissues (Little & Mullins, 2006; Zinski et al., 2017). BMP ligands are secreted ventrally and ultimately bind to two type I and two type II receptors to activate the signaling pathway. This receptor complex then allows the constitutively active type II receptors to phosphorylate the type I receptors (Wrana et al., 1994). The type I receptors phosphorylate Smad1, Smad5 and Smad8 (also called Smad9), which forms complex with Smad4 and accumulate in the nucleus, thereby inducing BMP target genes (Dutko & Mullins, 2011; Little & Mullins, 2006)



**Figure 5: Nodal and BMP Signalling.** Two of the effectors of TGF- $\beta$  pathway are Nodal and BMP signaling. Nodal ligands namely *ndr1*, *ndr2* and *ndr3* bind to its receptor complex, so does BMP ligands such as *bmp2*, *bmp4* and *bmp7* bind to its respective receptor complex. Type I receptors phosphorylate Smad2/3 and Smad1/5/8 of Nodal and BMP signaling respectively. Smad2/3 and Smad1/5/8 then forms a complex with Smad4 and accumulate in the nucleus to induce transcription of respective target genes. Smad 7 can inhibit Nodal signaling whereas Smad 6 can inhibit BMP signaling (Zinski et al., 2017).

Ligands: Zebrafish mutants defective in dorsoventral patterning have helped identify the genes encoding BMP signaling components including *swirl/bmp2b*, *snailhouse/bmp7a*, *somitabun/smad5*, *lost-a-fin/alk8*, *mini fin/tolloid*, *chordin/chordino* and *ogon/sizzled*. Loss of either *bmp2* or *bmp7* causes a loss of all ventral tissues leading to embryonic lethality during somitogenesis whereas loss of *bmp4* has a much milder effect on DV patterning (Langdon & Mullins, 2011).

Intracellular effectors: Smad1, Smad5 and Smad8 are the known intracellular transducers or effectors of the BMP signaling pathway in vertebrates, which get phosphorylated by the type I receptors. Smad1/5/8 then forms a complex with Smad4 and accumulate in the nucleus to induce transcription of BMP target genes (Wrana et al., 1994; Little & Mullins, 2006; Dutko & Mullins, 2011).

Antagonists: The dorsal organizer or the Spemann-Mangold organizer is the region where the gastrulation movements begin. The dorsal organizer expresses the BMP antagonists and transcriptional repressors essential to repress BMP signaling in the dorsal region of the embryo (Thisse & Thisse, 2015). BMP antagonists include Chordin, Noggin and Follistatin that bind to BMP ligands in the extracellular space, preventing BMP signaling dorsally. These antagonists are opposed by ventrally expressed metalloproteases such as Tolloid and Bmp1, which cleave Chordin and release the BMP ligand (Langdon & Mullins, 2011; Zinski et al., 2017)

## **AIMS OF THE STUDY**

The major aim of this thesis was to advance our understanding of the mechanisms involved in non-selective and selective autophagy by investigating the role of lipid-binding proteins, in particular the PX-domain containing protein HS1BP3 and the proteins NIPSNAP1 and NIPSNAP2 found to interact with the FYVE domain containing protein ALFY. The specific aim of this thesis was to investigate the in vivo function of these proteins using zebrafish as a model organism.

### **Paper I: HS1BP3 negatively regulates autophagy by modulation of phosphatidic acid levels.**

The PX-domain containing protein HS1BP3 was found to negatively regulate autophagy in mammalian cells and zebrafish. The study was aimed at unravelling the mechanisms by which HS1BP3 affects autophagy, throwing light on its lipid binding properties and localization, thereby providing knowledge on the overall role of lipids in autophagy.

### **Paper II: NIPSNAP1 and NIPSNAP2 facilitate mitophagy to inhibit ROS production and neuronal death.**

The NIPSNAP domain containing proteins NIPSNAP1 and NIPSNAP2 were identified as interactors of the PI(3)P-binding autophagy adaptor protein ALFY, different autophagy receptors and ATG8 family proteins. The aims of the study were to understand the functional significance of these interactions and the role of NIPSNAP proteins in cells and zebrafish.

### **Paper III: Lack of Nipsnap1 causes gastrulation arrest in zebrafish.**

In paper II we elucidated the role of NIPSNAP1 and -2 as facilitators of mitophagy in mammalian cells and showed that Nipsnap1-mediated mitophagy in zebrafish brain is important for neuronal health. The aim of this study was to further characterize the role of Nipsnap1 during early zebrafish embryogenesis.

### **Paper IV: Studying autophagy in zebrafish.**

In this invited review, we discuss the past, present and future of zebrafish as a model organism to analyze autophagy.

## SUMMARY OF INCLUDED PAPERS

### **Paper I: HS1BP3 negatively regulates autophagy by modulation of phosphatidic acid levels.**

In a previous study, our lab performed a siRNA screen aimed to identify novel lipid-binding proteins involved in the membrane modulations and regulations happening during the process of autophagy (Knævelsrud et al., 2013). This screen yielded many interesting candidates, including the PX-domain containing protein HS1BP3, which was identified as a negative regulator of autophagy. In the current study, we show that depletion of HS1BP3 increases LC3 lipidation and LC3 spot formation both under basal and starved conditions. Autophagic flux was found to be increased in cells lacking HS1BP3, as analyzed by long lived protein degradation (LLPD) and by the degradation of autophagy cargo receptor SQSTM1/p62. Autophagy was also inhibited in zebrafish larvae at 2dpf, where autophagic flux estimation showed a significant increase in Lc3 spots in the trunk area upon morpholino induced knockdown of Hs1bp3, an effect that was rescued by co-injection of human Hs1bp3 mRNA. This indicates a conserved role of Hs1bp3 across species.

HS1BP3 was shown to colocalize with structures positive for ATG9 and ATG16L1, but not with DFCP1 or WIPI2. These structures were also positive for transferrin receptor (TfR), suggesting they are recycling endosome membrane. Furthermore, using live cell imaging it was observed that HS1BP3-positive structures fuse with LC3-positive structures, suggesting that HS1BP3 localizes to recycling endosome derived membranes and contributes to membrane formation at a stage after the initial omegasome is formed.

Interestingly, by overexpressing different parts of HS1BP3, we found that the PX domain inhibited autophagy to a similar degree as WT, indicating that lipid binding through the PX domain is responsible for its negative effect on autophagy. PX-domain proteins are known to bind to PI(3)P and also other phosphoinositides (Teasdale & Collins, 2012). Using lipid coated strips and liposome floatation assays we show that HS1BP3 binds to PA and several other phosphoinositides, which led us to speculate that HS1BP3 might be affecting autophagy through PA or other lipids. To get an unbiased view on the total cellular lipid content in starving cells depleted of HS1BP3, we performed a lipidomic analysis. PA was found to be the single most

significantly upregulated lipid in a pool of other lipids, thus confirming the idea that it is through PA that HS1BP3 affects autophagy.

Next, we explored a role of HS1BP3 in the various pathways of PA production and found that the activity of the PA-producing enzyme PLD1 was increased with HS1BP3 depletion. By imaging studies we determined that PLD1 also localizes to ATG16L1-positive recycling endosome membranes and that its recruitment to these membranes is inhibited by overexpression of HS1BP3, indicating that HS1BP3 negatively regulates autophagy by modulation of PLD1 activity and PA levels at autophagy precursor membranes.

## **Paper II: NIPSNAP1 and NIPSNAP2 facilitate mitophagy to inhibit ROS production and neuronal death.**

NIPSNAP1 and -2 were identified in two independent screens aimed at identifying interacting partners of the autophagy adaptor protein ALFY and the autophagy receptor protein SQSTM1/p62, through immunoprecipitation and mass spectroscopy analysis. The interactions were confirmed by GFP-pulldown assays, wherein NIPSNAP1 was found to interact with both ALFY and p62, independent of each other. NIPSNAP1 and -2 were also found to directly associate with the autophagy receptors p62, NBR1, NDP52 and TAX1BP1 and shown to interact with human ATG8 family proteins, thus confirming earlier studies identifying NIPSNAP1 and -2 in the LC3 interactome (Behrends et al., 2010; Rigbolt et al., 2014). Together, these interaction studies suggested a possible role of NIPSNAP1 and -2 in selective autophagy.

NIPSNAP1 and -2 were both found to be mitochondrial matrix proteins. Cloning and imaging experiments showed that the N-terminal 23 amino acids of NIPSNAP1 were sufficient and essential for import into the mitochondrial matrix. Interestingly, deletion of this N-terminal signal resulted in NIPSNAP1 recruitment to the outer mitochondrial surface and we could show that amino acids 24-59 were sufficient to localize EGFP to the mitochondrial surface. Thus we have characterized two MTS in NIPSNAP1 – amino acid 1-23 being required for mitochondria import and amino acids 24-59 being required for efficient targeting to the mitochondria surface. In line with NIPSNAP1 having a signal for binding to the mitochondria surface we observed a fraction of NIPSNAP1 on the mitochondrial surface, which was increased upon mitochondrial depolarization, thus suggesting the need of a mitochondrial membrane potential for mitochondria import of NIPSNAP1, but not for its recruitment to the surface. Mitochondrial stressors like

hypoxia and uncouplers (e.g. CCCP) led to an increased interaction between NIPSNAP1 and -2 with autophagy receptors p62 and NDP52 and between NIPSNAP1 and GABARAP, suggesting the role of NIPSNAPs as “eat-me” signals for mitophagy.

CRISPR/Cas9 based knockout of NIPSNAP1 or -2 alone in HeLa PARKIN cells did not inhibit mitophagy, as analysed by TIM23 or COXII degradation upon treatment with OA or CCCP, although mitophagy was inhibited in ATG7 KO cells. However double knockouts (N1/N2 DKO) blocked both OA and CCCP induced mitophagy, which could be rescued by re-expression of either NIPSNAP1 or -2 in these DKO cells. We also showed that recruitment of NIPSNAP2 to the mitochondrial surface is sufficient for rescue. Furthermore, our data show that NIPSNAP1/2 are required for recruitment of autophagy receptors to mitochondria, as this was considerably reduced in the N1/N2 DKO cells, although recruitment of PARKIN and ubiquitination of OMM was unaffected. Taking together, we conclusively show that NIPSNAP1 and -2 are required for PARKIN-dependent mitophagy in mammalian cells and have a redundant function.

To investigate the functional significance of NIPSNAP1/2-mediated mitophagy in vivo we used zebrafish having *nipsnap1* depleted by CRISPR/Cas9 or a *nipsnap1* mutant line. Indeed, our data further indicate the importance of Nipsnap1 for mitophagy in zebrafish brain, as analyzed by transient expression of a tandem-tagged mitochondrial protein construct microinjected in *nipsnap1* mutant zebrafish embryo and control (WT) zebrafish embryo. Nipsnap1 ablation in zebrafish larvae showed phenotypes characteristic of parkinsonism, including high levels of oxidative stress, loss of dopaminergic neurons and locomotor defects. Locomotor defects could be rescued by exogenous addition of L-Dopa. Thus we conclude that NIPSNAP1 and -2 facilitate mitophagy to inhibit ROS production and neuronal death.

### **Paper III: Lack of Nipsnap1 causes gastrulation arrest in zebrafish.**

In earlier studies *Xenopus Nipsnap1* was suggested to be a direct target of BMP signaling pathway (Peiffer et al., 2005) and shown to be important for trans-differentiation from cornea to lens in *Xenopus* (Day & Beck, 2011). This suggested a probable role of Nipsnap1 during early embryogenesis. Indeed, zebrafish *nipsnap1* KO larvae showed a high mortality rate by 1dpf indicating its importance during early zebrafish embryogenesis. The surviving larvae were highly dorsalized with varied phenotypes, prominent being cyclopia. Dorsal, endodermal and mesodermal markers were highly upregulated in Nipsnap1 KO zebrafish larvae as compared to

control during the shield stage of development, as shown by *in situ* hybridization and qRT PCR experiments. We also found total Smad5 (effector of BMP pathway in complex with Smad1 and Smad8/9) and Smad2/3 (effector of Nodal pathway) levels to be low, indicating that Nipsnap1 causes gastrulation arrest in zebrafish.

We have preliminary data showing upregulation of mitochondrial serine/threonine-protein kinase, PINK1 in Nipsnap1 KO zebrafish larvae at the shield stage, suggesting mitophagy may be affected at this stage and that Nipsnap1 is important for this. Further experiments are needed to conclude what pathway and which processes are affected.

#### **Paper IV: Studying autophagy in zebrafish.**

This review outlines a brief history on autophagy and the core components involved. It also tabulates all zebrafish autophagy genes, past, present and future of DNA, RNA and protein based techniques used to analyze autophagy in zebrafish accompanied with review of literature of instances using the particular technique for the same. The review also documents different antibodies that can be used for zebrafish autophagy studies, different drugs and optimal concentrations that one can use and different autophagy based transgenic lines that could be advantageous for autophagy studies. The field of autophagy would benefit tremendously from using zebrafish, owing to their immense potential in identifying therapeutics against diseases and this review gives a brief “peek” into what have been done and possible future directions.

## DISCUSSION

The major outcome of this thesis is the identification and characterization of three proteins, the PX-domain containing protein HS1BP3 and the two NIPSNAP domain containing proteins NIPSNAP1 and NIPSNAP2, in regulation of non-selective and selective autophagy, respectively. HS1BP3 was identified as a negative regulator of autophagy from a siRNA-based imaging screen targeting all human PX-domain proteins to identify new regulators of autophagy. NIPSNAP1 and NIPSNAP2 were identified as unique interactors of the FYVE-domain containing protein ALFY and of the well-known autophagy receptor p62, using mass spectrometry analysis of the anti-ALFY antibody precipitates from ALFY<sup>+/+</sup> and ALFY<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) (Dragich et al., 2016) or from GFP-TRAP pulldown of HEK293 cells stably expressing GFP-p62. The common link between the different studies in this thesis is the interconnection between lipids and lipid-binding proteins involved in regulation of autophagy. The phosphoinositide PI(3)P is important for the initiation of autophagy and for localizing effector proteins to the site of autophagosome formation. The PX-domain is a lipid binding domain with a preference for PI(3)P, but also can mediate binding to other phosphoinositides. PX-domain proteins such as PLD1 (Dall'Armi et al., 2010), *SNX18* (Knævelsrud et al., 2013), *Vam7p* (Xu Liu et al., 2016), *Atg24* (Ano et al., 2004), *Kip98a* (Mauvezin et al., 2016) and FYVE-domain proteins such as *DFCPI* (Axe et al., 2008), FYCO1 (Pankiv et al., 2010), PIKFYVE (Ikonov et al., 2002), RUFY4 (Terawaki et al., 2015) and ALFY (Simonsen et al., 2004; Filimonenko et al., 2010; Lystad et al., 2014; Clausen et al., 2010) are amongst the few that have been shown to regulate autophagy.

### Negative regulation of autophagy

The pleiotropic roles of autophagy in living organisms can be attributed to its function as a double edged sword – too little and too much can both have serious consequences. While autophagy is indispensable for protecting cells to metabolic stresses and other immune challenges (Kaushik, Singh, & Cuervo, 2010; Deretic et al., 2013), excessive autophagic activation or imbalanced degradation and recycling can give rise to situations where critical cellular constituents are strained, ultimately leading to cell degeneration and toxicity (Levine & Yuan, 2005). One such example is the role of autophagy in cardiovascular systems. It has been shown consistently that autophagy is indispensable for cardiogenesis (Hongxin & Lin, 2017), but

it has also been shown that excessive autophagy plays a crucial part during the transition from cardiac hypertrophy to decompensated heart failure, owing to loss of cardiac mass (De Meyer & Martinet, 2009). Another example is the role of autophagy both as a tumor suppressor and as a tumor promoter, e.g. *Becn1* heterozygous mutant mice are more prone to develop liver and lung tumors (Yue et al., 2003), whereas cell-autonomous autophagy supports tumor growth (Katheder et al., 2017). Thus, depending on the cell type and/or disease condition, autophagy may be a protective response or a detrimental process.

Given the potential detrimental function of autophagy it is important that the regulation and execution of autophagy is under tight negative regulation. In paper I of this thesis we found that the PX-domain containing protein HS1BP3 negatively regulate autophagy, as its depletion increased LC3 spots both in mammalian cell lines and in zebrafish, elevated LC3-lipidation and increased overall autophagic flux. HS1BP3 regulate autophagy by a negative-feedback mechanism, involving its binding to phosphatidic acid (PA) and inhibition of the PA-generating enzyme PLD1. The RAB11-binding protein TBC1D14 is another negative regulator of autophagy, found to regulate trafficking of core autophagy proteins through the recycling endosome (RE) (Longatti et al., 2012). The best characterized negative regulator of autophagy is the kinase mTOR that constitutes an important signaling hub involved in regulation of cellular metabolism, protein synthesis and suppression of autophagy (Neufeld, 2010). Under nutrient-rich conditions,, the PI(3)K-Akt signaling pathway, stimulated by growth factors, activates mTORC1 leading to suppression of autophagy by phosphorylation of ULK1 Ser-737 (Bjornsti & Houghton, 2004; Neufeld, 2010). During starvation mTORC1 is repressed by AMP-activated protein kinase (AMPK), leading to de-phosphorylation of the mTOR site on ULK1 and concomitant ULK1 phosphorylation by AMPK at Ser-555, and subsequent induction of autophagy (Hosokawa et al., 2009; Wong et al., 2015). The PI(3)P phosphatase *jumpy*, also known as MTMR14, is another negative regulator of autophagy. Ablation of MTMR14/Jumpy increases PI(3)P levels and PI(3)P -dependent recruitment of the early autophagic protein WIPI1 to sites of autophagosome formation, leading to significantly increased basal and starvation-induced autophagy. Thus, Jumpy regulates autophagy negatively by stimulating PI(3)P hydrolysis to control excessive PI(3)P-mediated signaling (Vergne et al., 2009). Similarly, anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL and Bcl-w negatively regulate autophagy by targeting the ER-associated PIK3C3 complex to restrict PI(3)P production (Pattingre et al., 2005;

Erlich et al., 2007).

In our study, we showed that PLD activity and PLD1 localization to ATG16L1-positive vesicles were elevated in HS1BP3 depleted cells, suggesting that HS1BP3 acts as a sensor and regulator of local PA levels, the product of PLD activity (Oliveira & Paolo, 2010). Thus regulating the levels of lipids during early stages of autophagosome formation is critical to maintain right amount of autophagy. FLIP (FLICE-like inhibitory protein) inhibits autophagy by competing with LC3 for binding to ATG3, thus perturbing LC3 lipidation during membrane elongation, thereby negatively regulating autophagy (Lee et al., 2009). Two Beclin 1-binding proteins, ATG14L and Rubicon, reciprocally regulate autophagy at different stages. ATG14L is a subunit of the PIK3C3 complex required for autophagosome biogenesis, while Rubicon negatively regulates autophagosome maturation by joining the UVRAG, Beclin1, and PIK3C3 complex (Matsunaga et al., 2009; Zhong et al., 2009). Calpain, Ca<sup>2+</sup>-dependent non-lysosomal cysteine proteases exert negative regulation of autophagy by cleaving off full-length ATG5, on apoptotic stimulus, thus obliterating its autophagic activity and activating apoptosis by associating to Bcl-xL on mitochondria (Yousefi et al., 2006). Inhibition of autophagy is as important as its induction and thus it is very important to envisage autophagy as a tightly regulated dynamic process. Any perturbation in negative regulation of autophagy can cause cell toxicity, tissue injury, and predispose the host to many atrophic diseases (Sandri, 2010).

### **Role of recycling endosomes in autophagy**

The importance of recycling endosomes (RE) in autophagosome formation was first studied by Sharon Tooze and co-workers who found that the early acting autophagy proteins ATG9 and ULK1 localize to TfR-positive RE, upon overexpression of TBC1D14 leading to RE tubulation and inhibition of autophagosome formation (Longatti et al., 2012). Furthermore, it was also shown that ATG16L1 and ATG9 meet in RE after their trafficking via the plasma membrane, from where they are internalized into different populations of clathrin-coated vesicles (Puri et al., 2013). In other words, under conditions of starvation, RE feed membranes to the phagophore or isolation membrane for growth of the autophagosome. In our study (paper I), we identified HS1BP3 as a negative regulator of autophagy and found it to localize to ATG9 and ATG16L1 positive RE membrane, with little or no colocalization with the early phagophore/omegasome markers WIPI2, ATG14L or DFCP1. Nor were there any difference in the number of WIPI2 or

DFCP1 spots upon ablation of HS1BP3, suggesting that HS1BP3 acts downstream of autophagosome initiation and nucleation. Intriguingly, although HS1BP3 largely localized to structures positive for ATG9 and ATG16L1, the number of ATG16L1 spots were not altered on HS1BP3 depletion, suggesting HS1BP3 might affect the composition and/or distribution of ATG16L1 spots. The vesicles that were positive for ATG16L1, ATG9 and HS1BP3 were also positive for TfR and were found to fuse to autophagosomes marked with LC3, thus suggesting that these are RE derived vesicles contributing to the forming autophagosomes. We have previously shown that SNX18 promotes LC3 lipidation and tubulation of recycling endosomes to provide membrane for phagophore expansion (Knævelsrud et al., 2013) and that it regulates ATG9 trafficking from RE by binding to Dynamin-2, thus facilitating budding of ATG9A and ATG16L1 containing membranes from RE for traffic to sites of autophagosome formation (Søreng et al., 2018). In contrast, TBC1D14 negatively controls delivery of membranes from RAB11-positive recycling endosomes to forming autophagosomes (Longatti et al., 2012). Interaction between TBC1D14 and TRAPIII activates RAB1, which is required for trafficking of RE from the periphery to the early Golgi, thus help maintaining the cycling pool of ATG9 required for initiation of autophagy (Lamb et al., 2016). Furthermore, it was shown that ATG9 is not incorporated into the autophagosome, but may traffic elsewhere to recruit more membrane and/or autophagy proteins (Orsi et al., 2012) for delivery to growing autophagosome. Recent studies have shown that sorting motifs in the N-terminal cytosolic stretch of ATG9A interacts with adaptor proteins (AP-1, AP-2 and AP-4) (Zhou et al., 2017; Mattera et al., 2017; Imai et al., 2016) and that this interaction is important for ATG9A trafficking from RE for the formation of autophagosomes. Mutations in the sorting motifs, lead to the accumulation of Atg9A in RE and aggravated to autophagic defects (Imai et al., 2016). Thus trafficking of RE from and through TGN to the periphery and to the phagophore site is critical for proper delivery of membranes aiding to the formation of autophagosomes.

### **PA and PLD1 in autophagy**

In our studies, we showed that both full-length HS1BP3 and the PX-domain bind phosphatidic acid (PA) and other 3-phosphorylated phosphoinositides. Knockdown of HS1BP3 in HEK293 cells via siRNA yielded 2-fold increase of the total PA content. The activity of the PA-producing enzyme PLD1 was also increased upon HS1BP3 depletion. Furthermore, it was observed that

PLD1 localizes to the same autophagic precursor membranes as HS1BP3 and that PA generated by PLD1 is important for autophagy. PA is a minor class of membrane lipids, but an important class of lipid messengers. The cellular PA levels are dynamic, as it is produced and metabolized by several enzymatic reactions including different phospholipases, lipid kinases and phosphatases (Liu, Su, & Wang, 2013). The role of PA in autophagy is somewhat contradictory and controversial (Laplante & Sabatini, 2012). PA interacts with mTOR in a manner that is competitive with rapamycin (Fang et al., 2001; Chen et al., 2003). PA stabilizes mTOR whereas rapamycin disrupts mTOR complexes (Toschi et al., 2009), that is, PA enhances mTOR signaling and thus leads to inhibition of autophagy. In contrast our experiments showed induced autophagy and increased PA levels in cells lacking HS1BP3, with no effect on mTOR activity, as analyzed by immunoblotting for phosphorylated S6-kinase, a target of mTOR. PLD1 and PLD2 are the two mammalian PLD isoforms, the functions of which are poorly understood (Jenkins & Frohman, 2005). Both catalyze the hydrolysis of phosphatidylcholine (PC) to PA and choline. Our results showed increased PLD1 activity (and thus elevated PA-content) in HS1BP3 depleted cells, contradictory to studies where inhibition of PA-producing enzyme PLD1 induced autophagy signaling (Jang, Choi, & Min, 2014). In line with our studies, it was observed that PLD1 can be recruited to LC3-positive autophagosomes upon starvation in a PI(3)P -dependent manner to promote autophagy (Dall'Armi et al., 2010)

PLD1 activity has also been found to be important for selective autophagic degradation of  $\alpha$ -synuclein protein aggregates (Bae et al., 2014). The role of PLD1 in the pathogenesis of PD is now a vociferously studied area. It was shown that WT  $\alpha$ -synuclein overexpression strongly reduces PLD1 levels, resulting in degenerative-like phenotype of neurofilaments (NFL). It was also observed that pharmacological inhibition of PLD1, but not PLD2, activity in non-transfected IMR-32 neurons affect NFL levels (Conde et al., 2018). The level of NFL in the cerebrospinal fluid has been proposed as a marker of PD progression and other neurodegenerative diseases (Hansson et al., 2017). Thus it would be highly desirable to study the effect of HS1BP3 in selective autophagy and how it affects neuronal health.

PA is an anionic, cone shaped lipid, which owing to its small head group attached to a wide backbone generates negative membrane curvature when inserted into membranes (Zimmerberg & Kozlov, 2006; Kooijman et al., 2005). The phagophore membranes are highly curved and cone

shaped lipids like PA and PE can contribute to curvature generation, also aided by the fact that they generate less packaging of the surrounding lipid bilayer, thus facilitating insertion of membrane proteins (Van Den Brink-Van Der Laan, Antoinette Killian, & De Kruijff, 2004). The E2-like enzyme ATG3 senses the highly curved ends of the growing phagophore where it facilitates lipidation of LC3/GABARAP (Nath et al., 2014). In our study, HS1BP3 depletion in cells resulted in elevated levels of LC3-lipidation and increased cellular PA, where the latter could be responsible for making negatively curved membrane stress.

### **Mitochondria targeting sequences (MTS)**

As elaborated in the introduction part, the two proteins PINK1 and Parkin are involved in mitophagy upon mitochondrial depolarization. PINK1 is first stabilized on the OMM, leading to phosphorylation of ubiquitin (P-Ub) chains and recruitment of Parkin to the OMM owing its high affinity to P-Ub and binding to phosphorylated Mitofusin2 that acts as receptor for Parkin. Parkin, an E3 ubiquitin ligase, acts as an amplifier – magnifying the ubiquitination process. This helps recruit autophagy receptors like OPTN and NDP52. The autophagy receptors through their LIR recruit LC3 together with other autophagy machinery members, resulting in the expansion and elongation of the autophagic membrane around the damaged mitochondria to be delivered to lysosomes for degradation. In the second paper of this thesis, we characterized the selective autophagic role of two mitochondrial matrix proteins, NIPSNAP1 and NIPSNAP2. NIPSNAP1 and 2 were shown to be redundant in their function as ‘eat-me signals’ during Parkin-mediated mitophagy in mammalian cell lines. Nipsnap1 was also shown to regulate mitophagy in the brain of the zebrafish.

The MTS, or the pre-sequence, is cleaved off after efficient protein import (Greene et al., 2012) and defects in proper cleavage can lead to disease (Driest et al., 2005). The PINK1 N-terminal MTS is translocated across the outer and inner membrane upon targeting to healthy mitochondria, which is then later cleaved by MPP in the matrix (Greene et al., 2012). In our study, we characterize two distinct mitochondrial targeting signals for NIPSNAP1. The first 20 amino acids of NIPSNAP1 were found to effectively target GFP protein inside the mitochondria, whereas NIPSNAP1 lacking the first 23 amino acids was recruited to the mitochondrial surface, but not imported into the mitochondrial matrix. Thus, our data show that the N-terminal part of NIPSNAP1 is both sufficient and essential for intra-mitochondrial localization. Interestingly, a

very recent study identified additional internal MTS-like signals (iMTS-Ls) in the mature or internal part of several mitochondrial precursor proteins. These are similar to MTS in respect to their length and other properties and it was shown that these iMTS-Ls failed to target protein to the matrix, but instead mediated recruitment to the outer mitochondrial surface where they interact with proteins on the surface (Backes et al., 2018). This is in line with what we observe in our study, where NIPSNAP1 amino acids between 24 and 59 fused to EGFP was sufficient to localize EGFP to the mitochondrial surface. Cytochrome C1 is another inner membrane protein known to have two MTSs (Arnold et al., 1998).

Pre-sequence-containing precursor proteins can be divided into two classes: (1) precursors that completely translocate across the inner membrane into the matrix and (2) those that are released by the translocase into the lipid phase of the inner membrane. The driving force for most precursor transport across the inner membrane is the mitochondrial membrane potential ( $\Delta\psi$ ) that acts on the positively charged MTSs (Schleyer, Schmidt, & Neupert, 1982; Roise & Schatz, 1988; Schulz, Schendzielorz, & Rehling, 2015; Truscott, Brandner, & Pfanner, 2003) although there are reports of mitochondrial precursor transport, independent of  $\Delta\psi$  (Turakhiya et al., 2016). Mitochondrial depolarization opens up the mitochondrial membrane permeability transition pores, which results in the dissipation of the mitochondrial membrane potential ( $\Delta\psi$ ). This has shown to be the cause of cytochrome c release from the mitochondria to the cytosol during apoptosis (Chlu & Olelnick, 2001). Mitochondrial matrix proteins generally do not decorate the OMM on depolarization. Here, we show that NIPSNAP1 accumulates on the surface of the mitochondria upon depolarization. Using a range of different in vitro and in vivo approaches, we convincingly show that cytosolic NIPSNAP1 accumulates on mitochondria upon depolarization, but the possibility of intra-mitochondrial NIPSNAP1 and -2 being exported to the surface on depolarization cannot be excluded. The lack of a positive membrane potential, owing to depolarization, might be the reason for cytosolic NIPSNAP1 being stabilized on the surface of mitochondria. It would be desirable to check for any likelihood of already imported and cleaved NIPSNAP1 being exported on depolarization cues, though it would seem difficult to analyze such a phenomenon since mitochondrial export mechanisms have not yet been characterized.

### **Recruitment of autophagy receptors**

One of the important phases in PINK1/PARKIN-mediated mitophagy is the recruitment of

autophagy receptors to the site of mitochondrial degradation. Autophagy receptors connect the ubiquitinated cargo and the membrane conjugated ATG8 proteins through their LIR. Though different autophagic receptors are implicated in mitophagy, including p62/SQSTM1 (Geisler et al., 2010), optineurin (Wong & Holzbaur, 2014), NBR1 (Hollville et al., 2014), NDP52 (Heo et al., 2015) and TAX1BP1 (Moore & Holzbaur, 2016b), it was found that NDP52, OPTN and to a lesser extent TAX1BP1 are the primary receptors for PARKIN-dependent mitophagy in HeLa cells (Lazarou et al., 2015). Here in paper II, we find a role of NIPSNAP1 and -2 upstream to the recruitment of autophagy receptors. NIPSNAP1 and -2 interacts with autophagy receptors and this interaction increased upon depolarization of mitochondria. The recruitment of autophagy receptors NDP52, p62, OPTINEURIN and TAX1BP1 to the damaged mitochondria was dramatically reduced in NIPSNAP1 and -2 double knock outs (N1/N2 DKO) cells as compared to control (WT) cells. Also interesting was the fact that both Parkin recruitment and ubiquitination of the mitochondria was unaltered. This implies that recruitment of NIPSNAP1 to the surface of damaged mitochondria is indispensable for PINK1/PARKIN-mediated mitophagy. In line with this, NIPSNAP2 lacking the first 24 amino acids (MTS) can rescue lack of mitophagy in N1/N2 DKO cells, on induction of mitophagy by CCCP or OA.

Recently, the IMM protein Prohibitin 2 (PHB2) was found to act as a mitophagy receptor during Parkin-mediated mitophagy in mammalian cells and for paternal clearance of mitochondria during *C. elegans* embryogenesis (Wei et al., 2017). Upon depolarization, proteasome activity leads to rupture of the OMM, which exposes the IMM. PHB2 establishes interaction with LC3 through a LIR motif, thereby promoting mitochondrial degradation (Wei et al., 2017). Thus, this study shows a concerted effect both by proteasome and autophagy in degrading the mitochondria. In our study, NIPSNAP1 and -2 decorates the OMM of the defective mitochondria, where they act as “eat-me” signals for mitophagy. It is likely that both processes occur upon depolarization-induced PARKIN-dependent mitophagy.

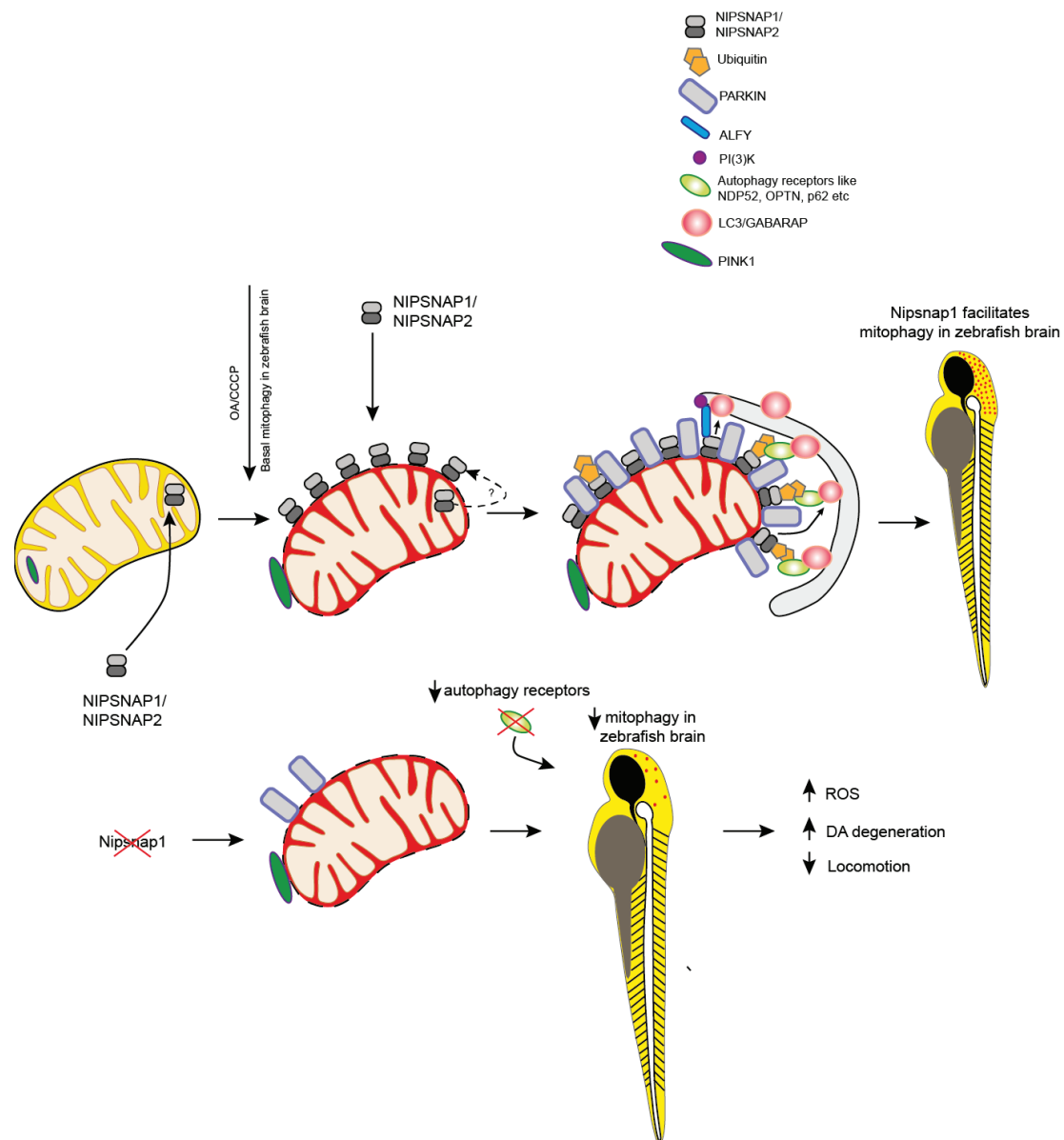
Efficient targeting of dysfunctional mitochondria for mitophagy involves several layers of specific interactions between mitochondrial proteins and the autophagy machinery. We confirm our mass-spectroscopy data wherein NIPSNAP1 and -2 were shown to interact with the autophagy adaptor ALFY by immunoprecipitation assays showing that both the autophagy receptor p62 and the adaptor ALFY interacted with NIPSNAP1 independent of each other.

ALFY interacts with p62 and is known to facilitate recruitment of the autophagy membrane for selective autophagy through its binding to PI(3)P and GABARAP (Clausen et al., 2010; Filimonenko et al., 2010; Lystad et al., 2014). NIPSNAP1 and -2 also interacts with ATG8 family proteins, with GABARAP being the preferred ATG8 protein, also confirming an earlier study where NIPSNAP1 and -2 were identified in an ATG8 interactome network (Behrends et al., 2010) (Figure 6)

### **PINK1/Parkin-mediated mitophagy and Parkinson's disorder (PD)/Parkinsonism**

Familial cases of PD facilitated the identification of different genes linked to PD, including PINK1 and PRKN (Parkin), both autosomal recessive (Klein & Westenberger, 2016). Seminal work by Richard Youle and colleagues identified the roles of these two proteins in mitophagy. As described earlier, PD is characterized by the progressive degeneration of dopaminergic neurons of the SNpc and a resulting decrease of dopamine levels in the dorsal striatum owing to dysfunctional turnover of mitochondria in dopaminergic cells (Gautier et al., 2014; Pickrell & Youle, 2015).

In paper II of this thesis, we show defective mitochondrial turnover upon depletion of NIPSNAP1 and -2 in mammalian cells. We further validated this in zebrafish larvae lacking functional Nipsnap1, where we see lack of basal mitophagy in the brain as compared to the control (WT) larvae at 3 dpf (Figure 6). Post-mitotic structure such as neurons rely heavily on OXPHOS for energy production and any disruption in the mitochondrial homeostasis can render it dysfunctional and becomes a source of oxidative stress through unbalanced production of ROS. This can prove fatal and be a cause for neurodegeneration. Evidently, zebrafish larvae lacking Nipsnap1 showed a significant loss of dopaminergic neurons in the diencephalospinal tract of the brain. These Nipsnap1 mutant larvae also showed defective locomotor function at 7 dpf as compared to controls at the same age, which could be rescued by adding L-Dopa to the E3 water they are in, the drug used for treating PD patients (Figure 6) Nipsnap1 mutant larvae also had significantly elevated ROS levels compared to the control larvae at 3 dpf. Taken together our data showing defective mitophagy, increased production of ROS, degeneration of dopaminergic neurons, locomotor defects and rescue by L-Dopa strongly indicate that Nipsnap1 mutant larvae have a parkinsonian phenotype. PD is however much more complex and clinical implications extend beyond motor symptoms where one has to take into account the non-motor features



**Figure 6: NIPSNAP1 and -2 facilitates PINK1/Parkin-mediated mitophagy in mammalian cells; Nipsnap1 facilitates mitophagy in zebrafish brain to prevent ROS production and dopaminergic neurodegeneration.** NIPSNAP1 and -2 are mitochondrial matrix proteins that decorate the OMM on depolarization. Mitochondrial depolarization stabilizes PINK1 on the OMM and further phosphorylations help recruit Parkin as well. Parkin ubiquitinates NIPSNAP1 and -2 on the OMM (Sarraf et al., 2013) and also interacts with NIPSNAP1 on depolarization. NIPSNAP1 and -2 mediates recruitment of the autophagy receptors to the mitochondria. The receptors through its LIR, binds to LC3/GABARAP and recruits further autophagy machinery for mitochondrial degradation. Nipsnap1 facilitates mitophagy in zebrafish brain. Nipsnap1 ablation in zebrafish leads to reduced mitophagy resulting in parkinsonian syndromes.

including neuropsychiatric disturbances (Aarsland, Marsh, & Schrag, 2009). PD is an adult onset

disorder with the majority of cases being sporadic (90%) that may arise from complex interactions between environmental exposure and genetic susceptibility (Teismann, 2012). The youngest recorded human juvenile case was diagnosed with PD at the age of 12 (Yamamura et al., 2010). That is approximately at the 15% mark of the total human lifespan (assuming the average lifespan is 80). This would implicate that a 3 – 4 month old zebrafish could be the earliest to show hallmarks of PD. In this study we are looking at 3 day old zebrafish larvae. It is highly unlikely that zebrafish so young would start manifesting all of the PD hallmarks. Moreover, PD is also highlighted by the presence of cytoplasmic protein inclusions called Lewy bodies within the affected neurons, having  $\alpha$ -synuclein protein as their main component (Dauer & Przedborski, 2003). We did not look for  $\alpha$ -synuclein in our mutant model. Thus, based on the symptoms that we observe, we use the term ‘Parkinsonism’, which can be defined as a collection of symptoms that are characteristically observed in PD, but that are not necessarily due to PD (Singer et al., 2016). It is often associated with a dopaminergic deficit and characterized by two or more of the cardinal signs of PD, such as bradykinesia, tremor, rigidity and postural instability (Jankovic, 2008)

Interestingly, NIPSNAP1 was identified as the gene being most significantly downregulated in a report analyzing genome-wide gene expression data of PD diseased and control samples (Fu & Fu, 2015). Moreover, NIPSNAP1 was also shown to be downregulated in a comparative 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 dopaminergic neurons (Choi et al., 2014). MPTP inhibits the mitochondrial complex I in the electron transport chain, leading to increased ROS production (Calvo & Mootha, 2010; Perfeito, Cunha-Oliveira, & Rego, 2012). Furthermore, mouse Nipsnap1 was shown to be highly expressed in the dopaminergic neurons in midbrain and noradrenergic neurons in the brainstem (Nautiyal et al., 2010). These observations, along with our data strongly indicate a role for Nipsnap1 in maintaining dopaminergic neuronal health by regulating mitochondrial homeostasis, failure of which can lead to symptoms pointing to parkinsonism. Interestingly, a genetically engineered mouse model lacking NIPSNAP1 was characterized by neurodegeneration in the cortex of the mouse brain. The study shows that Nipsnap1 deficiency increased neuronal apoptosis resulting in increased neuron specific death (Baggett, K. A. 2016)

The discovery of loss of function attributed with autosomal recessive mutations in PINK1 and PRKN (Parkin) led to many independent labs generating PINK1 and Parkin KO animal models to study the function of PINK1 and Parkin in vivo, respectively. *Pink1* and *Parkin*-KO mouse models have however turned out to be quite disappointing as they did not recapitulate the phenotypes seen in human PD patients. Most of the *Pink1*-KO and *Parkin*-KO models exhibit only mild phenotypes, such as the disruption of fine motor skills and slight abnormalities in dopamine metabolism and release and no significant dopaminergic loss (Goldberg et al., 2003; Itier et al., 2003; Von Coelln et al., 2004; Perez & Palmiter, 2005; Kitada et al., 2007). A likely explanation is that mice compensate for the loss of *Pink1* and *Parkin* or that the effects caused by their absence are below the threshold for causing detrimental phenotypes. Post-natal conditional *Parkin* KO does result in loss of nigral dopaminergic neurons, supporting the idea of compensatory mechanisms in the development of germline mouse knockouts, but it is unclear that if the DA loss led to locomotor defects (Shin et al., 2011; Stevens et al., 2015a). Studies in *Drosophila* were the first to show a role for Parkin in mitochondrial maintenance and that PINK1 and Parkin act in the same pathway. Ablation of *PINK1/Parkin* in *Drosophila* lead to accumulation of enlarged and damaged mitochondria in sperm, flight muscle and dopaminergic neurons, suggesting lack of mitochondrial quality control in energetically demanding tissues (Clark et al., 2006; Park et al., 2006; Greene et al., 2003).

Zebrafish knock-down (KD) models of *pink1* and *parkin* display similar phenotypes to what we observe in paper II and also corroborate with *Drosophila* models of *Pink1* and *parkin*. *Pink1* depleted zebrafish display shunted development and moderate decrease in dopaminergic neurons with considerable alterations of mitochondrial function, increased ROS levels and locomotor defects (Anichtchik et al., 2008; Xi et al., 2010; Sallinen et al., 2010; Priyadarshini et al., 2013; Soman et al., 2017). Recent studies in *pink1*<sup>-/-</sup> mutants, showed a very interesting feature in that *pink1*<sup>-/-</sup> mutants alone showed no decrease in mitochondrial ATP content despite exhibiting reduced complex I activity and membrane potential. However, treatment with rotenone induced a synergistic loss of mitochondrial ATP content, reduced basal and maximum oxygen consumption and further permanently reduced the number of midbrain 5, 6, and 11 DA neurons in these *Pink1*-deficient zebrafish larvae (Zhang et al., 2017). This further indicate that compensatory pathways are induced to help protect the *pink1*<sup>-/-</sup> zebrafish larvae from developing detrimental phenotypes, but that they are highly sensitized to toxins of mitochondria. It was earlier observed

that knockdown of *pink1* highly sensitized the zebrafish larvae to sub-effective doses of MPTP, which caused locomotor deficit and further loss of dopaminergic neurons (Sallinen et al., 2010). We did not test for sensitization to mitochondrial toxins in our *nipsnap1* mutant zebrafish model. Parkin KD in zebrafish leads to a significant decrease in the number of ascending dopaminergic neurons in the posterior tuberculum, which elevated further on treatment with MPP<sup>+</sup> (an oxidized product of MPTP on action by the enzyme MAO-B). The effects were shown to be specific for dopaminergic neurons, as neither serotonergic nor other motor neurons were affected (Flinn et al., 2009). In contrast, another study showed there were no loss of diencephalic dopaminergic neurons on *parkin* ablation (Fett et al., 2010). In line with a role for mitophagy in neuronal homeostasis, it was found that *atg5* expression during zebrafish embryogenesis is critical for the neural development of the organism (Hu, Zhang, & Zhang, 2011) and that its down-regulation caused a pathological locomotor behavior, loss of dopaminergic neurons and accumulation of  $\alpha$ -synuclein aggregates, which were reversed by overexpression of Atg5 in an MPTP-induced PD model of zebrafish (Hu et al., 2017). Thus, based on our data and from the literature we propose that PINK1-Parkin-Nipsnap1 are critical core players of the same pathway, with PINK1-Parkin being upstream of Nipsnap1 and abrogation of any in zebrafish does have an effect on the dopaminergic system, more sensitization to sub-minimal levels of mitochondrial toxins and locomotory defect. It would be greatly desirable to look at synuclein profiles in these mutants or morphants.

A long standing question regarding PINK1/Parkin-mediated mitophagy is how physiological relevant is this process? Our current understanding of this pathway is mainly based on studies from cultured cells where mitophagy is induced by depolarization of the mitochondria membrane potential, using high concentrations of protonophores such as CCCP/FCCP. Moreover, such studies are often performed on cultured cells overexpressing Parkin. While it may be important to use such artificial scenarios to elucidate the molecular mechanisms involved, it is essential to investigate their relevance under physiological conditions. It is also possible to circumvent the use of cell culture. Vincow and co-workers used a classical and innovative proteomic approach to test the hypothesis that PINK1 and Parkin promote mitophagy *in vivo*. They employed quantitative mass spectrometry to monitor mitochondrial protein turnover in *Drosophila* heads of *Pink1* and *parkin* mutants compared to wild-type flies. They speculated that under normal basal conditions, mitochondrial components would be degraded and replaced at a constant rate to

maintain homeostasis and the accumulation of heavy isotope-labelled mitochondrial proteins was therefore monitored. These experiments revealed that the half-life of many mitochondrial proteins was significantly increased in *parkin* mutants in a pattern that closely resembled that of autophagy-defective *Atg7* mutants, thus supporting a role for Parkin in mitophagy (Vincow et al., 2013). A very recent study from Ian Ganly and colleagues revealed the prevalence of mitophagy in a variety of organ systems and cell types of high metabolic demand in *Pink1* KO mouse with no difference from the corresponding WT controls (McWilliams et al., 2018). It should be mentioned here that the knockout was germline induced and thus as mentioned before, compensatory pathways can step in or as the authors discuss *PINK1* dependent mitophagy can be highly context dependent. All of the data procured from zebrafish *pink1-parkin* models and from the *nipsnap1* mutant model used in this study were under normal physiological steady-state conditions and showed signs characteristic of PD or parkinsonism, thus strongly propagating the mechanism of mitophagy being defective in PD conditions. More and more studies highlight the complexity of the disease, suggesting interconnection of multiple pathways as causality. Some studies also point to defective mitophagy being downstream of the disease. PD causality still remains a jigsaw puzzle, but likely to be solved in near future.

### **Nipsnap1 and zebrafish embryogenesis**

In paper III of this thesis, we further characterize the role of Nipsnap1 during zebrafish embryogenesis. We show that complete abrogation of *nipsnap1* and -2 in zebrafish results in dorsalization of zebrafish embryos – extreme dorsalization in the former. We also see very high mortality rates by 1dpf in *nipsnap1* KO embryos, throwing light on the lethality of the gene. Furthermore, Nipsnap1 ablation leads to alterations in the expression of dorsal and ventral markers during the gastrulation stage of development, affecting the stability of Smad proteins.

The possibility of Nipsnap1 playing a key role during the gastrulation stage of embryogenesis was first put forward by Cho and colleagues in *Xenopus* embryos. Animal cap assays in combination with *Xenopus* DNA microarrays were employed to identify genes that are directly induced by BMP signals (Peiffer et al., 2005). The authors claimed Nipsnap1 to be a direct target of BMP signaling, being upregulated 7.9 times fold, on blocking translation by cyclohexamide and proceeding with induction of BMP signaling by BMP2 protein on the gastrula stage animal cap explants. *Xenopus Nipsnap1* spatial expression pattern partially resembled that of *BMP4*

expression pattern and it was found to be expressed in the brain, eye, otic vesicle, and weakly in the branchial arches of *Xenopus* embryos. In paper III, we see *nipsnap1* spatial expression to be ubiquitous and uniform across the cells of shield stage (mid-gastrula) zebrafish embryo with no specific enrichment in either the ventral or dorsal domain, thus discarding the idea of similar expression pattern with zebrafish *bmp4*. In paper II of this thesis, we show spatial expression pattern of *nipsnap1* in the brain, eyes and other endodermal/mesodermal derived organs thus corroborating with the *Nipsnap1* expression in organs of *Xenopus* embryo (Peiffer et al., 2005). In another interesting study, *Nipsnap1* was found to be associated with cornea to lens transdifferentiation in *Xenopus*. The study aimed at identifying functional pathways upregulated during differentiation from cornea to lens in *Xenopus laevis* tadpoles and observed massive upregulation of *Nipsnap1*, thus concluding a role of BMP signaling during the process (Day & Beck, 2011). As mentioned earlier, though we see *nipsnap1* transcripts in the eyes of the zebrafish larvae, we did not look for a possible role of *Nipsnap1* there. Indeed we do see eye defects in *nipsnap1* ablated zebrafish embryos from 1 dpf, highlighted in paper III of this thesis. The majority of the *Nipsnap1* KO zebrafish larvae were associated with the phenotype “cyclopia”. As the name suggests, these are related to mutations around the *cyclops* gene in zebrafish, a nodal ligand or in general linked to perturbation of the nodal pathway (Blader & Strähle, 1998). There are also studies linking cyclopia to Wnt signaling pathway in zebrafish (Pei & Feldman, 2009).

There are enormous studies that throw light on regulators and effectors of Smad2/3-mediated Nodal signaling and Smad1/5/8-mediated BMP signaling in zebrafish. They uniquely and distinctly control the germ layer induction and patterning of vertebrate embryos. The BMP signaling pathway runs parallel to the Nodal signaling pathway, with the former responsible for patterning tissues along the DV axis of the blastula and gastrula embryo (Langdon & Mullins, 2011) and the latter responsible for patterning tissues along the animal-vegetal axis and to break symmetry around the left-right axis of the embryo (Zinski et al., 2017). The scrutiny of a mitochondrial role during developmental processes in zebrafish is quite vague. There have been observations in echinoderms supporting a direct role of mitochondria during axis specification (Coffman & Denegre, 2007). Bcl-2, acknowledged as the key regulator of apoptosis (Youle & Strasser, 2008), is a mitochondrial protein that have been shown to shape the embryo body through sustained cell homeostasis and tissue morphogenesis. Bcl-wav, a Bcl-2 related protein,

was found to orchestrate morphogenetic movements during gastrulation of zebrafish embryo by regulating  $\text{Ca}^{2+}$  trafficking (Prudent et al., 2013). Nr2 is another Bcl-2 family homolog protein in zebrafish, found to participate in early development of zebrafish embryo by controlling cell movements through  $\text{Ca}^{2+}$  fluxes inside the embryo (Popgeorgiev et al., 2011). In paper III of this thesis, we show a marked upregulation of dorsal, endodermal and mesodermal markers on *nipsnap1* depletion at the shield stage (mid-gastrulation) of zebrafish development. This was found to attenuate total Smad5 stability, which can be attributed to the high expression of dorsal markers leading to dorsalization of the zebrafish larvae. We have not looked at calcium levels in the *nipsnap1* knockout embryo, but it would be highly desirable to do so, as Nipsnap1 was found to regulate transient receptor potential vanilloid channels 5 and 6 (TRPV5/6) – known  $\text{Ca}^{2+}$  channels of TRP superfamily of ion channels (Schoeber et al., 2008). Ablation of Nipsnap2 had much milder effects on zebrafish larvae and probably is more important during a later time point as evident from data in paper II of this thesis, where temporal expression pattern of *nipsnap2* showed lower expression during early zebrafish embryogenesis, but increased from 3 dpf with enriched expression in the skeletal muscles as shown by *in situ* hybridization. Nipsnap2 has also been shown to regulate L-type  $\text{Ca}^{2+}$  channels (Brittain et al., 2012).

We have two major hypotheses for the role of Nipsnap1 during zebrafish embryogenesis that we will pursue in the near future. First, we propose that Nipsnap1 is a direct target of either the Nodal signaling pathway or the BMP signaling pathway. As described earlier, Nipsnap1 has been suggested to be a direct target of BMP pathway in *Xenopus* and our data show the upregulation of dorsal markers and downregulation of ventral markers (majority of them being BMP ligands) upon CRISPR-mediated Nipsnap1 depletion. This suggests that Nipsnap1 may directly regulate the ventral determinants and helps antagonize the dorsal mediators. A very recent study connects BMP/SMAD signaling to development of mammalian dopaminergic neurons. The main findings highlight the role of BMP/SMAD signaling as a novel essential pathway in regulating the development of mammalian midbrain dopaminergic (mDA) neurons *in vivo* (Jovanovic et al., 2018). A very recent review also highlights the importance of BMP pathway as a possible therapeutic approach in PD (O’Keeffe, Hegarty, & Sullivan, 2017). This might also suggest a possible role of Nipsnap1 in the development of dopaminergic neurons through BMP signaling pathway.

Nipsnap1 and nodal connection arises through the “cyclopia” phenotype. There have been reports of nodal regulators affecting the dorso-ventral patterning as well, thus causing dorsalization of the zebrafish embryos (Xingfeng Liu et al., 2013). Moreover, Smad2 does not bind to DNA directly and hence it requires a co-factor that associate to DNA to regulate transcription. During mesendodermal patterning, the important co-factor is FoxH1 (Fan & Dougan, 2007). We have initial findings from our *in silico* work that shows consensus sequences identical to the ones that FoxH1 is shown to bind to. These sequences are found in the intronic region between exon1 and exon2 of zebrafish *nipsnap1* gene. Furthermore, E3 ubiquitin ligase Siah2 (Parkin is an E3 ubiquitin ligase) enhances Nodal signaling activity in zebrafish and FoxH1 was shown to be absolutely required for the Siah2 dependent Nodal augmentation (Kang et al., 2014).

Second, based on paper II findings, where we demonstrate the importance of Nipsnap1 in PINK1/Parkin-mediated mitophagy in human cell lines and in the brain of zebrafish larvae, we hypothesize that Nipsnap1 regulates mitophagy either a) during late blastula – early gastrula stage of zebrafish embryogenesis or b) during elimination of paternal mitochondria. The role of mitochondria is extremely important during early embryogenesis in all organisms – vertebrate or invertebrate – where the undifferentiated pluripotent cells differentiate to more specific cell types. This marks a metabolic switch from a more inactive glycolytic phase to a more vibrant oxidative phosphorylative phase (OXPHOS). In zebrafish, this switch should happen sometime during gastrulation, as this is when pluripotent marginal cells start differentiating (Onichtchouk & Driever, 2016). We propose that lack of Nipsnap1 inhibits the mitochondrial “switch” and have some initial data suggesting that mitophagy might be attenuated upon depletion of Nipsnap1 during gastrulation of zebrafish. Moreover, microarray analysis of *pink1* knockdown zebrafish larvae identified TGF- $\beta$  signaling pathway as one of the five canonical pathways being affected, the others being, hypoxia-inducible factor (HIF) signaling, mitochondrial dysfunction, retinoic acid receptor (RAR) activation and biogenesis of mitochondria (Priyadarshini et al., 2013).

Uniparental inheritance of mitochondria is nearly universal in animals. Recently it was shown in mice that PARKIN and Mitochondrial Ubiquitin ligase 1 (MUL1) work synergistically to promote paternal mitochondrial elimination (Rojansky, Cha, & Chan, 2016). Several studies in

*Caenorhabditis elegans* embryos have demonstrated that fertilization-triggered autophagy is essential for removal of paternal mitochondria and maintenance of maternally inherited mtDNA (Sato & Sato, 2011; Zhou, Li, & Xue, 2011). In a very recent study it was shown that prohibitin 2 plays an essential role *in vivo* in eliminating the transmission of paternally derived mitochondrial DNA to offspring in *C. elegans*, via mitophagy (Wei et al., 2017). The reasons and cues that trigger paternal mitochondrial elimination are still speculative and no real consensus has been reached.

As described in the sections above, defective mitophagy can be a source for aberrant ROS, but not all ROS is bad. Owing to the large amount of evidence showing that ROS can regulate fundamental cellular processes, a function of ROS in development is likely, although too much ROS can be detrimental. In paper II, we show highly increased ROS in Nipsnap1 mutants and Nipsnap1 knockout zebrafish larvae as compared to controls at 2dpf. We will look for the levels of ROS at earlier time points of Nipsnap1 KO larvae, which could be the decisive factor in causing gastrulation arrest. Oligomycin and antimycin A are used to induce mitophagy as an alternative for depolarizing agents such as CCCP/FCCP, owing to off-target effects it may have (Lazarou et al., 2015). In a very interesting study, it was found that treatment of zebrafish larvae with Oligomycin (a complex I inhibitor) caused arrest of gastrulation, but albeit abnormal, embryos underwent organogenesis and survived past 80 hpf (Pinho et al., 2013). One could easily speculate a very important role of mitophagy here.

## EXPERIMENTAL CONSIDERATIONS

The conclusions derived from the papers in this thesis have been primarily studies conducted on mammalian cell culture (*in vitro* system) and then later validated those using zebrafish as our *in vivo* model. Here I will focus on methods involving zebrafish only as that is what I have used throughout my PhD period.

### Zebrafish lines

The use of model organisms helps add a ‘physiological’ dimension to the scientific studies being done using *in vitro* systems. Many genes and gene functions are conserved between simpler organisms to humans and to study the effect of a gene in a living organism, provides a helpful tool to better understand a possible role for the gene or the protein in the human body. We use zebrafish as a model organism to validate our *in vitro* studies owing to the large advantages that it possesses. More of the advantages of zebrafish for research and especially for autophagy is elaborated in our review entitled “Studying autophagy in Zebrafish” (Mathai et al., 2017) (paper IV of thesis).

In this thesis we mainly used the AB strain of wild type, two transgenic and one mutant zebrafish lines. In paper I, we used the Tg(CMV:EGFP-map1lc3b) transgenic line (He et al., 2009) to quantify GFP-LC3 puncta. In paper II, we used Nipsnap1 mutant line (*nipsnap1*<sup>sal14357</sup>) procured from Zebrafish Mutation Project (ZMP) (Kettleborough et al., 2013) to understand the *in vivo* importance of the gene Nipsnap1. We also generated the tandem tagged transgenic line Tg(CMV:MLS-GFP-mCherry) to study the process of mitophagy in zebrafish. Furthermore, we used CRISPR/Cas9 technology to knockout genes in zebrafish. These were not grown to adulthood, but used for experimentation on respective days, after injection with Cas9 mRNA/protein and guide RNA.

Fish (WT strains, Nipsnap1 mutant lines and GFP-LC3 transgenic line) 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 CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, equilibrated to pH 7.0). From 12 hpf, 0.003% (w/v) 1-phenyl-2-thiourea (Sigma-Aldrich) was used to inhibit pigmentation for *in situ* hybridization experiments. 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).

## Microinjections

One of the common ways of studying the function of a protein of interest is by either eliminating it permanently or transiently using knock-out or knock-down techniques, respectively. Knockdown in zebrafish can be achieved by various methods. One of the standard approaches that have been in practice for a considerable time is the use of modified antisense oligonucleotides called morpholinos (Summerton, 1999; Corey & Abrams, 2001). Morpholinos are injected into 1-4 celled embryo and would in an ideal situation bind to its target mRNA and block expression. But this is not always the case and recent studies have shown that the effects of morpholinos are not specific (off-target effects) and that up to 80% observed morpholino-induced phenotypes are due to false-positives (Kok et al., 2015), mostly induced by p53-dependent apoptosis although p53-independent non-specificity has also been shown (Robu et al., 2007). Several precautions should be taken to conclude results from a morpholino based knockdown experiment, including validation of results using two different types of morpholinos – translational morpholino and splice junction morpholino – and co-depletion of p53 along with your respective gene of interest. In paper I, we injected approximately 17 ng of both types of morpholinos to knockdown Hs1bp3. For rescue experiments, like as in paper I, capped full-length human wildtype Hs1bp3 mRNA was transcribed from linearized pSP64 Poly (A) Vector (Promega) using mMessage mMachine (Ambion) and 50–150 pg was co-injected with the Hs1bp3 morpholino as described (Chi et al., 2010).

Knockouts in zebrafish can be achieved by ZFNs (Doyon et al., 2008), TALENs (Huang et al., 2011) and the most recent CRISPR/Cas9 technology (Jinek et al., 2012; Hwang et al., 2013). We used CRISPR/Cas9 to introduce indels into our gene of interest. In paper II, we microinjected 50-200 ng/μL sgRNA (designed using the CHOPCHOP tool) (Montague et al., 2014) against *nipsnap1*, mixed with capped and poly-adenylated Cas9 mRNA (300 pg/μL) (in-vitro transcribed from pCS2-nls-zCas9-nls (Addgene plasmid ID 47929)), into one-celled zebrafish embryo. In paper III, we microinjected 50-200 ng/μL sgRNA (for *nipsnap1* and *nipsnap2*) mixed with Cas9

nuclease (EnGen Cas9 NLS, NEB) having dual NLS for improved transport to the nucleus. The advantage of using Cas9 protein over Cas9 mRNA (as used in paper II) is that the protein is immediately available for the cell to use for editing as it does not require translation as in the case of using mRNA. Moreover, the protein is quickly turned over by the cell and thus lowers the chance of off-target effects. Prior to injection, this complex was incubated for 5-6 minutes at room temperature and then immediately placed back on ice until pipetted into the capillary needle used for microinjection. Elaborate CRISPR/Cas9 protocols can be found elsewhere (Yin et al., 2015; Jao et al., 2013).

### **Autophagy assays in zebrafish**

The different ways to study autophagy in zebrafish have been reviewed earlier (Mathai et al., 2017). In this thesis, we have mainly used transgenic zebrafish lines to look at general autophagy and mitophagy. In paper I, we used Tg(CMV:EGFP-map1lc3b) transgenic line (He et al., 2009) to look at Lc3 puncta formations after depletion or rescue of Hs1bp3. An increased number of Lc3 puncta can either indicate increased autophagosome formation or a block in autophagosome maturation. Thus, to distinguish between these two possibilities, we included an inhibitor of lysosomal degradation to quantify the autophagic flux. In paper I, we used 10 $\mu$ M chloroquine for 6 hours to inhibit lysosomal degradation in both control and *hs1bp3* depleted zebrafish larvae. Chloroquine in monoprotonated form diffuses into the lysosome, where it turns into diprotonated form and is trapped. Protonated chloroquine then changes the lysosomal pH, thereby inhibiting autophagic degradation in the lysosomes (Homewood et al., 1972). In paper II, we generated a Tg(CMV:MLS-GFP-mCherry) transgenic line to study mitophagy. The mitochondrial targeting signal (MTS) used here is from zebrafish CoxVIII (Kim et al., 2008). The principle of this assay is relatively simple. Mitochondria in normal situation would show both GFP and mCherry signals (yellow) but in the lysosome they would appear as mCherry positive only (red) owing to the fact that GFP is quenched in the highly acidic lysosomal compartment. Quantification of red only puncta would thus give an account of cells undergoing mitophagy in various organs of the zebrafish. This is a “first of its kind” transgenic zebrafish line to study mitophagy in zebrafish.

Another way of monitoring autophagic flux is by immunoblotting for the autophagic markers Lc3 and p62. During autophagy, Lc3 is conjugated to PE in the autophagic membrane and because the membrane bound form of Lc3 (Lc3-II) migrates slightly faster by SDS-PAGE than

the non-membrane form (Lc3-I), the conversion of Lc3-I to Lc3-II can be monitored by western blotting. Unfortunately in our hands, this has not worked for zebrafish embryos, even through multiple optimization trials, likely due to anti-human LC3 antibodies not cross-reacting with zebrafish Lc3. SQSTM1/p62 turnover can also serve as a marker for autophagic activity in zebrafish. An induction of autophagy would increase the degradation of SQSTM1/p62 while a decrease would result in its accumulation. All autophagic flux assays require that the experiments are performed in the absence and presence of lysosomal inhibitors. In paper III, we have immunoblotted for the mitophagy effector protein kinase PINK1 in zebrafish embryos at shield stage.

## **Microscopy**

In this thesis, I have used two microscopic systems to image zebrafish embryos or larvae, a stereomicroscope and confocal microscopes. A stereomicroscope has great working distance and depth of field, but compromises on the resolution. It differs from other light microscopes, as a stereo microscope most often uses reflected illumination rather than transmitted (diascopic) illumination. Confocal microscopy provides images from a single plane mediated by the exclusion of the background information above and below the plane, allowing high quality imaging. In paper I, we used the fluorescent stereomicroscope Leica DFC365FX with a 1.0x planapo lens and the Olympus FV1000 scanning confocal microscope (under a 60x/1.00 numerical aperture water immersion objective) for live imaging of Tg(CMV:EGFP-map1lc3b) larvae. We used the Zeiss Stemi 508 stereomicroscope (with an AxioCam MRc5) for *in situ* hybridization images and for larvae images (paper II and III) and the Zeiss LSM 780 confocal microscope using an Apochromat 40x/1.2 WC or 60x/1.2 oil DIC objective for imaging fixed Tg(CMV:MLS-GFP-mCherry) larvae (paper III). The punctas for both paper I and II were quantified manually.

## **Whole mount *in situ* hybridization and qRT PCR**

*In situ* hybridization is one of the oldest methods in zebrafish research. It enables the investigation of spatial gene expression patterns in whole embryo/larvae or in sections. During an *in situ* hybridization procedure, an antisense mRNA probe is designed to recognize and bind the endogenous transcript, which is later detected by a color- or fluorescence-based assay. In paper II, we used *in situ* hybridization to detect transcript levels of endogenous tyrosine

hydroxylase (*th1*), *nipsnap1* and *nipsnap2* at various embryonic developmental stages. In paper III, we used *in situ* hybridization to spatially stain for *nipsnap1*, as well as dorsal, ventral and mesodermal markers at the shield stage and endodermal markers at 75% epiboly stage. Even though, *in situ* hybridization helps answer the spatial localization of transcripts, it does not provide information about a particular biological activity. One would then need to look at the respective protein and e.g. its phosphorylation events. Temporal expression pattern of genes can be measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We have used qRT-PCR to analyze relative expression levels of various genes in paper II and III. In particular when analyzing autophagic flux (as described above), it is necessary to monitor the mRNA levels of respective autophagy genes (Klionsky et al., 2016).

### **Oxidative stress analysis**

Oxidative stress is caused by the imbalance between redox reactions (that produce free radicals) and the antioxidant mechanisms. This can lead to detrimental consequences if not taken care off. There have not been much advances in tools for measurement of oxidative stress in zebrafish, though some fluorescent probes have been tested here and there (Hermann et al., 2004; Rieger & Sagasti, 2011). In paper II, we measured reactive oxygen species (ROS) in zebrafish larvae by treating dissociated zebrafish cells with 10  $\mu$ M of CellRox. CellRox is a cell-permeant dye that is weakly fluorescent when in reduced state, but fluoresces brightly when oxidated by ROS with subsequent binding to DNA. It has an absorption/emission maxima of  $\sim 485/520$  nm. This can be analyzed on a Fluorescence Activated Cell Sorter (FACS). We also used 1  $\mu$ M propidium iodide (PI) to check for cell viability of the same samples used for measuring ROS. PI is a membrane impermeant dye that is generally excluded from viable cells with absorption/emission maxima of  $\sim 488/617$  nm.

### **Locomotory analysis**

A high degree of conservation of the nervous system in mammals and zebrafish make zebrafish very useful for comparative behavioral studies (Panula et al., 2010; Eklof-Ljunggren et al., 2012). The use of zebrafish larvae for behavioral studies have been rapidly rising owing to the availability of platforms designed to assess locomotor activity in larval zebrafish. In paper II of this thesis we have used an automated live video tracking system called the ZebraBox (ZebraLab, ViewPoint Life Sciences, France), which employs a high-speed infrared camera to

track and quantify zebrafish larval movement. Some of the advantages of the ZebraBox over other systems are i) Total temperature control allows hours of experimenting without the device heating up and thus negating temperature variations, ii) Strong light simulation to mimic circadian rhythm studies or other particular light conditions, iii) Sound proof, thus minimizing sound induced variations in data, iv) Real time tracking and visualization. Nipsnap1 mutant and control larvae at 7 dpf were placed into separate wells of a 48 well plate (BD Falcon GmbH, Germany). The outer wells were devoid of larvae to minimize false positives generated through reflections of larvae while swimming. Fry were gently pipetted into the plate 12-16 hours before the experiment started. The plates were placed into the ZebraBox and larvae were allowed to habituate for 1 hour before recording began. The experiment was run for 60 minutes, including 30 minutes of light cycle and 30 minutes of dark cycle. Infrared camera captured videos in 30 frames per second.

## **FUTURE PERSPECTIVE**

The work performed in this thesis provides a significant contribution to the field of autophagy. Our studies identified the PX-domain containing protein HS1BP3 as a novel negative regulator of non-selective autophagy and NIPSNAP domain containing proteins NIPSNAP1 and -2 as facilitators of mitophagy, a form of selective autophagy. Our understanding of the hierarchy of autophagy-related proteins have increased significantly over the last decades, but very little is still known about the lipids involved, their interaction with the autophagic protein machinery and how these are regulated under various metabolic conditions and in disease. We found that depletion of HS1BP3 results in increased levels of PA and that the PA generating enzyme PLD1 was required for autophagy. The exact role of PA in autophagy is however still obscure. PA is a cone shaped lipid that may contribute to membrane curvature and penetration of proteins into membranes. PA has also been found to have fusogenic properties, suggesting it could facilitate fusion of autophagosome precursor membranes with the growing phagophore or autophagosome maturation. Future studies are needed to elucidate whether the effects we see by HS1BP3 on PA and autophagy are due to effects on the phagophore lipid composition, the maturation of the phagophore or effects on the incoming membrane sources or several of these. Furthermore, it is highly desirable to study the cues that determine the levels of PA during autophagosome generation. The fusogenic properties of PA make it a possible candidate to study how membrane input fuses with the growing phagophores, the role of HS1BP3 during this process and if it involves other proteins like SNAREs.

The role of Hs1bp3 as a negative regulator of autophagy was validated in zebrafish, suggesting its conserved role across species. It will be interesting to investigate the phenotypes associated with Hs1bp3-mediated depletion in zebrafish, as well as a possible effect on Pld1 activity and PA levels. In a previous study it was found that Pld1 is required for the development of notochord and for intersegmental vessel in zebrafish. Ablation of Pld1 resulted in vasculature defects (Zeng et al., 2009). Thus it would be advantageous to study the role of Hs1bp3 during the development of zebrafish and the effects that it would have on its ablation or overexpression. Since knockdown of Pld1 causes vasculature defects, one could speculate that autophagy is considerably downregulated on the basis of its connection with autophagy and HS1BP3. Thus, future studies may reveal that HS1BP3-regulated autophagy could have a role in vascular

biology.

According to the literature, NIPSNAP domain containing proteins NIPSNAP1 and -2 are predicted to have varied roles in varied situations. It would be highly desirable to understand why they do have so many functions. NIPSNAP1 and -2 are found to be conserved from amoeba to higher eukaryotes excluding plants, with high level of conservation in their NIPSNAP domain region, suggesting a very important common function across species. They have been predicted to function as monooxygenases, but this is something that needs to be explored further. In our study we found that NIPSNAP1 and -2 get imported to the mitochondrial matrix. Their functions in the mitochondrial matrix are however unknown. We do not see any effect on mitochondrial respiration on their abrogation in mammalian cells.

NIPSNAP1 and -2 was found to mediate mitophagy both in mammalian cells and zebrafish brain. We show that upon mitochondria depolarization, newly synthesized NIPSNAP1 decorates the OMM surface. We cannot exclude the possibility of imported NIPSNAP1 being exported out to the surface on depolarization, but this is less likely since an export apparatus of proteins out of mitochondria has not been described. Furthermore, we find that the role of NIPSNAP1 and -2 in mitophagy is dependent on PARKIN. It will be important to further understand the relationship between PARKIN and NIPSNAP1 or -2.

We show that Nipsnap1 is important for mitophagy in the brain of zebrafish larvae and have some preliminary data suggesting Nipsnap2 being important for mitophagy in muscles of zebrafish larvae. Further studies are however needed to describe the effect of Nipsnap2 depletion and inhibited mitophagy in zebrafish muscles. We show that DA neurons are being degenerated upon ablation of Nipsnap1, most likely owing to dysfunctional mitophagy. It needs to be seen if Nipsnap1 is involved in the development of DA neurons in the first place, to exclude the possibility of developmental defects of DA neurons in the absence of Nipsnap1. Furthermore, it will be important to investigate whether Nipsnap1 absence has an effect on other neuronal population or brain regions, in particular the basal ganglia (with its connection to PD) or if it is specific to DA neurons only. Zebrafish lacking Nipsnap1 show a parkinsonian phenotype and it would be advantageous to look for  $\alpha$ -synuclein profiles in these mutants and for total dopamine levels as well.

Investigation of the role of Nipsnap1 during embryogenesis is still “work in progress” where we will try to answer if Nipsnap1 is directly involved in the pathways of gastrulation and thereby affecting the development of zebrafish larvae or if the high mortality and gross phenotypes that we observe are resultant from defective mitophagy.

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## **ORIGINAL PUBLICATIONS**







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# HS1BP3 negatively regulates autophagy by modulation of phosphatidic acid levels

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A fundamental question is how autophagosome formation is regulated. Here we show that the PX domain protein HS1BP3 is a negative regulator of autophagosome formation. HS1BP3 depletion increased the formation of LC3-positive autophagosomes and degradation of cargo both in human cell culture and in zebrafish. HS1BP3 is localized to ATG16L1- and ATG9-positive autophagosome precursors and we show that HS1BP3 binds phosphatidic acid (PA) through its PX domain. Furthermore, we find the total PA content of cells to be significantly upregulated in the absence of HS1BP3, as a result of increased activity of the PA-producing enzyme phospholipase D (PLD) and increased localization of PLD1 to ATG16L1-positive membranes. We propose that HS1BP3 regulates autophagy by modulating the PA content of the ATG16L1-positive autophagosome precursor membranes through PLD1 activity and localization. Our findings provide key insights into how autophagosome formation is regulated by a novel negative-feedback mechanism on membrane lipids.

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**A**utophagy targets intracellular components for lysosomal degradation to promote cellular and organismal health and homeostasis, and has been shown to protect against neurodegeneration and cancer, help remove invading pathogens and promote longevity<sup>1</sup>. Macroautophagy (here referred to as autophagy) is characterized by the formation of double-membrane autophagosomes from an expanding cargo-enwrapping phagophore and the subsequent fusion of autophagosomes with lysosomes. Autophagy is induced by stresses like starvation and also provides cellular quality control under basal conditions<sup>2</sup>. Autophagy must be tightly controlled at each step of the process; autophagosome formation without proper turnover is linked to neurodegenerative disorders such as Alzheimer's disease<sup>3</sup>, defective as well as excessive autophagy is detrimental for muscle health<sup>4</sup> and uncontrolled autophagy could potentially harm or even kill an otherwise healthy cell.

Nucleation of a phagophore and biogenesis of a functional autophagosome is regulated by several multi-subunit complexes, including the ULK1 complex, the integral membrane protein mATG9 and its associated proteins, the class III phosphatidylinositol (PI) 3-kinase (PI3K) complex and two ubiquitin-like conjugation systems, resulting in the conjugation of ATG12 to ATG5 and ATG8/LC3 family members to phosphatidylethanolamine (PE)<sup>5</sup>. ATG5–ATG12 further associates with ATG16L1 and the resulting complex is recruited to endoplasmic reticulum-associated PI(3)P-rich sites of phagophore nucleation (called omegasomes)<sup>6</sup> by the PI(3)P-binding protein WIPI2 (ref. 7). Further expansion of the phagophore to generate an autophagosome requires input from several membrane sources, including the endoplasmic reticulum<sup>8–10</sup>, mitochondria<sup>9,11</sup>, plasma membrane<sup>12</sup> and recycling endosomes<sup>13–16</sup>. Recycling endosome-derived membranes are positive for ATG9 and ATG16L1, and essential for autophagosome formation<sup>13–16</sup>.

The autophagic pathway involves lipids as signalling molecules, constituents and cargo of autophagosomes. However, the role of different lipids in autophagy is not clear<sup>17,18</sup>. PA was initially found to activate mammalian target of rapamycin (mTOR)<sup>19</sup>, a well-known inhibitor of autophagy, in a PLD1-specific manner<sup>20</sup>. Recent studies have also implicated PLD1-generated PA in autophagosome formation<sup>21,22</sup> and in autophagosome–lysosome fusion<sup>23</sup>. PI(3)P, the lipid product of the class III PI3K complex, has a central role in autophagy and several PI(3)P-binding proteins in autophagy have been identified<sup>17,24</sup>, including the FYVE domain proteins DFCEP1, a marker for omegasomes<sup>6</sup>, the scaffold protein ALFY that links cargo to the autophagic machinery for selective autophagy<sup>25,26</sup> and FYCO1, which is involved in trafficking of autophagosomes on microtubuli<sup>27</sup>. Furthermore, the WD-repeat protein WIPI2 also binds PI(3)P and is found at omegasomes<sup>28</sup>.

Another group of phosphoinositide-binding proteins are the PX domain-containing proteins, but little is known about their involvement in autophagy. Here we show that the PX domain protein HS1BP3 negatively regulates autophagosome formation, PA levels and PLD activity. HS1BP3 binds PA through its PX domain, which leads to the recruitment of HS1BP3 to PLD1- and ATG16L1-positive autophagosome precursor membranes. We propose that HS1BP3, through its binding to PA and inhibition of PLD1 activity, provides a novel negative-feedback mechanism to ensure the proper regulation of autophagosome biogenesis.

## Results

**HS1BP3 is a negative regulator of autophagy.** To identify PX domain proteins involved in autophagy, we recently performed an imaging-based short interfering RNA (siRNA) screen in HEK GFP-LC3 cells<sup>13</sup> and one of the candidate proteins was HS1BP3.

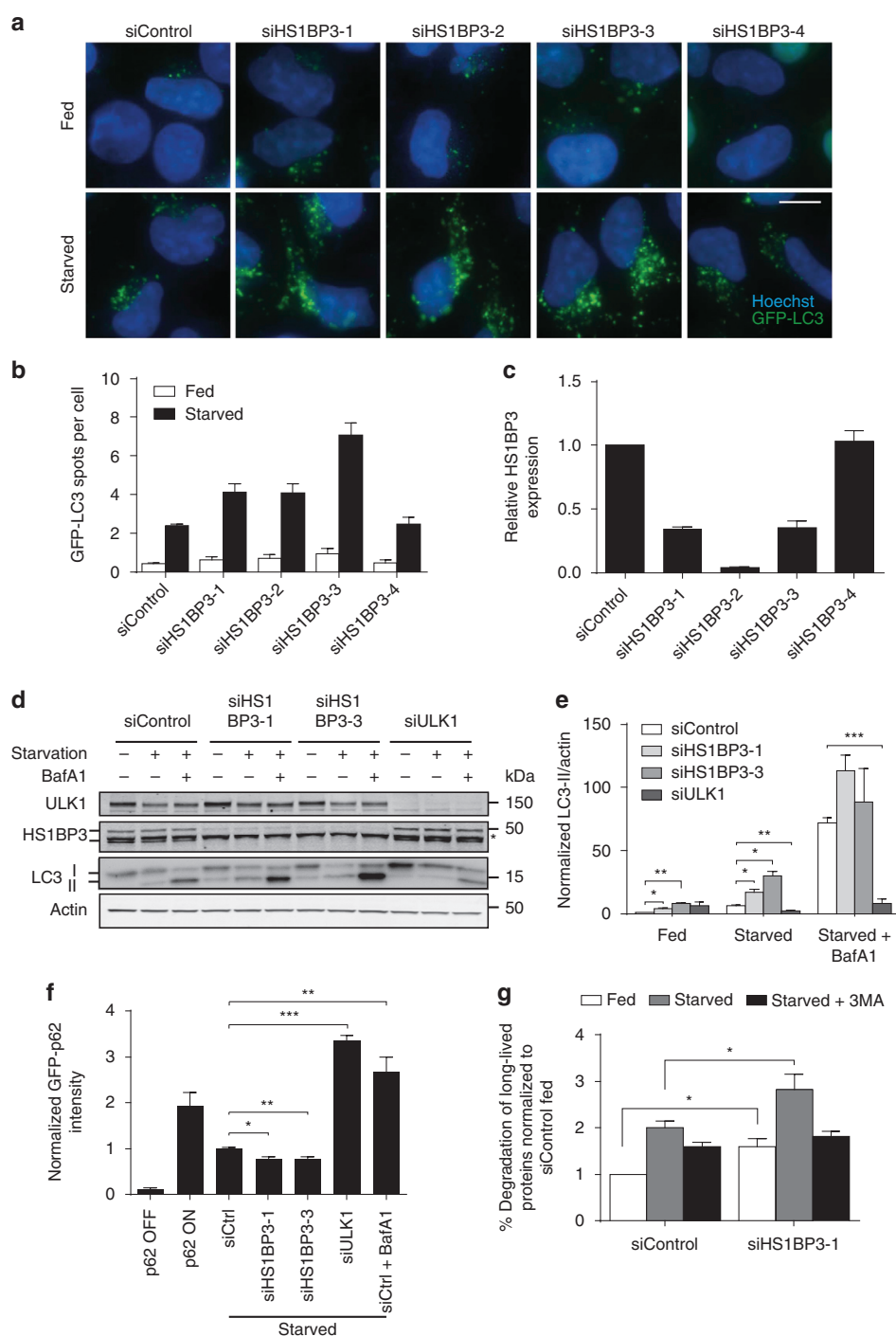
Using the individual siRNA oligos from the screen, we find that depletion of HS1BP3 results in increased amounts of GFP-LC3 spots (autophagosomes) both in complete (fed) and nutrient-deplete (starved) medium in correlation with knockdown levels (Fig. 1a–c). Depletion of HS1BP3 also increases the total intensity of endogenous LC3 spots in starved cells (Supplementary Fig. 1a).

Since depletion of HS1BP3 increased the number of autophagosomes, we next investigated whether this is due to the increased formation or inhibited maturation and turnover of autophagosomes. To this end, cells were starved in the presence or absence of the lysosomal proton pump inhibitor Bafilomycin A1 (BafA1; which inhibits autophagosome maturation and lysosomal degradation) and autophagic flux monitored by quantification of the level of PE-conjugated LC3 (LC3-II)<sup>29</sup>. We find that LC3-II levels are significantly increased in HS1BP3-depleted cells both in complete medium and after starvation (Fig. 1d,e), and increase further in the presence of BafA1, indicating that autophagosome formation is increased on HS1BP3 depletion. As expected, LC3 lipidation is strongly inhibited in ULK1-depleted cells. We verified that LC3 messenger RNA (mRNA) levels are not significantly affected by HS1BP3 depletion (Supplementary Fig. 1b).

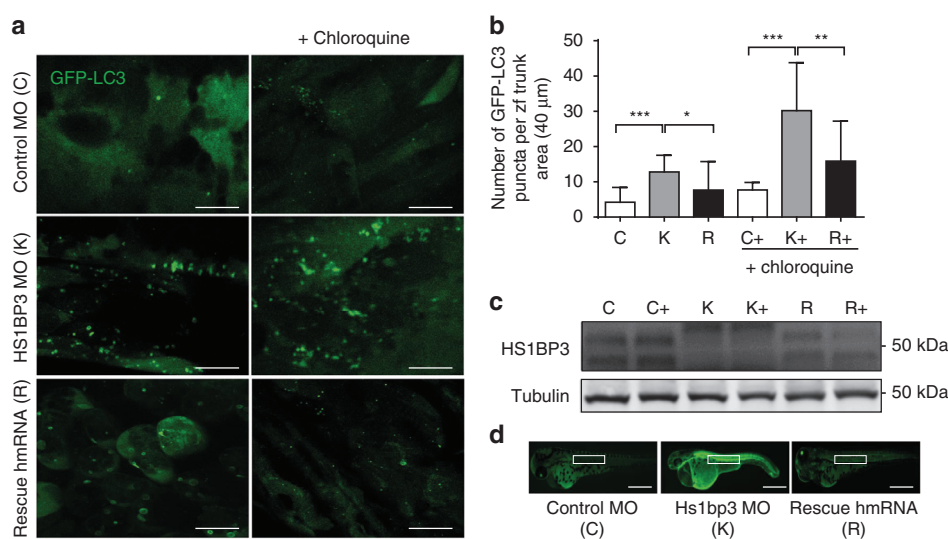
To further determine if depletion of HS1BP3 activates autophagy, we studied the degradation of the cargo receptor protein p62 (also known as Sequestosome-1), which is itself an autophagy substrate<sup>30,31</sup>. GFP-p62 expression was shut off in a stable cell line<sup>32</sup> and the amount of GFP-p62 remaining after starvation was measured by flow cytometry. Whereas about half of the initial GFP-p62 is degraded in control cells, GFP-p62 strongly accumulates in ULK1-depleted cells, as well as in cells treated with BafA1 (Fig. 1f). Consistent with an increase in GFP-LC3 spots and LC3 lipidation, GFP-p62 degradation increases by 20% in cells depleted of HS1BP3 (Fig. 1f), indicating increased autophagic flux. This was further confirmed by assessing the degradation of long-lived proteins, which preferentially happens through autophagy and is inhibited by the PI3K inhibitor 3-methyladenine. As shown in Fig. 1g, the release of free <sup>14</sup>C-valine from the degradation of previously radiolabelled long-lived proteins is increased in HS1BP3-depleted cells compared with control cells both in fed and starved conditions, further indicating that HS1BP3 is a negative regulator of autophagy.

To analyse a possible role of HS1BP3 in regulation of autophagy *in vivo*, we employed transient silencing of *Hs1bp3* in the zebrafish line Tg(CMV:EGFP-map1lc3b)<sup>33</sup>, using a translational-blocking morpholino targeting the start site of the *Hs1bp3* mRNA. The overall homology between human and zebrafish *Hs1bp3* is 36%, with the PX domain being highly conserved (67% homology; Supplementary Fig. 1c). At 2 days post fertilization (dpf), abundant GFP-LC3 puncta are present in the trunk region of the morphant compared with the control embryos (Fig. 2a–d) and this difference is even more pronounced after chloroquine treatment, known to block autophagosome degradation in zebrafish<sup>34,35</sup>. On injection of *in vitro*-transcribed-capped human *Hs1bp3* mRNA alongside the *Hs1bp3* morpholino, we observe a partial rescue of the phenotype at 2 dpf both with and without chloroquine treatment (Fig. 2a–d; Supplementary Fig. 1d). These results suggest that autophagy is significantly elevated in *Hs1bp3* morphant zebrafish at 2 dpf and that *Hs1bp3* also regulates autophagy *in vivo*.

**HS1BP3 interacts with cortactin.** HS1BP3 was originally identified as an interaction partner of the actin cross-linking protein HS1 (ref. 36). HS1 is exclusively expressed in cells of hematopoietic lineage, whereas other cells express the homologous protein cortactin<sup>37</sup>. We therefore asked whether



**Figure 1 | HS1BP3 is a negative regulator of autophagy.** (a) HEK GFP-LC3 cells were transfected with four individual siRNA oligonucleotides against HS1BP3. 72 h post transfection the cells were starved or not for 2 h in EBSS, followed by fixation and fluorescence microscopy. Scale bar, 10  $\mu$ m. (b) The number of GFP-LC3 spots per cell in a was quantified by high-content analysis (mean  $\pm$  s.d. from two independent experiments in triplicates,  $\sim$  50,000 cells analysed per condition). (c) Relative expression of HS1BP3 after siRNA knockdown was measured by quantitative PCR with reverse transcription (mean  $\pm$  s.d.). (d) HEK GFP-LC3 cells were transfected with the indicated siRNA oligos and starved or not for 2 h in EBSS in the presence or absence of BafA1. \* Indicates an unspecific band in the HS1BP3 immunoblot. (e) The level of LC3-II/actin was quantified from immunoblots and normalized to siControl fed (mean  $\pm$  s.e.m.,  $n = 5$ ). (f) HEK GFP-p62 cells were transfected with siRNA against HS1BP3 or ULK1. Expression of GFP-p62 was induced by addition of tetracycline (compare ON versus OFF) for 48 h before expression was shut off and the cells were incubated in EBSS (starved) for 2.5 h to induce autophagic degradation of GFP-p62. GFP-p62 intensity was monitored by flow cytometry and normalized to starved siControl (siCtrl; mean  $\pm$  s.e.m.,  $n = 4$ ). (g) The degradation of long-lived proteins in HeLa cells transfected with control siRNA or siRNA against HS1BP3 was quantified as the release of  $^{14}$ C-valine after 4 h starvation in the absence or presence of 3-methyladenine (3MA) and normalized to the degradation in fed control cells (mean  $\pm$  s.e.m.,  $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , by Student's  $t$ -test.



**Figure 2 | HS1BP3 regulates autophagy in zebrafish.** (a) Representative confocal images of GFP-LC3 puncta (autophagosomes) in the trunk area of GFP-LC3 transgenic zebrafish embryos injected with control morpholino (C), Hs1bp3 translational-blocking morpholino (K), and the human Hs1bp3 mRNA coinjected with the morpholino (R) and imaged at 2 dpf with or without pre-treatment with chloroquine (10 μM) for 6 h. Scale bars, 10 μm. (b) GFP-LC3 puncta were counted in the trunk region (marked in d) of the transgenic zebrafish embryos at 2 dpf (mean ± s.e.m.,  $n = 3$ ). Total of 7–13 embryos were used for each condition per experiment. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , by Student's  $t$ -test. (c) Representative immunoblotting of Hs1bp3 and Tubulin in whole lysates of zebrafish embryos at 2 dpf, treated with or without chloroquine for 6 h before harvest. (d) Representative light fluorescent microscopy images of whole embryos at 2 dpf. Scale bars, 300 μm.

HS1BP3 also binds to cortactin and whether this interaction is involved in HS1BP3-mediated inhibition of autophagy. We find that endogenous cortactin co-immunoprecipitates with GFP-HS1BP3 (Supplementary Fig. 3a). The interaction is mediated by the SH3 domains of cortactin and HS1 (Supplementary Fig. 3b, lane 9 and 11), and is lost when a critical SH3 domain tryptophan is mutated to tyrosine (Supplementary Fig. 3b, lane 10 and 12). The HS1 and cortactin SH3 domains interact exclusively with the C-terminal part of HS1BP3 (HS1BP3-ΔPX, Supplementary Fig. 3c) containing four proline-rich regions (Fig. 4a). We can however not detect a role for cortactin in basal or starvation-induced autophagy (Supplementary Fig. 3d–e), indicating that binding of HS1BP3 to cortactin is not essential for its inhibitory function in autophagy.

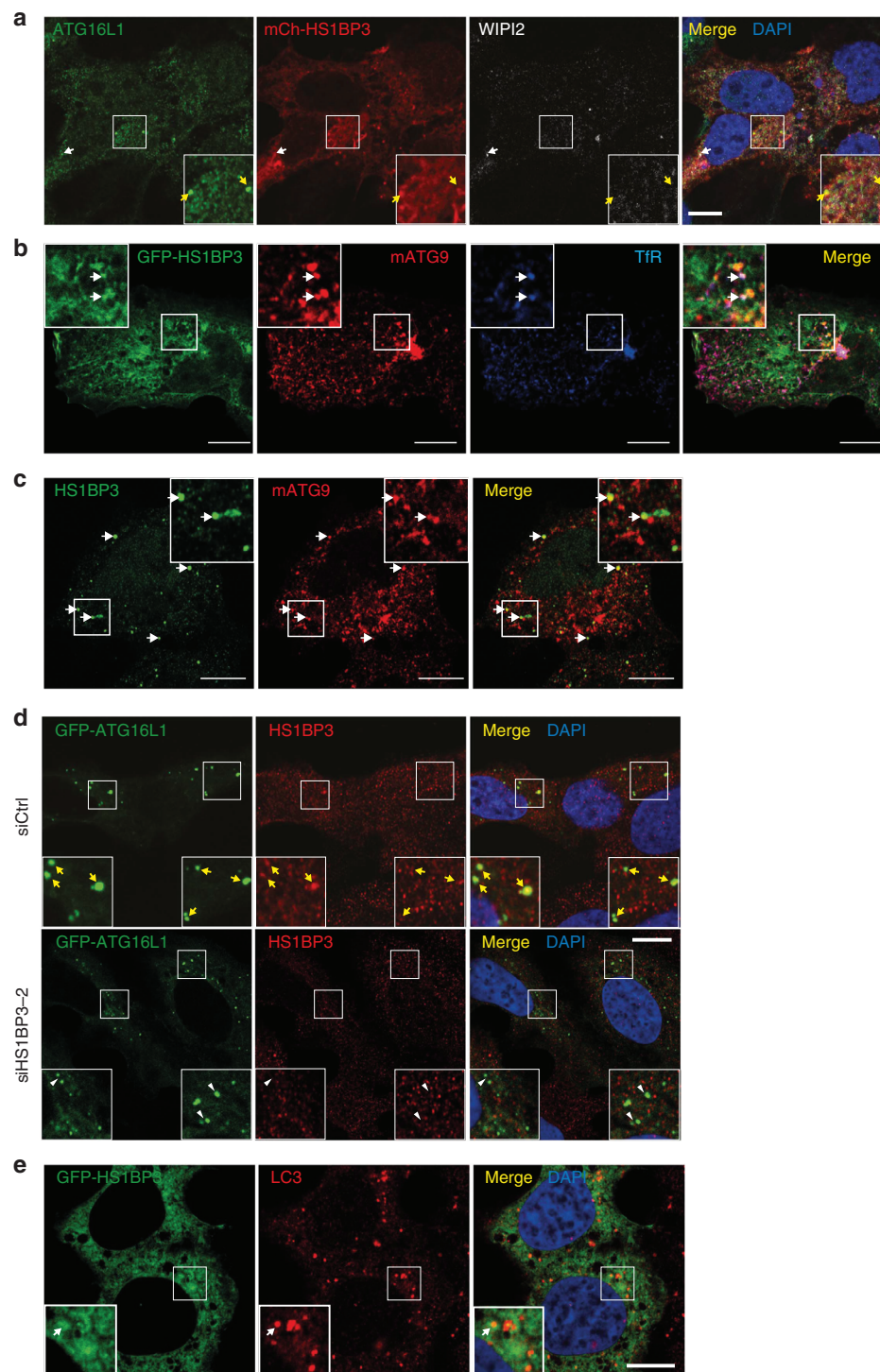
#### HS1BP3 localizes to ATG9–ATG16L1-positive membranes.

To identify the mechanisms underlying the role of HS1BP3 as a negative regulator of autophagy, the localization of HS1BP3 to autophagy-related membranes in HEK293 and U2OS cells was investigated. Cells were transfected with GFP- or mCherry-tagged HS1BP3 and their co-localization with WIPI2, ATG9, ATG16L1 or LC3 analysed by confocal imaging. While HS1BP3 is only occasionally detected on WIPI2-positive structures (Fig. 3a, white arrows), it co-localizes well with ATG9 and ATG16L1-positive membranes (Fig. 3a,b). Endogenous HS1BP3 also clearly co-localizes with endogenous ATG9 (Fig. 3c) and with GFP-ATG16L1-positive vesicles (Fig. 3d), and the co-localization is lost after HS1BP3 depletion (Fig. 3d, white arrowheads), demonstrating the specificity of the HS1BP3 antibody. In contrast, HS1BP3 does not show much co-localization with LC3-positive structures (Fig. 3e; Supplementary Fig. 2a) and does not interact with LC3 or GABARAP proteins (Supplementary Fig. 2b). Moreover, no co-localization of endogenous HS1BP3 with GFP-p62, -DFCP1 or -ATG14 is detected (Supplementary Fig. 2a).

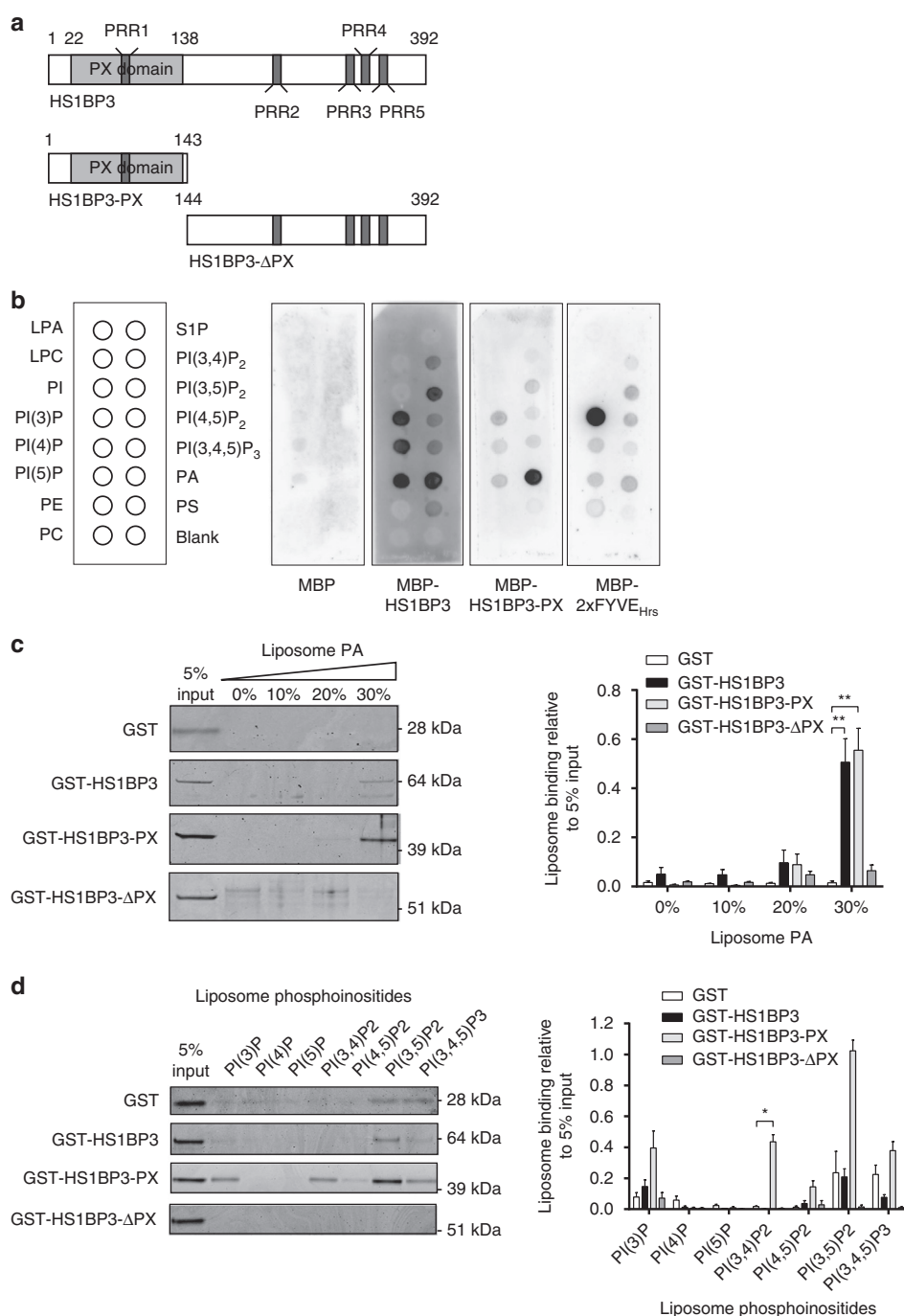
Trafficking of ATG16L1 and ATG9 through recycling endosomes is important for autophagosome biogenesis<sup>13–16</sup>. Using confocal and live cell imaging, we find that the HS1BP3-, ATG9- and ATG16L1-positive membranes contain transferrin and transferrin receptor (TfR; Fig. 3b; Supplementary Fig. 5c) and seem to fuse with LC3-positive structures (Supplementary Movies 1–3), indicating they are recycling endosome-derived membranes. Depletion of HS1BP3 does not cause any quantitative changes in the early phagophore/omegasome markers GFP-DFCP1, WIPI2 and ATG16L1. Neither the total intensity of GFP-DFCP1 spots (Supplementary Fig. 2c) nor the number of endogenous WIPI2 or ATG16L1 spots (Supplementary Fig. 2d–e) are affected by HS1BP3 depletion. Taken together, we find that HS1BP3 localizes to ATG9 and ATG16L1-positive recycling endosome-derived membranes that contribute membrane to the forming autophagosome at a stage after omegasome formation, indicating that HS1BP3 regulates autophagy downstream or in parallel of the initial phagophore nucleation step.

**HS1BP3 binds PA and regulates cellular PA levels.** To investigate a possible role for HS1BP3 in regulation of membrane trafficking and/or biogenesis at the expanding phagophore, we first set out to characterize the lipid-binding specificity of the HS1BP3 N-terminal PX domain (Fig. 4a). PX domain proteins are known to mainly bind PI3P, but also other phosphoinositide-binding preferences have been described<sup>38,39</sup>. Using lipid-coated membrane strips, we find the HS1BP3 PX domain to bind strongly to PA, whereas full-length HS1BP3 binds PA, as well as monophosphorylated phosphoinositides (Fig. 4b; Supplementary Fig. 4a). The lipid specificities of purified HS1BP3 proteins (full length or PX domain) was further explored using liposome flotation experiments. We find that the binding of both HS1BP3 and the PX domain to PA increase with increasing concentrations of liposome PA (Fig. 4c).

Using liposomes containing various phosphoinositide species, we could confirm that the PX domain of HS1BP3 also has



**Figure 3 | HS1BP3 localizes to ATG16L1- and ATG9-positive vesicles.** HEK293 and U2OS cells expressing the indicated proteins were starved for 2 h before fixation and immunostaining with the indicated antibodies. Confocal micrographs show: **(a)** HEK cells expressing mCherry-HS1BP3 stained for endogenous ATG16L1 and WIPI2. Yellow arrows mark HS1BP3- and ATG16L1-positive structures. White arrow marks HS1BP3-, ATG16L1- and WIPI2-positive structure. **(b)** Co-localization of GFP-HS1BP3 with endogenous ATG9 and TfR (white arrows show triple co-localization) in U2OS cells. **(c)** Co-localization of endogenous HS1BP3 with endogenous ATG9 in HEK cells. **(d)** Control or HS1BP3-depleted U2OS cells expressing GFP-ATG16L1 stained for endogenous HS1BP3. Yellow arrows indicate ATG16L1-positive structures that are positive for HS1BP3, while white arrow heads indicate ATG16L1-positive structures that are not positive for HS1BP3. Note that in addition to the specific staining (co-localization with ATG16L1), the HS1BP3 antibody also recognizes other proteins non-specifically both on immunofluorescence and western blotting (Fig. 1d). **(e)** HEK cells expressing GFP-HS1BP3 stained for endogenous LC3. Scale bars, 10  $\mu$ m.



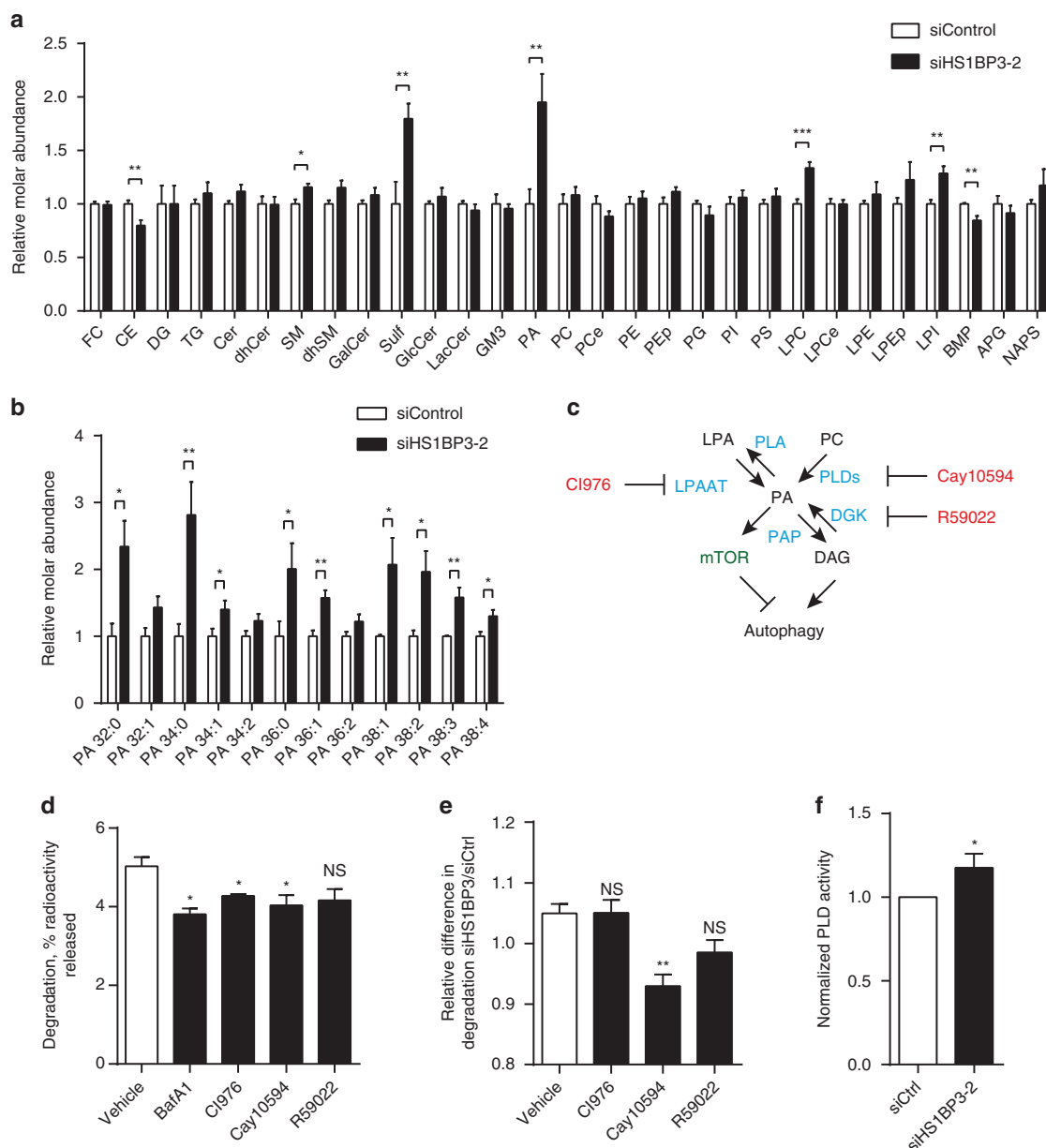
**Figure 4 | HS1BP3 binds PA through its PX domain.** (a) Domain structure of HS1BP3: an N-terminal PX domain followed by an unstructured C terminus. The positions of five proline-rich regions (PRRs) are indicated. HS1BP3 truncations lacking the C-terminal (HS1BP3-PX) or the PX domain (HS1BP3-ΔPX) are shown. (b) Membranes spotted with the indicated lipids were incubated with  $1 \mu\text{g ml}^{-1}$  of the indicated recombinant MBP-tagged proteins in a lipid protein overlay assay and bound proteins were detected with anti-MBP immunoblotting. (c,d) Liposomes with the indicated molar ratios of dioleoyl-phosphatidic acid (DOPA) (c) or the indicated phosphoinositides (d) were incubated with GST or GST-tagged HS1BP3 protein constructs. Protein binding to liposomes was analysed by a lipid floatation assay. Representative coomassie-stained gels are shown and quantified from three independent experiments (mean  $\pm$  s.e.m.). Significance is calculated as compared with GST control. If significance is calculated as compared to GST-HS1BP3-ΔPX, then GST-HS1BP3-PX shows significantly increased binding to PI(3)P, PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. \* $P < 0.05$ , \*\* $P < 0.01$ , by Student's *t*-test.

increased affinity for PI(3)P, PI(3,4)P<sub>2</sub> and PI(3,5)P<sub>2</sub> as compared with the GST control (Fig. 4d). The full-length HS1BP3 protein also shows affinity for phosphoinositides and if compared with GST-HS1BP3-ΔPX it binds several of the

same phosphoinositides as the PX domain alone, most notably PI(3)P and PI(3,5)P<sub>2</sub> (Fig. 4d). The discrepancy between the data obtained using lipid strips and liposomes is most likely due to differences in the context that the lipids are presented,

where the liposomes more closely resemble the context proteins bind to membranes in cells. We conclude that HS1BP3 binds to PA and several phosphoinositides and we speculate that HS1BP3 has phosphoinositide affinity *in vivo* for PI(3,5)P2 and PI(3)P. Interestingly, the HS1BP3 PX domain co-localizes with ATG16L1 (Supplementary Fig. 4b), suggesting that the lipid affinity of the PX domain contributes to its specific recruitment to ATG16L1-positive membranes.

To get an unbiased quantification of a wide range of lipids in HS1BP3-depleted cells compared with control cells under starvation conditions, the total cellular lipid content was measured by lipidomics (Fig. 5a). Interestingly, whereas the quantities of several lipids are significantly altered (Fig. 5a; Supplementary Fig. 4c); the quantitatively biggest effect is on PA levels, as depletion of HS1BP3 causes a twofold increase in the total abundance of the lipid (Fig. 5a). In total, the levels of 9 out of



**Figure 5 | Autophagy is dependent on PA synthesis and HS1BP3 affects PLD activity.** (a) HEK cells were treated with non-targeting or HS1BP3 siRNA and starved for 2 h. The total lipid content was extracted and analysed by mass spectrometry. Only lipid species that were present in all experimental runs were included. The measured lipid concentrations were first normalized to total lipid per sample to determine molar percentages for each lipid subclass and species, before normalizing to the average molar percentage of controls (mean  $\pm$  s.e.m.,  $n = 6$ ). For a full list of all lipids with abbreviations, see Supplementary Table 1. The lipidomics data sets have been deposited in the Dryad Digital Repository (doi:10.5061/dryad.gq3fk). (b) The relative molar abundance of all 12 PA species is shown. (mean  $\pm$  s.e.m.,  $n = 6$ ). (c) Schematic overview of the enzymes (blue) governing the generation and turnover of PA from other lipid species (black) and the drugs (red) used to inhibit these pathways. (d) Degradation of long-lived proteins in HEK cells was quantified as the release of  $^{14}\text{C}$ -valine after 4 h starvation in the presence of the indicated inhibitors (mean  $\pm$  s.e.m.,  $n = 3$ ). (e) The relative difference in long-lived protein degradation between HEK cells with siCtrl and siHS1BP3 was measured for the indicated inhibitors (mean  $\pm$  s.e.m.,  $n = 3$ ). (f) PLD activity was measured in HEK lysates of cells transfected with non-targeting or HS1BP3 siRNA (mean  $\pm$  s.e.m.,  $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , by Student's *t*-test.

12 PA species are increased by HS1BP3 depletion (Fig. 5b). Interestingly, many of these PA species have been shown to be products of PLD activity<sup>40,41</sup>.

PA is a cone-shaped lipid that has been found to stimulate both autophagosome biogenesis<sup>21,22</sup> and autophagosome-lysosome fusion<sup>23</sup>. On the other hand, PA has been shown to activate mTOR signalling<sup>19</sup>, which inhibits autophagy. We therefore asked whether depletion of HS1BP3 might stimulate autophagy through changes in mTOR activity. Phosphorylation of the mTORC1 substrate S6 Kinase (pS6K) is however not affected by HS1BP3 depletion (Supplementary Fig. 4d), indicating that the increased PA levels seen on HS1BP3 depletion is not inducing autophagy through decreased mTORC1 signalling.

### HS1BP3 regulates PA levels and autophagy through PLD1.

Several metabolic pathways lead to PA formation (Fig. 5c)<sup>42</sup>. To first determine which of these contribute to increased autophagic flux, cells were treated with inhibitors specific for each pathway followed by quantification of autophagic degradation of long-lived proteins (Fig. 5d). We find that inhibitors of PLD activity (Cay10594, inhibits both PLD1 and PLD2) and lysophosphatidic acid acyltransferases (LPAATs; CI-976) inhibit autophagic flux, whereas an inhibitor of diacylglycerol kinase (R50922) has no significant effect (Fig. 5d). These observations taken together with the increased levels of PA and autophagy on HS1BP3 depletion suggested that HS1BP3 may be inhibiting autophagy as a negative regulator of one of these PA-generating pathways. We reasoned that if HS1BP3 depletion increased the activity of one of the pathways, then chemical inhibition of this pathway should abolish the increased autophagy seen in HS1BP3-depleted cells. Indeed, the relative inhibition of autophagy by the PLD inhibitor Cay10594 is significantly larger in cells depleted of HS1BP3, compared with control cells and cells treated with inhibitors of LPAATs or diacylglycerol kinase (Fig. 5e), suggesting that HS1BP3 may regulate PA levels through PLDs. In line with this, we find that the total activity of PLD enzymes is increased in cell lysates of HS1BP3-depleted cells compared with control cells (Fig. 5f), in line with our data showing that HS1BP3 depletion leads to increased PA levels (Fig. 5a).

To determine which of the PLD enzymes are contributing to the HS1BP3 phenotype, we analysed the localization of GFP-PLD1 and -PLD2 in relation to the autophagy markers LC3 and ATG16L1. GFP-PLD2 staining is concentrated at the plasma membrane with no co-localization with either ATG16L1 (Fig. 6a) or LC3 (Supplementary Fig. 5a). In contrast, GFP-PLD1 shows extensive co-localization with ATG16L1-positive puncta (Fig. 6a), and is often seen in close proximity to LC3-positive puncta (Supplementary Fig. 5a,c). The majority of the vesicles stained by GFP-PLD1 and ATG16L1 contain transferrin and TfR (Fig. 6b), and seem to fuse with Cherry-LC3B-positive structures (Supplementary Fig. 5c; Supplementary Movies 1 and 2), indicating that they are recycling endosome-derived vesicles destined for autophagosome biogenesis. HS1BP3 is also detected at the TfR- and PLD1-positive structures (Supplementary Fig. 5b). Because PLD1, and not PLD2, localizes to ATG16L1-positive membranes (Fig. 6a), we conclude that the effect of HS1BP3 on PLD activity is most likely through the regulation of PLD1 on recycling endosomes or vesicles derived thereof.

To get some mechanistic insight into how HS1BP3 regulates PLD1 activity at the Atg16L1-positive membranes, we investigated the localization of PLD1 to ATG16L1-positive structures in the absence or presence of HS1BP3. Interestingly, there is a significant increase in the amount of ATG16L1-positive vesicles with GFP-PLD1 staining in HS1BP3-depleted cells compared

with control cells (Fig. 6c), indicating that HS1BP3 may regulate PLD1's access to these vesicles.

As both HS1BP3 and PLD1 have a PX domain with similar lipid-binding specificities (Fig. 4; ref. 43), we hypothesized that they might compete for binding to lipids in ATG16L1-positive membranes. In line with this notion, the co-localization between HA-PLD1 and endogenous ATG16L1 is significantly decreased in cells expressing the full length or PX domain of GFP-HS1BP3 compared with control cells expressing GFP only, but not in cells expressing HS1BP3 lacking the PX domain (GFP-HS1BP3  $\Delta$ PX; Fig. 6d,e).

We further looked for protein-protein interactions between these proteins that could explain their apparent co-localization and functional relationship, but were unable to detect any interactions between over-expressed or endogenous PLD1, HS1BP3 and ATG16L1 under the conditions tested (Supplementary Fig. 6a). Taken together, our data indicate that HS1BP3 prevents access of PLD1 to ATG16L1-positive vesicles and we speculate that the two proteins compete for binding to lipids in the ATG16L1-positive membranes.

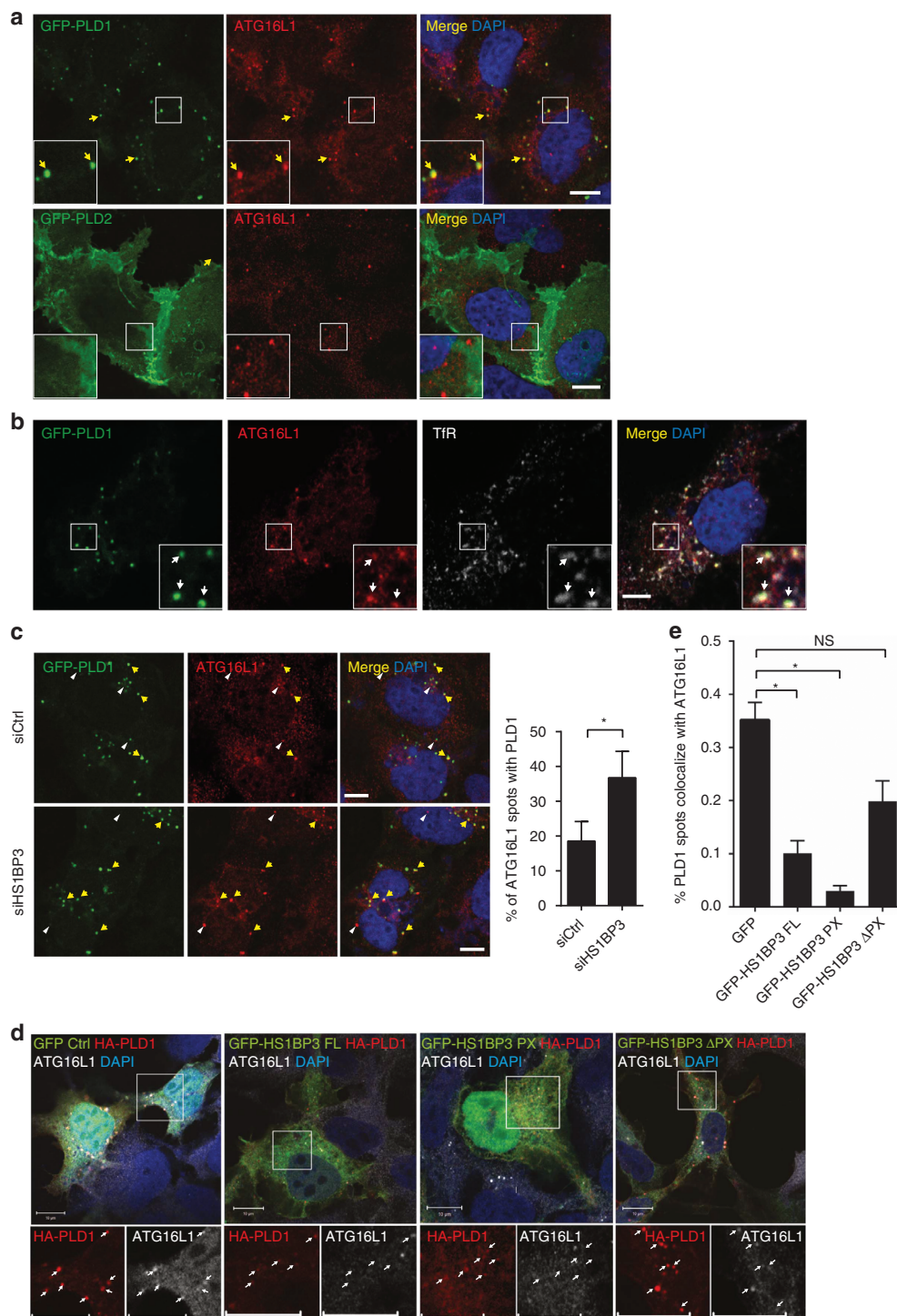
To further map the functional relationship between HS1BP3 and PLD1 in regulation of autophagy, endogenous LC3 puncta were quantified in cells depleted of HS1BP3 and/or PLD1, and at the same time transfected with GFP, GFP-HS1BP3 or GFP-PLD1 (Fig. 7a). While depletion of HS1BP3 increases the number of LC3 spots and LC3-II levels (in line with data in Fig. 1), this effect is reversed in cells co-depleted of PLD1 (Fig. 7a; Supplementary Fig. 6b), indicating that the increase in autophagy seen with HS1BP3 depletion is dependent on PLD1, in line with our previous observation of the HS1BP3-mediated increase in autophagy being reverted by the use of PLD inhibitors (Fig. 5e). Moreover, overexpression of GFP-PLD1 causes a significant increase in the amount of LC3-positive spots (Fig. 7a), with no further increase on concurrent depletion of HS1BP3, further supporting that HS1BP3 and PLD1 are part of a common mechanism, where HS1BP3 acts on autophagy through regulation of PLD1.

Taken together, our observation that HS1BP3 inhibits the localization of PLD1 to ATG16L1-positive vesicles suggests that the effect of HS1BP3 on autophagy is through the regulation of PLD1-generated PA on ATG16L1-positive autophagosome precursors (Fig. 7b). In light of the binding of HS1BP3 to PA, we propose a mechanism by which HS1BP3 regulates PLD1 by providing negative feedback on PA production through regulating the access of PLD1 to target membranes.

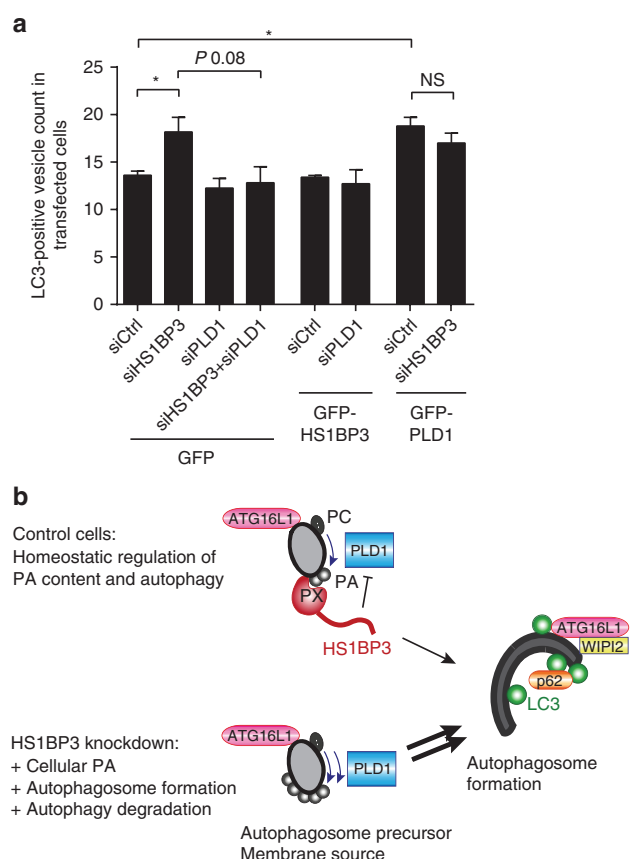
### Discussion

Autophagosome formation must be properly regulated to balance the need to ensure cellular quality control and nutrient availability during starvation, with the necessity to prevent excessive autophagosome formation and detrimental degradation of crucial cellular components. We here identify the PX domain protein HS1BP3, as an inhibitor of the autophagosome formation and autophagic degradation through a negative-feedback mechanism, involving the regulation of PLD1 activity, and hence PA levels, in ATG16L1-positive membranes. Depletion of HS1BP3 affects both PLD activity in lysates and PLD1 localization to ATG16L1-positive vesicles, suggesting that HS1BP3 acts as a sensor and regulator of local PA levels. On its recruitment, HS1BP3 will inhibit PLD1 activity on ATG16L1-positive autophagosome precursors, thereby reducing their PA content and autophagosome formation (Fig. 7b).

Many PX domains bind PI(3)P, but there are also other demonstrated lipid-binding specificities<sup>38,39</sup>. We found that both the full-length protein and the PX domain of HS1BP3 bind to



**Figure 6 | PLD1 co-localization with ATG16L1 is affected by HS1BP3.** (a) HEK cells were transfected with GFP-tagged PLD1 or PLD2. After starvation and fixation the cells were immunostained for ATG16L1 and analysed by confocal microscopy. (b) HEK cells were transfected with GFP-PLD1, starved, fixed and immunostained for ATG16L1 and TfR. (c) HEK cells were first treated with non-targeting or HS1BP3 siRNA, then transfected to express GFP-PLD1, starved, fixed and immunostained for ATG16L1. Yellow arrows indicate ATG16L1 vesicles positive for PLD1 and white arrow heads indicate ATG16L1 vesicles negative for PLD1. Co-localization of GFP-PLD1 to ATG16L1 vesicles was quantified in transfected cells using the ImageJ plugin Squash, using 10 pictures of each condition from three independent experiments (mean  $\pm$  s.e.m.,  $n = 3$ ). (d) HEK cells were transfected with HA-PLD1 together with GFP, GFP-HS1BP3 full-length, -PX or  $\Delta$ PX constructs, starved and stained for endogenous ATG16L1. Arrows indicate co-localization between ATG16L1 and HA-PLD1. (e) Co-localization of HA-PLD1 with endogenous ATG16L1 vesicles was quantified in transfected cells in d with the Zen software (Zeiss) using 10 pictures of each condition from three independent experiments (mean  $\pm$  s.e.m.,  $n = 3$ ). Scale bars, 10  $\mu$ m. \* $P < 0.05$ , by Student's  $t$ -test.



**Figure 7 | HSI1BP3 regulates autophagy through PLD1.** (a) HEK cells were first treated with the indicated siRNA and then transfected with the indicated GFP-tagged construct. Cells were starved and fixed before immunostaining for endogenous LC3. LC3 spots were counted only in transfected cells, minimum 200 transfected cells per condition in three independent experiments (mean  $\pm$  s.e.m.,  $n = 3$ ). \* $P < 0.05$ , by Student's  $t$ -test. (b) Model for the role of HSI1BP3 in autophagy. PLD1 generates PA on ATG16L1-positive autophagosome precursor membranes. HSI1BP3 is recruited to these membranes by the generated PA, inhibiting PLD1 activity and displacing it from the ATG16L1 vesicles. HSI1BP3 thus provides a negative feedback on PA generation on these vesicles. If HSI1BP3 is depleted from the cells, this negative feedback is lost, causing the PA concentrations of these membranes to increase and thereby drive increased autophagosome formation.

PA and phosphoinositides. PA binding by PX domains has previously been reported. A study of the PX domain of PLD1 demonstrated binding to PI(3,4,5)P<sub>3</sub>, PI(3)P, as well as other PI species and a moderate affinity for PA in a separate binding pocket on the PX domain<sup>43</sup>. Interestingly, the simultaneous binding of both sites was shown to increase the membrane affinity of the PX domain<sup>43</sup>. Similarly, the PX domain of the p47 subunit of NADPH oxidase was found to simultaneously bind PI(3,4)P<sub>2</sub> and PA in separate binding pockets, increasing its membrane affinity<sup>44</sup>. Strikingly, we observed a competition between HSI1BP3 and PLD1 for binding to ATG16L1-positive precursors. Having a similar lipid-binding specificity may be the basis of this competition between HSI1BP3 and PLD1 for membrane binding, and this is something that will be interesting to explore further in future studies.

We also found that the LPAAT pathway of PA generation contributes to autophagy, demonstrating that the involvement of PA in autophagy is not limited to PLD1. The exact mechanism(s)

underlying the role of PA in stimulation of autophagosome biogenesis is not clear and might be related to its role as a second messenger, but could also be associated with PA having a direct structural role in membrane curvature and/or fusion due to the unique characteristics of PA in a lipid bilayer. PA is the only anionic phospholipid that induces negative membrane curvature due to its cone shape under physiological conditions<sup>45</sup>. Cone-shaped lipids such as PA also facilitate penetration of proteins into the membrane, since the lipid head groups are more loosely packed<sup>46</sup>, as shown to be important for insertion of ATG3 in the forming phagophore and subsequent lipidation of LC3/GABARAP<sup>47</sup>.

Another relevant characteristic of PA is the demonstrated fusogenic properties of this lipid. PLD activity has been demonstrated as essential for various vesicle fusion events, such as sporulation in yeast<sup>48</sup>, mitochondrial fusion<sup>49</sup> and exocytosis<sup>50</sup>. We speculate that the increased autophagy seen in HSI1BP3-depleted cells might be facilitated by PA-mediated changes in the fusogenic properties of the ATG16L1-positive autophagosome precursors. Homotypic fusion of ATG16L1-positive vesicles has previously been found to facilitate their contribution to autophagosome formation and this was demonstrated to be dependent on the SNARE protein VAMP-7 (ref. 51). Intriguingly, VAMP-7 was recently described as an effector of PLD1 in neurite outgrowth<sup>52</sup>, suggesting a possible mechanism by which PLD1-generated PA could affect autophagy. HSI1BP3 is detected on ATG16L1 vesicles that also contain ATG9 and TfR, suggesting they are of recycling endosome origin. We recently identified the PX-BAR protein SNX18 as a positive regulator of autophagosome biogenesis by tubulation of recycling endosome membrane for the delivery to phagophore nucleation sites<sup>13</sup>. The RAB11-binding protein TBC1D14 is another negative regulator of autophagy found to regulate the recycling endosome membrane remodelling<sup>15</sup>. It will be interesting to explore how these membrane-associated proteins regulate the recycling endosome dynamics and how potential qualitative changes in these vesicles affect autophagy through homotypic and possibly heterotypic fusion processes.

In conclusion, we have identified HSI1BP3 as a novel negative regulator of autophagy and cellular PA levels. We propose that PLD1 generates PA on ATG16L1-positive autophagosome precursor membranes and that HSI1BP3 is recruited to these membranes by binding to PA (Fig. 7b). HSI1BP3 affects the ability of PLD1 to generate PA, as well as regulating the access of PLD1 to ATG16L1-positive vesicles, changing the properties of these membranes and thereby providing a homeostatic regulation of autophagy. HSI1BP3 thus functions as a negative-feedback mediator of PA levels to regulate autophagosome formation.

## Methods

**Cell lines and inhibitors.** HeLa, HEK and U2OS cells were from American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 5 U ml<sup>-1</sup> penicillin and 50  $\mu$ g ml<sup>-1</sup> streptomycin. The HEK 293A GFP-LC3 cell line<sup>53</sup> was a kind gift from S. Toozé, Cancer Research UK, London, UK. The HEK GFP-DFCP1 cell line<sup>6</sup> was a kind gift from N. Ktistakis, Babraham Institute, Cambridge, UK. The HEK GFP-p62 cell line was a kind gift from G. Bjørkøy, HiST, Trondheim, Norway. All cell lines have been tested negative for mycoplasma. Bafilomycin A1 (Enzo Lifesciences) was used at 100 nM. CI 976 (Tocris Bioscience), Cay10594 (Cayman Chemical) and R59022 (Tocris Bioscience) were used at 20  $\mu$ M. Glass support was coated by 20  $\mu$ g ml<sup>-1</sup> fibronectin (Sigma) before plating HEK cell lines to avoid the cells from detaching from the surface. For starvation in nutrient-deplete medium, the cells were incubated in Earls Balanced Salt Solution (EBSS; Invitrogen), with the exception of the HEK GFP-DFCP1 cells that were starved as described previously<sup>6</sup> in 140 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose and 20 mM Hepes, pH 7.4.

**Antibodies and dyes.** The following primary antibodies were used: mouse anti-cortactin (Upstate, 05-180, 1:1,000), mouse anti-GFP (Clontech, 632381,

1:1,000), mouse anti-Flag (Sigma, F1804, 1:500), mouse anti-MBP (NEB, e8032S, 1:10,000), rabbit anti-ULK1 (Santa Cruz, sc-33182, 1:250), rabbit anti-HS1BP3 (GeneTex, GTX107715, 1:10,000 for WB and 1:500 for IF), rabbit anti-LC3 (Cell Signaling, 27755, 1:1,000 for WB), mouse anti- $\beta$ -actin (Sigma, SAB1305567 1:20,000), mouse anti-myc (DSHB, 9E10, 1:20), mouse anti- $\alpha$ -tubulin (Sigma, T5168, 1:20,000), rabbit anti-LC3 (MBL, PM036, 1:500 for IF), mouse anti-p62 (BD biosciences, 610833, 1:1,000 for WB), goat horseradish peroxidase (HRP)-conjugated anti-GST (Abcam, ab58626, 1:10,000 for phosphatidylinositol phosphate (PIP) strips), rabbit anti-phospho-AKT Ser473 (Cell Signaling, 4060, 1:2,000), rabbit anti-phospho-p70-S6K Thr389 (Cell Signaling, 9202, 1:1,000), rabbit anti-p70-S6K (Cell Signaling, 9205, 1:1,000), rabbit anti-ATG16L1 (MBL, PM040, 1:200), mouse anti-TfR CD71 (Santa Cruz, sc-65877, 1:200), mouse anti-WIP1 (kind gift from Sharon Tooze, 1:2,000), rabbit anti-PLD1 (Cell Signaling, 3832S, 1:200), mouse anti-HA (Abcam, ab18181, 1:200), hamster anti-mAtg9 (kind gift from Sharon Tooze, 1:1,000). HRP- and Cy2/3/5-conjugated secondary antibodies were obtained from Jackson Immunolabs. Far-red fluorophore-conjugated secondary antibodies were from LI-COR. Transferrin-Alexa 647 was from Invitrogen.

**Transfection of siRNA oligonucleotides and western blotting.** siRNA oligonucleotides were Dharmacon ON-TARGET plus; HS1BP3-1J-013029-09 AAGAAGGAGUGACCGUAU, HS1BP3-2J-013029-10 UGAAGAGGCUUUCG ACUUU, HS1BP3-3J-013029-11 GAGCCUGAAGGCGGAGGAU, HS1BP3-4J-013029-12 UCCCAAAGUGGCGUGAAA, ULK1 J-005049-06 CCACGAG-GUGCAGAACUA, cortactin CCCGAAAGACUAUGUGAAGGG<sup>54</sup> and PLD1 SmartPool consisting of four oligonucleotides pooled together. An amount of 20–100 nM siRNA was delivered to the cells by Lipofectamine RNAi max (Invitrogen). To demonstrate specific protein knockdown and monitor LC3 levels, the cells were lysed in 25 mM Hepes pH 7.5, 125 mM K-acetate, 2.5 mM Mg-acetate, 5 mM EGTA, 1 mM DTT and 0.5% NP-40 supplemented with Complete protease inhibitor (Roche). Protein concentration was measured by Biorad Protein Assay to run equal amounts of cell lysate on SDS-polyacrylamide gel electrophoresis (PAGE), followed by western blotting using specified primary antibodies and secondary antibodies for enhanced chemiluminescent (ECL) detection or far-red fluorescence. For ECL detection: membranes were incubated with HRP-conjugated secondary antibodies detected by the Supersignal West Dura Extended Duration Substrate kit (Pierce). Imaging and quantification of protein levels were performed using the Syngene gel documentation unit, Genesnap acquisition software and GeneTools analysis software. For far-red fluorophores: membranes were incubated with far-red fluorophore-conjugated secondary antibodies, and detection and analysis was performed by LI-COR Odyssey imaging. Uncropped scans of western blots are found in Supplementary Fig. 7.

**Plasmids and transfection for ectopic expression.** HS1BP3 complementary DNA (cDNA) was amplified by PCR using primers 5'-ATAGTCGA-CATGCAGTCCCCGCGGTGCTC-3' and 5'-ATAGCGGCCGCTCAGAAGA-GACTGGGGGCGG-3' from a cDNA library made by reverse transcription (Biorad iScript) from mRNA isolated from HEK cells, TA cloned into pCR2.1-TOPO (Invitrogen) and subcloned into pENTR1A (Invitrogen) using *Sall* and *NotI* restriction sites. From there, sequences coding for HS1BP3-PX and HS1BP3- $\Delta$ PX were amplified by PCR using primers 5'-ATAGTCGACATGCAGTCCCCGCGG TGCTC-3' and 5'-ATAGCGGCCGCTCAGGATCTGGTACCTAAGAACTC-3' or 5'-ATAGTCGACGCTGACGGGCTCACCAGCAG-3' and 5'-ATAGCGGCCGCT CAGAAGAGACTGGGGGCGG-3', respectively, and cloned into pENTR1A (Invitrogen). Tagged variants were made by Gateway LR cloning (Invitrogen) into respective pDEST vectors (Invitrogen). See Supplementary Table 2 for a full list of plasmids used in this study. To transfect cells with plasmids encoding GFP- or mCherry-tagged HS1BP3 variants, the plasmids were delivered to the cells by forward transfection with FuGene (Roche) or Lipofectamine 2000 (Invitrogen) before further treatment as described.

**Microscopy.** siRNA-treated HEK GFP-LC3 cells grown on glass support, were starved or not for 2 h, pre-permeabilized on ice with 0.05% saponin in 80 mM K-Pipes pH 6.8, 5 mM EGTA and 1 mM MgCl<sub>2</sub> before fixation in 3% paraformaldehyde (PFA) or fixed directly in methanol for 10 min at  $-20^{\circ}\text{C}$ . The nuclei were counterstained with 1  $\mu\text{g ml}^{-1}$  Hoechst in phosphate-buffered saline or mowiol. The number of GFP-LC3 spots was quantified using the automated Olympus ScanR microscope equipped with a ULSAPO 40 $\times$  objective and the corresponding analysis program or by an automated Zeiss CellObserver equipped with a 40 $\times$  EC Plan Neofluar objective, and using the physiology module of the Zeiss Assaybuilder software. For immunostaining and confocal analysis, cells were grown on glass cover slips and after the described treatments, fixed in 3% PFA for 15 min on ice or in methanol for 10 min at  $-20^{\circ}\text{C}$  and mounted in mowiol containing 1  $\mu\text{g ml}^{-1}$  Hoechst or 4,6-diamidino-2-phenylindole to stain the nuclei. Confocal images were taken on an Olympus confocal microscope equipped with a UPlanSApo 60 $\times$  objective. Co-localization of stainings of interest from confocal images was quantified using the ImageJ-based Squash plugin<sup>55</sup>. The plugin subtracts background, segments the image into vesicles in each channel and co-localization is quantified as degree of signal intensity overlap in the segmented

regions. Cell mask thresholding was used to include vesicles only in transfected cells.

For live cell imaging, HEK293A cells were plated in complete media in wells of Lab-Tek II chambered coverglass ( $2 \times 10^4$  cells per well), precoated with poly-D-lysine and transfected with indicated constructs. Complete media was removed 24 h after transfection, cells were washed  $2 \times$  in phosphate-buffered saline, then starved for 1 h in EBSS containing 5  $\mu\text{g ml}^{-1}$  of transferrin-Alexa Fluor 647 conjugate and imaged live with Zeiss LSM710 confocal microscope ( $63 \times$  1.4 plan-apochromat objective, single plane).

**Quantitative PCR.** siRNA-transfected cells were frozen dry at  $-80^{\circ}\text{C}$ , RNA isolated by RNeasy plus kit (Qiagen), cDNA synthesized by reverse transcription (Biorad iScript) and quantitative real-time PCR performed using SYBRGreen (Qiagen), and pre-designed Quantitect (Qiagen) primer sets for the described targets relative to SDHA or TBP as housekeeping genes on a Lightcycler 480 (Roche Applied Science) or on a CFX96 (Bio-Rad).

**GFP-p62 measured by flow cytometry.** The GFP-p62 flow cytometry assay was described previously<sup>32</sup>. Briefly, HEK GFP-p62 cells in 24-well plates were transfected with siRNA and 24 h later induced with 1  $\text{ng ml}^{-1}$  doxycyclin to express GFP-p62 for 48 h. GFP-p62 expression was shut off and the cells were starved in EBSS for 2.5 h or treated as indicated. The cells were then trypsinized and passed through cell strainer caps (BD Biosciences) to obtain single-cell suspensions. Cells were analysed on a FACSARIA cell sorter running FACSDiva software version 5.0 (BD Biosciences) using the blue laser for excitation of GFP. GFP fluorescence was collected through a 530/30 nm band-pass filter in the E detector. Data were collected from a minimum of 10,000 singlet events per tube, and the median GFP-p62 value was used for quantification.

**Long-lived protein degradation.** To measure the degradation of long-lived proteins by autophagy, proteins were first labelled with 0.25  $\mu\text{Ci ml}^{-1}$  L-<sup>14</sup>C-valine (Perkin Elmer) for 24 h in GIBCO-RPMI 1640 medium (Invitrogen) containing 10% FBS. The cells were washed and then chased for 3 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). 10% Trichloroacetic acid was added to the cells before incubation at  $4^{\circ}\text{C}$  to precipitate radioactive proteins. Ultima Gold LSC cocktail (Perkin Elmer) was added to the samples and protein degradation was determined by measuring the ratio of trichloroacetic acid-soluble radioactivity relative to the total radioactivity detected by a liquid scintillation analyser (Tri-Carb 3100TR, Perkin Elmer), counting 3 min per sample.

**In vitro interaction pull-down assays and immunoprecipitation.** Recombinant GST- or MBP-tagged proteins were expressed and purified from *Escherichia coli*. 1  $\mu\text{g}$  of proteins of interest were incubated together in NETN buffer (50 mM Tris pH 8, 100 mM NaCl, 6 mM EDTA, 6 mM EGTA, 0.5% NP-40, 1 mM DTT, Roche Complete protease inhibitor) followed by GST pull-down by glutathione sepharose (GE Healthcare). For GST pull-down from cell lysate, cells were lysed in 10 mM TrisHCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, protease inhibitor (Roche) and phosphatase inhibitor (Sigma), and the cell lysate incubated with recombinant glutathione sepharose-bound GST proteins. The resulting pull-downs were analysed by immunoblotting. For *in vitro* translation, indicated GFP fusion proteins were *in vitro* translated in TNT T7-coupled reticulocyte lysate (Promega L4610) in the presence of <sup>35</sup>S-methionine (PerkinElmer) and precleared on glutathione-sepharose before incubation in NETN buffer together with glutathione-sepharose-bound recombinant GST-tagged LC3B or GABARAP proteins expressed in and purified from *E. coli* according to manufacturer's instructions. The resulting pull-downs were separated by SDS-PAGE. The gels were Coomassie blue stained and the *in vitro*-translated co-purified proteins were detected by autoradiography on a Typhoon phosphorimaging scanner (GE Healthcare). For immunoprecipitation from lysates, GFP, GFP-HS1BP3 or GFP-PLD1 were immunoprecipitated by GFP trap (Chromotek) following the manufacturer's protocol. The resulting immunoprecipitates or pull-downs were separated by SDS-PAGE and analysed by western blotting.

**Lipid-binding assays.** PIP strips or membrane lipid strips (Echelon biosciences) were blocked in 3% fatty acid-free bovine serum albumin (Sigma) in TBS-T (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween) before incubation with 1  $\mu\text{g ml}^{-1}$  recombinant MBP proteins in TBS-T. After repeated washing in TBS-T, bound protein was immunodetected with chemiluminescence. Liposomes were prepared by mixing different molar ratios of palmitoyl-oleyl-phosphatidylcholine, dioleoyl-phosphatidic acid, dioleoyl-PE-rhodamine and the PIPs in chloroform and drying the lipids to a thin film. Lipids were reconstituted in a buffer containing 20 mM Hepes, pH 7.4, 150 mM NaCl and 1 mM MgCl<sub>2</sub> and exposed to seven cycles of flash-freezing in liquid nitrogen and thawing in a  $37^{\circ}\text{C}$  water bath, before extruding the lipid mixtures through two polycarbonate filters with 200 nm pores a total of 21 times. Lipid binding to liposomes was

analysed by a floatation assay, where 5  $\mu$ M of GST-tagged protein was incubated with 2 mM total lipid and 1 mM DTT in a buffer composed of 20 mM Hepes pH 7.4, 150 mM NaCl and 1 mM  $MgCl_2$  for 20 min at 25 °C. To separate liposomes and bound protein from free protein, this mixture was subjected to a Nycodenz liposome floatation assay as described in ref. 47. Gradients were centrifuged at 48,000 r.p.m. (280,000g) in a SW55Ti rotor (Beckman) for 4 h at 4 °C, and the liposomes and bound protein were recovered from the top 80  $\mu$ l of the gradient. The lipid recovery from the gradient was determined by measuring dioleoyl-PE-rhodamine fluorescence using a SpectraMax fluorescence spectrometer. Floatation reactions were analysed on a 12% bis-Tris gel (Novex) by SDS-PAGE, where 10% of the total lipid from each floatation reaction was run together with a protein control containing 0.5% of the total protein. The proteins were visualized with Coomassie Blue stain per the manufacturer's instruction (Imperial Protein Stain, Thermo Scientific).

**High-performance liquid chromatography-mass spectrometry.** Lipid extracts were prepared from total cell lysates (after 2 h starvation) using a modified Bligh/Dyer extraction procedure as previously described<sup>56</sup>. Samples were analysed using an Agilent Technologies 6490 Ion Funnel LC/MS Triple Quadrupole system with front end 1260 Infinity HPLC. Phospholipids and sphingolipids were separated by normal-phase high-performance liquid chromatography (HPLC), while neutral lipids were separated using reverse-phase HPLC. For normal-phase analysis, lipids were separated on an Agilent Rx-Sil column (i.d. 2.1  $\times$  100 mm) using a gradient consisting of A: chloroform/methanol/ammonium hydroxide (89.9:10.0:1) and B: chloroform/methanol/water/ammonium hydroxide (55:39:5.9:0.1), starting at 5% B and ramping to 70% B over a 20 min period before returning back to 5% B. Neutral lipids were separated on an Agilent Zorbax XDB-C18 column (i.d. 4.6  $\times$  100 mm), using an isocratic mobile phase chloroform:methanol:0.1 M ammonium acetate (100:100:4) at a flow rate of 300  $\mu$ l min<sup>-1</sup>. Multiple reaction monitoring transitions were set up for quantitative analysis of different lipid species and their corresponding internal standards as described previously<sup>56</sup>. Lipid levels for each sample were calculated relative to the spiked internal standards and then normalized to the total amount of all lipid species measured and presented as relative mol %. Data are presented as mean mol % for three samples of each condition.

**PLD activity assay.** PLD activity was measured using the Amplex Red Phospholipase D Assay Kit (Invitrogen) according to the manufacturer's instructions. Cell lysates were made of six-well plates using the assay reaction buffer with 1% Triton X-100. For each replicate, 20  $\mu$ g of lysate was diluted to 50  $\mu$ l of lysis buffer and added to 50  $\mu$ l of reaction mixture. Four technical replicates per sample were distributed in a black half-area 96-well plate (Corning). The plate was covered and the reaction proceeded for 30 min at 37 °C before fluorescence was measured.

**Zebrafish work.** Experimental procedures followed the recommendations of the Norwegian Regulation on Animal Experimentation. All experiments were conducted on GFP-LC3 transgenic larvae<sup>57</sup> under 5 dpf. Translation-blocking antisense morpholino oligonucleotides for *Hs1bp3* (5'-TTcTaATACcTC CcTCTcACATTGT-3') or a scrambled-sequence morpholino (5'-TTGTTAT ACGTCCGCTGACATTGT-3') were designed according to the manufacturer's recommendations (Gene Tools, Philomath, OR, USA) and 16.86 ng of either was injected into embryos at the one-cell stage. Capped full-length human wild-type *Hs1bp3* mRNA was transcribed from linearized pSP64 Poly (A) Vector (Promega; Supplementary Table 2) using mMessage mMachine (Ambion) and 50–150 pg was coinjected with the *Hs1bp3* morpholino as described<sup>58</sup>. Microscopic visualization, screening and imaging of the fish were performed on a stereomicroscope Leica DFC365FX with a 1.0  $\times$  planapo lens. Control morpholino, *Hs1bp3* morpholino and human *Hs1bp3* mRNA injected live embryos were anaesthetized with tricaine at 2 dpf and mounted in low-melting point agarose for imaging with Olympus FV1000 scanning confocal microscope (under a 60  $\times$  /1.00 numerical aperture water immersion objective). Injected embryos were treated or not with 10  $\mu$ M chloroquine at 28 °C for 6 h and then followed by immunoblot analysis and imaging. Embryos at 2 dpf were deyolked and then homogenized in lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)). All experiments were replicated at least three times with 7–13 embryos per condition. Embryos were randomly distributed to receive the described treatments. No blinding was used and no animals were excluded from the analysis.

**Statistics.** The *P* values were derived from two-tailed *t*-test from Excel (Microsoft) for paired samples, and considered statistically significant at *P*  $\leq$  0.05. In some cases, values were log-transformed to obtain a normal distribution.

**Data availability.** The lipidomics datasets have been deposited in the Dryad Digital Repository (DOI: doi:10.5061/dryad.gq3fk).

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## Author contributions

H.K. and A.S. conceived the idea. H.K., P.H. and K.S. designed and conducted the experiments, analysed the data and generated figures. S.W.S. performed the ultra-structural studies. A.H.L. and T.J.M. performed the liposome interaction studies. S.P., S.R.C., V.H.L. and G.T.B. helped perform experiments. B.J.M. designed and performed the zebrafish experiments. R.B.C., B.Z. and G.D.P. performed and analysed the lipidomics study. K.L. contributed to the statistical analysis. H.K., P.H. and A.S. wrote the manuscript with input from all co-authors.

## Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

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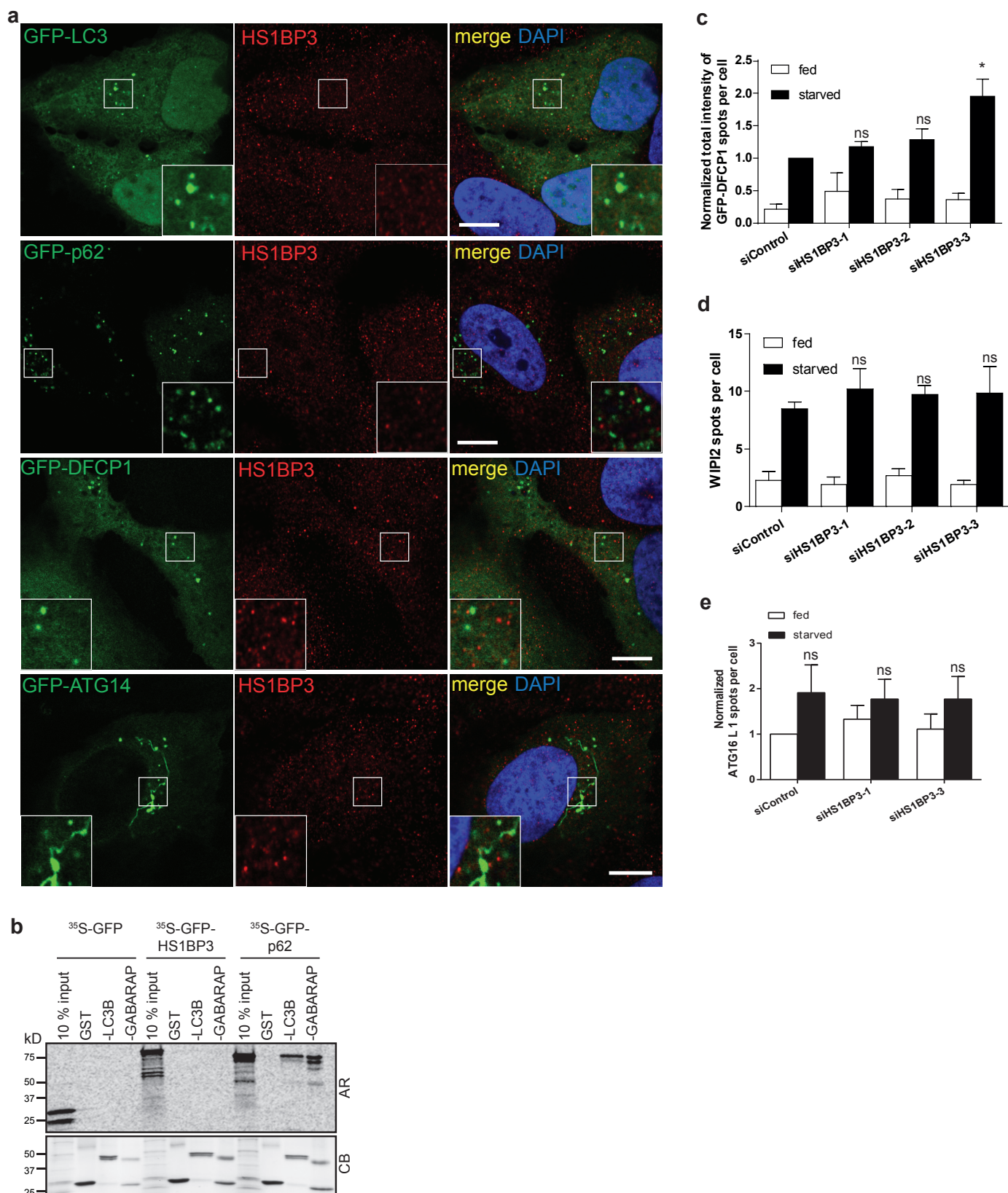
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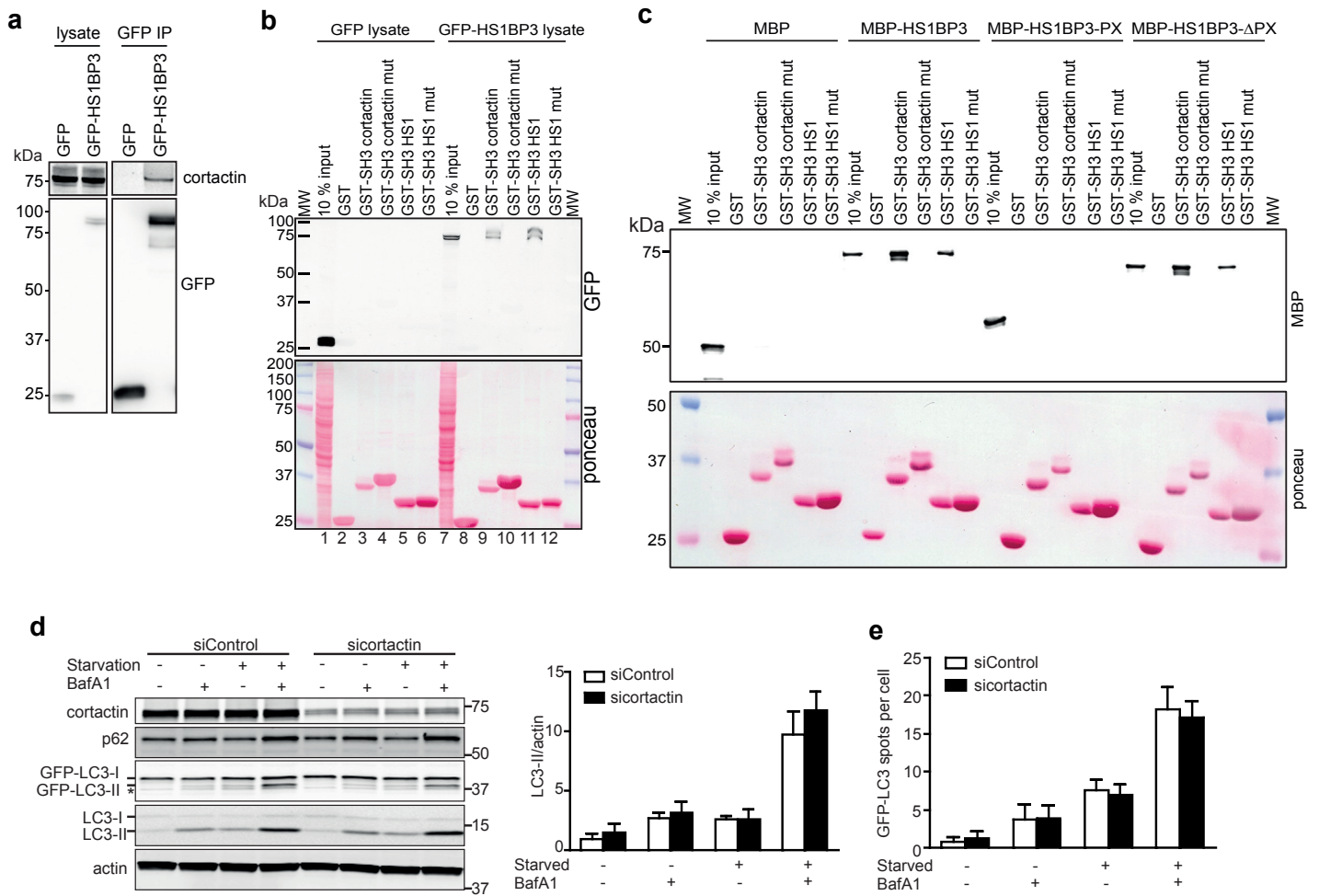
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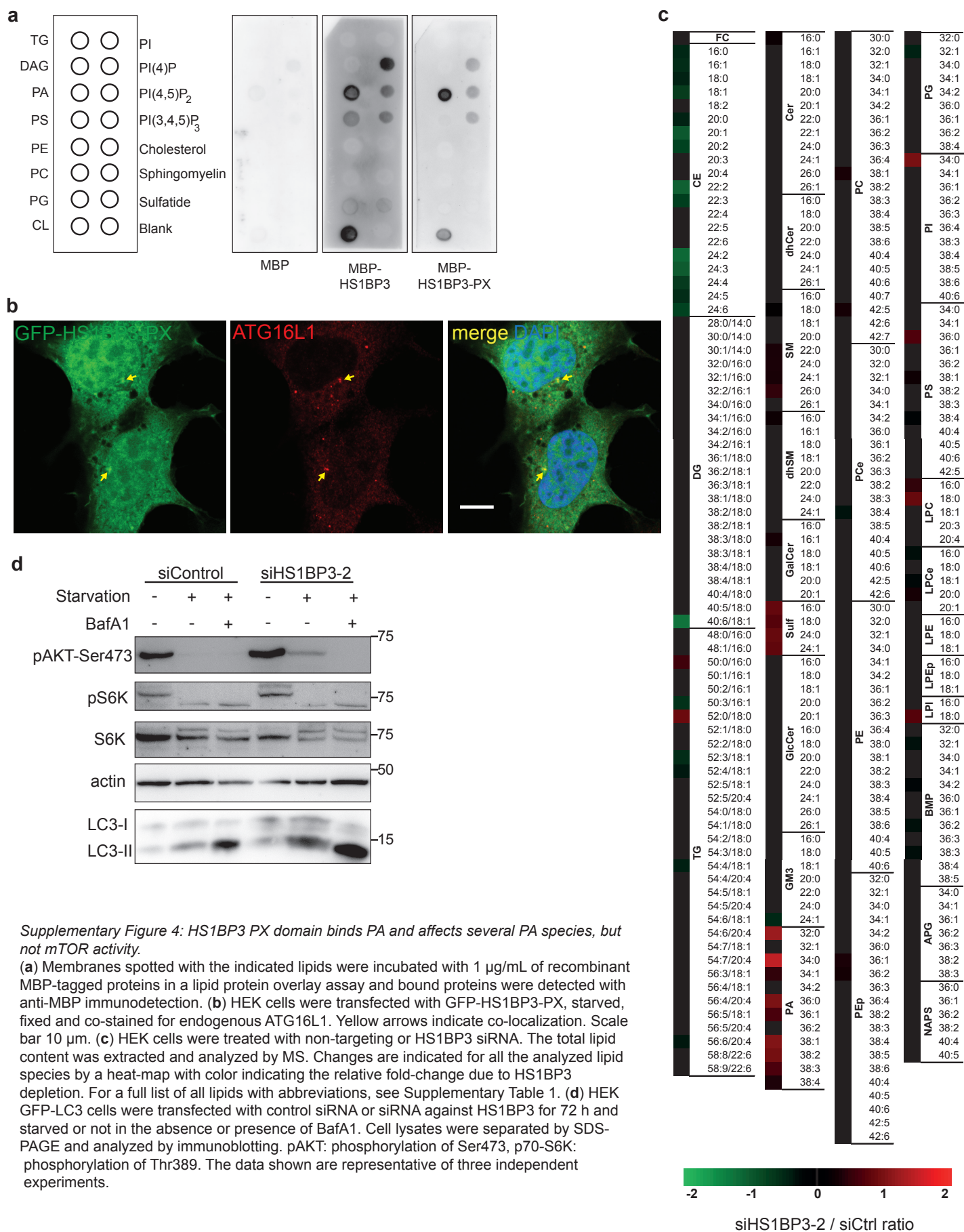
**Supplementary Figure 2: Localization of endogenous HS1BP3 and its effects on early phagophore markers.**

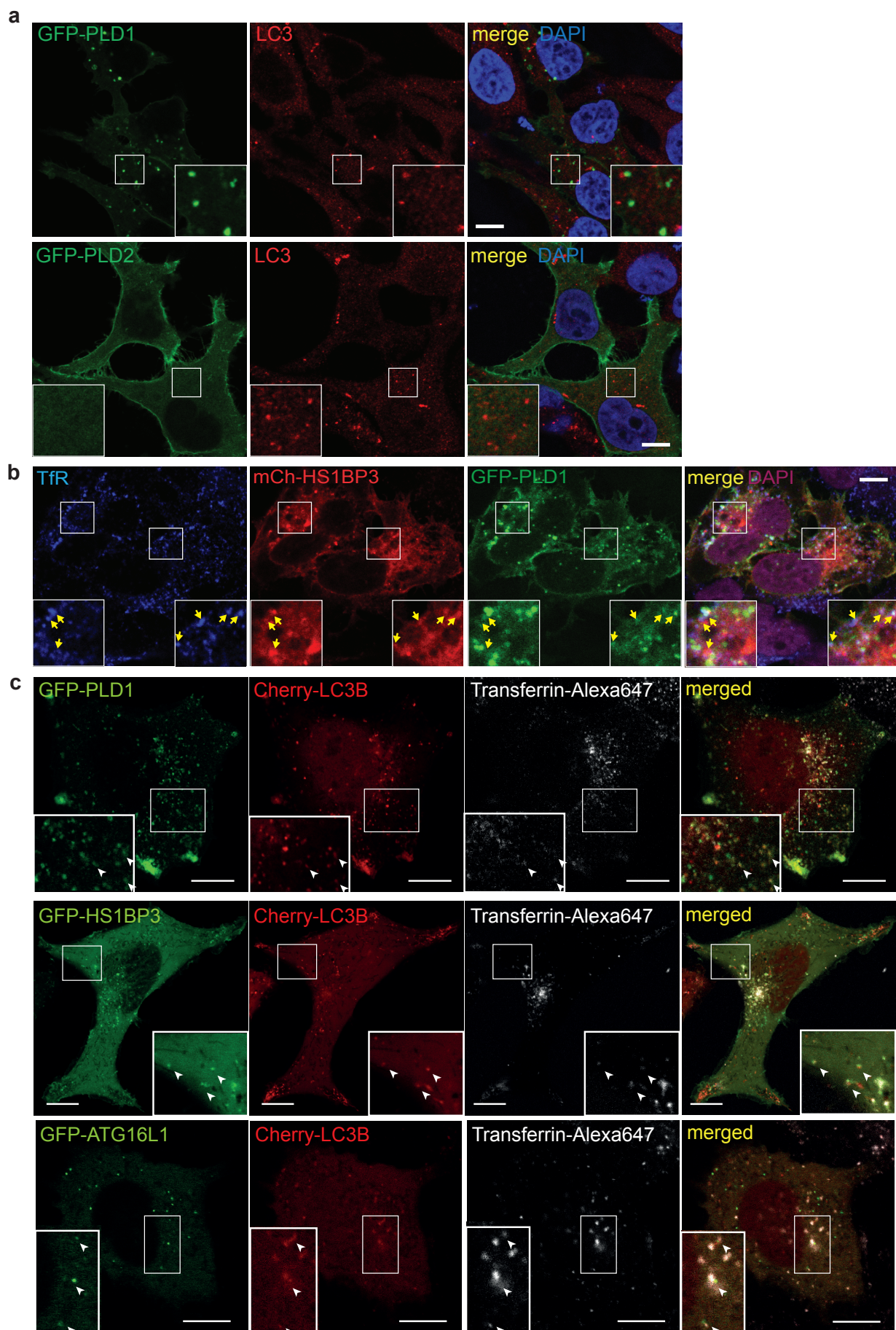
(a) HEK cells were transfected with the indicated GFP-tagged autophagy markers, starved, fixed and co-stained for endogenous HS1BP3. Scale bars 10  $\mu$ m. (b) In vitro translated GFP, GFP-HS1BP3 or GFP-p62 was incubated with recombinant GST-tagged LC3B or GABARAP. Following GST pull-down, bound proteins were detected by autoradiography (AR) and GST proteins by Coomassie blue staining (CB). (c) HEK GFP-DFCP1 cells were transfected with siRNA against HS1BP3 and starved or not for 50 min before fixation and imaging. The total intensity of GFP-DFCP1 spots per cell was quantified and normalized to that of starved siControl cells (mean  $\pm$  S.E.M.,  $n = 3$ ). 1500 cells were analyzed per condition. (d) HEK GFP-DFCP1 cells were treated as in c, stained for endogenous WIPI2 and the number of WIPI2 spots per cell was quantified (mean  $\pm$  S.E.M.,  $n = 3$ ). (e) HeLa cells were transfected with either control or HS1BP3 siRNA, starved or not for 2 h before fixation and staining for endogenous ATG16L1. The number of ATG16L1 spots per cell was quantified (mean  $\pm$  SEM,  $n = 3$ ): \* $p < 0.05$ , ns: non-significant.



**Supplementary Figure 3: HS1BP3 interacts with cortactin independently of its role in autophagy**

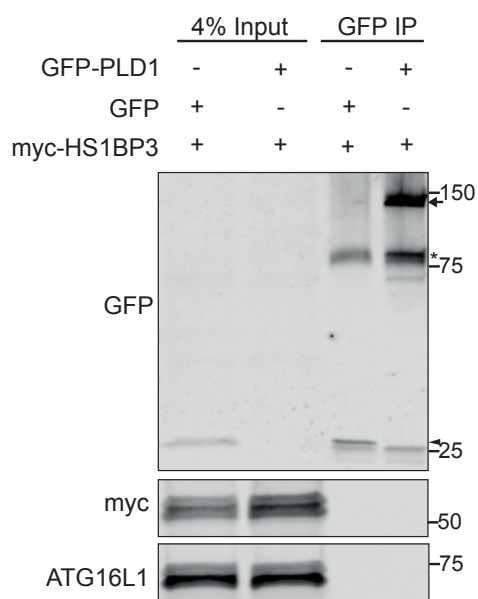
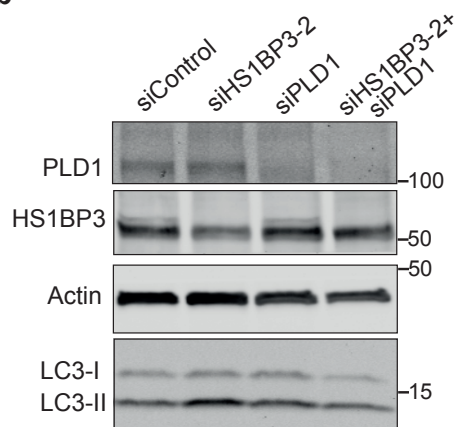
(a) HeLa cells stably expressing tet-on GFP or GFP-HS1BP3 were induced by 50 ng/mL tetracycline for 16 h followed by immunoprecipitation using GFP trap. The resulting immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting as indicated. (b) Lysates from HeLa GFP or GFP-HS1BP3 cells induced by 50 ng/mL tetracycline for 16 h were incubated with recombinant GST-tagged wild-type or mutated SH3 domain of cortactin or HS1, followed by pull-down using glutathione sepharose, separation by SDS-PAGE and immunoblotting against GFP. The membrane was stained with Ponceau S to visualize the GST-proteins and input lysate. (c) Recombinant MBP-tagged HS1BP3 full length or deletion mutants (HS1BP3-PX or HS1BP3-ΔPX) were incubated with recombinant GST-tagged wild-type or mutated SH3 domain of cortactin or HS1 followed by pull-down using glutathione beads, separation by SDS-PAGE and immunoblotting or Ponceau S staining as indicated. (d) HEK GFP-LC3 cells were transfected with siRNA against cortactin. 72 h later the cells were starved or not for 2 h in EBSS in the presence or absence of BafA1. Cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. LC3-II/actin was quantified from immunoblots (mean  $\pm$  S.E.M.,  $n = 3$ ). (e) HEK GFP-LC3 cells treated as in d were fixed and analyzed by fluorescent microscopy and high-content image analysis. The number of GFP-LC3 spots per cell was quantified (mean  $\pm$  S.E.M.,  $n = 3$ ). Around 1500 cells were quantified per condition. Scale bars are 10  $\mu$ m.



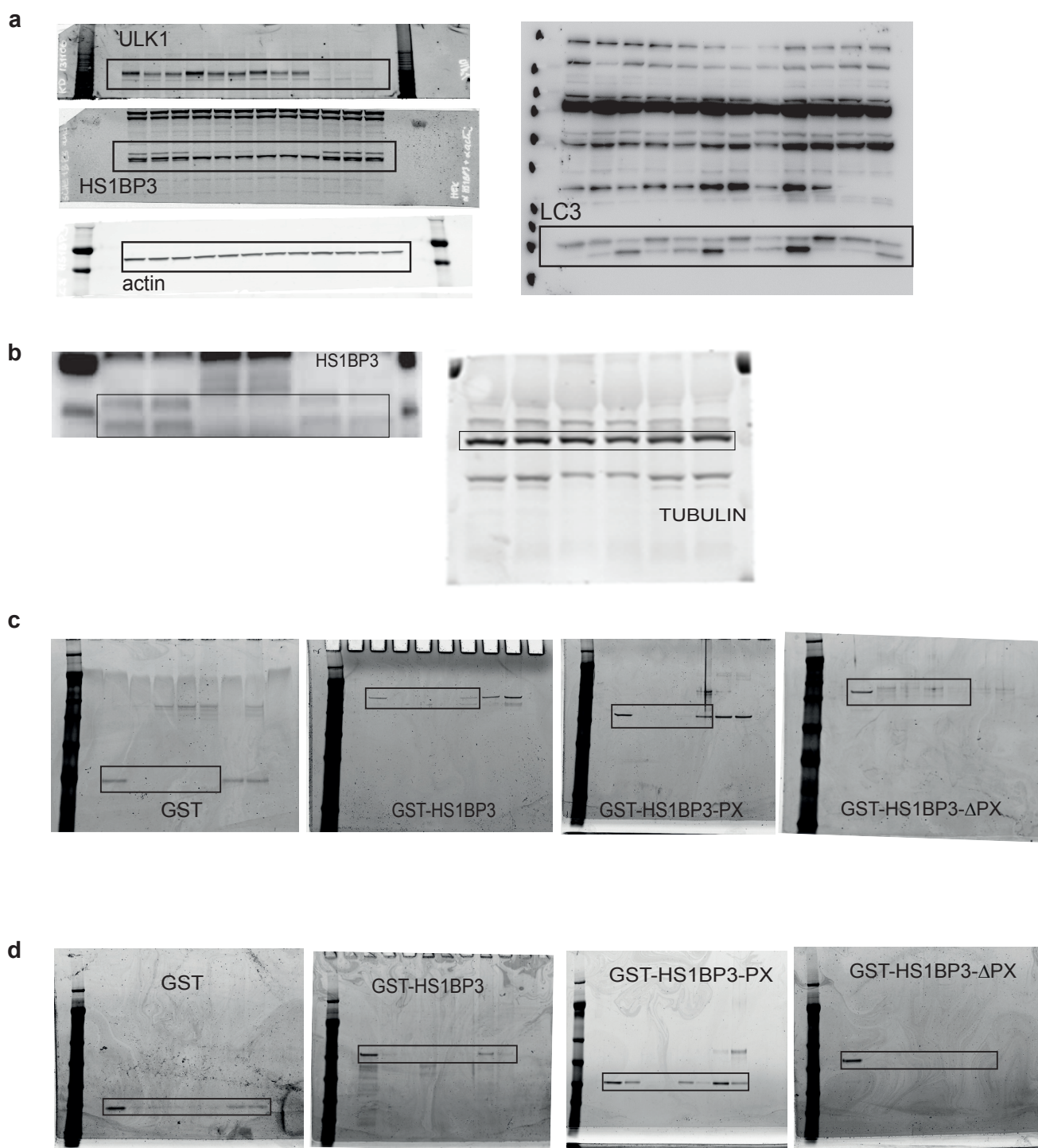


*Supplementary Figure 5: HS1BP3 affects PLD1 on autophagy precursor membranes*

(a) HEK cells were transfected to express GFP-tagged PLD1 or PLD2, starved and fixed then co-stained for endogenous LC3 and analyzed by confocal microscopy. (b) HEK cells were transfected to express GFP-tagged PLD1 and mCherry-HS1BP3, starved and fixed then co-stained for endogenous Transferrin receptor (TfR) and analyzed by confocal microscopy. All scale bars are 10  $\mu$ m. (c) HEK293A cells expressing the indicated constructs were starved for 1h in EBSS containing 5 $\mu$ g/ml of transferrin-Alexa Fluor 647 conjugate and imaged live with Zeiss LSM710 confocal microscope. Shown are still image frames from live scan. Arrowheads point to structures positive for all three proteins. All scale bars are 10 $\mu$ m.

**a****b**

**Supplementary Figure 6: HS1BP3 affects PLD1 on autophagy precursor membranes, but not through protein-protein interactions**  
**(a)** Immunoprecipitation of GFP or GFP-PLD1 from HEK cells transfected with GFP and myc-HS1BP3 or GFP-PLD1 and myc-HS1BP3. An interaction between GFP-PLD1 and myc-HS1BP3 or ATG16L1 was investigated by western blotting. On the GFP blot the arrow indicates GFP-PLD1, arrowhead GFP and the star an unspecific band. **(b)** HEK cells transfected with the indicated siRNA were starved then lysed and analyzed by western blotting with the indicated antibodies. The cells were treated in parallel with the ones analyzed by microscopy in Figure 7a.



Supplementary Figure 7: Original scans used in the main text figures

- (a) Blots from figure 1d
- (b) Blots from figure 2c
- (c) Gels from figure 4c
- (d) Gels from figure 4d

*Supplementary Table 1: Lipidomics data*

Lipid species		Average normalized mol %		SEM normalized mol %		t-test
Abbrev.	full name	siCtrl	siHS1 BP3-2	siCtrl	siHS1 BP3-2	
FC	Free cholesterol	1	0.99	0.02	0.03	0.857
CE	Cholesteryl esters	1	0.80	0.03	0.05	0.007
DG	Diglycerides	1	1.00	0.17	0.17	0.995
TG	Triglycerides	1	1.10	0.04	0.10	0.391
Cer	Ceramide	1	1.12	0.03	0.06	0.112
dhCer	Dihydroceramide	1	1.00	0.07	0.07	0.967
SM	Sphingomyelin	1	1.16	0.04	0.03	0.017
dhSM	Dihydrosphingomyelin	1	1.15	0.03	0.07	0.069
GalCer	Galactosylceramide	1	1.08	0.03	0.07	0.287
Sulf	Sulfatide	1	1.80	0.21	0.14	0.010
GlcCer	Glucosylceramide	1	1.07	0.03	0.08	0.434
LacCer	Lactosylceramide	1	0.94	0.03	0.06	0.369
GM3	Monosialodihexosylganglioside	1	0.96	0.09	0.04	0.685
PA	Phosphatidic acid	1	1.95	0.14	0.26	0.010
PC	Phosphatidylcholine	1	1.08	0.09	0.08	0.490
PCe	Phosphatidylcholine ether	1	0.88	0.07	0.05	0.219
PE	Phosphatidylethanolamine	1	1.05	0.07	0.07	0.583
PEp	Plasmalogen PE	1	1.11	0.06	0.04	0.154
PG	Phosphatidylglycerol	1	0.90	0.03	0.08	0.249
PI	Phosphatidylinositol	1	1.06	0.07	0.07	0.523
PS	Phosphatidylserine	1	1.07	0.04	0.07	0.401
LPC	Lysophosphatidylcholine	1	1.34	0.04	0.06	0.001
LPCe	Lysophosphatidylcholine ether	1	1.00	0.05	0.04	0.976
LPE	Lysophosphatidylethanolamine	1	1.09	0.03	0.11	0.456
LPEp	Plasmalogen LPE	1	1.23	0.06	0.17	0.225
LPI	Lysophosphatidylinositol	1	1.29	0.04	0.07	0.004
BMP	Bis(monoacylglycero)phosphates	1	0.85	0.01	0.04	0.005
APG	Acylphosphatidylglycerol	1	0.92	0.08	0.07	0.430
NAPS	N-acylphosphatidylserines	1	1.17	0.04	0.15	0.290

*Supplementary Table 2: Plasmids used in this study.*

<b>Plasmid</b>	<b>Primers</b>	<b>Cloning</b>
pEGFP-HS1BP3	5'-ATAGAATTCATGCAGTCCCCGGCGGTGCTC-3' 5'-ATAGTCGACTCAGAAGAGGCTGGGGGCGG-3'	Into pEGFP.C2 using EcoRI and Sall restriction sites
pENTR-HS1BP3	5'-ATAGTCGACATGCAGTCCCCGGCGGTGCTC-3' 5'-ATAGCGGCCGCTCAGAAGAGACTGGGGGCGG-3'	Amplification from cDNA library, into pENTR using Sall and NotI restriction sites
pENTR-HS1BP3-PX	5'-ATAGTCGACATGCAGTCCCCGGCGGTGCTC-3' 5'-ATAGCGGCCGCTCAGGATCTGGTACCTAAGAATC-3'	Amplification from pENTR-HS1BP3, into pENTR using Sall and NotI restriction sites
pENTR-HS1BP3-ΔPX	5'-ATAGTCGACGCTGCAGGGCTCACCAGCAG-3' 5'-ATAGCGGCCGCTCAGAAGAGACTGGGGGCGG-3',	Amplification from pENTR-HS1BP3, into pENTR using Sall and NotI restriction sites
pTH1-HS1BP3 (MBP tag)	-	Gateway LR cloning from pENTR-HS1BP3
pTH1-HS1BP3-PX (MBP tag)	-	Gateway LR cloning from pENTR HS1BP3-PX
pTH1-HS1BP3-ΔPX (MBP tag)	-	Gateway LR cloning from pENTR HS1BP3-ΔPX
pDEST-mCherry-HS1BP3	-	Gateway LR cloning from pENTR-HS1BP3
pTH1-2xFYVE-Hrs (MBP tag)	-	Gateway LR cloning from pENTR-2xFYVE-Hrs
pGEX-2xFYVE-Hrs	-	<sup>1</sup>
pCI2Flag Cortactin	-	Kindly provided by J. K. Burkhardt
pGEX kG hHS1 SH3	-	Kindly provided by J. K. Burkhardt
pGEX kG hHS1 SH3 W->Y	-	Kindly provided by J. K. Burkhardt
pGEX kG hcortactin SH3	-	Kindly provided by J. K. Burkhardt
pGEX kG hcortactin SH3 W->Y	-	Kindly provided by J. K. Burkhardt
pCGN-HA-hPLD1b	-	Kindly provided by M. Frohman <sup>2</sup>
pEGFP-C1-hPLD1b	-	Kindly provided by M. Frohman <sup>2</sup>
pEGFP-C1-mPLD2	-	Kindly provided by M. Frohman <sup>2</sup>
pDEST15-LC3B	-	<sup>3</sup>

(GST tag)		
pDEST15-GABARAP (GST tag)	-	<sup>3</sup>
pDEST15-HS1BP3-PX (GST tag)	-	Gateway LR cloning from pENTR HS1BP3-PX
pDEST53-p62 (in vitro translation)	-	<sup>3</sup>
pDEST53-HS1BP3 (in vitro translation)	-	Gateway LR cloning from pENTR-HS1BP3
pSP64 Poly (A)-Hs-Hs1bp3	5' taaaAAGCTTATGCAGTCCCCGGCGGTG 3' 5' taaaGAGCTCTCAGAAGAGGCTGGGGGC 3'	Expression of HS1BP3 in zebrafish
pEGFP-C2 DFCP1	-	Kindly provided by Nicholas Ktistakis
pEGFP – p62	-	-
pDEST-EGFP-LC3B	-	-
pEGFP(C1)-Atg16L	-	-
pEGFP-Atg14L	-	Kindly provided by Tamotsu Yoshimori

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Review

# Studying Autophagy in Zebrafish

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**Abstract:** Autophagy is an evolutionarily conserved catabolic process which allows lysosomal degradation of complex cytoplasmic components into basic biomolecules that are recycled for further cellular use. Autophagy is critical for cellular homeostasis and for degradation of misfolded proteins and damaged organelles as well as intracellular pathogens. The role of autophagy in protection against age-related diseases and a plethora of other diseases is now coming to light; assisted by several divergent eukaryotic model systems ranging from yeast to mice. We here give an overview of different methods used to analyse autophagy in zebrafish—a relatively new model for studying autophagy—and briefly discuss what has been done so far and possible future directions.

**Keywords:** autophagy; zebrafish; GFP-Lc3; confocal microscopy; mitophagy; aggrephagy; xenophagy

## 1. Introduction

Over the past few decades we have seen a dramatic surge in research on a basic and fundamental cellular process called autophagy. Autophagy is defined as the lysosomal degradation of cytoplasmic materials (proteins, lipids, organelles, etc.), and three major types of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy [1]. This review will focus on macroautophagy (hereafter referred to as autophagy), which involves the sequestration of cytoplasmic components in a double membranous structure, the autophagosome, followed by its fusion to the acidic lysosome, resulting in cargo degradation and release of simple biomolecules that can be reused for varied cellular purposes (Figure 1A). Thus, autophagy is an adaptive catabolic process leading to substrate formation for further anabolic energy-generating processes, to ultimately maintain homeostasis at the cell, tissue and organism levels.

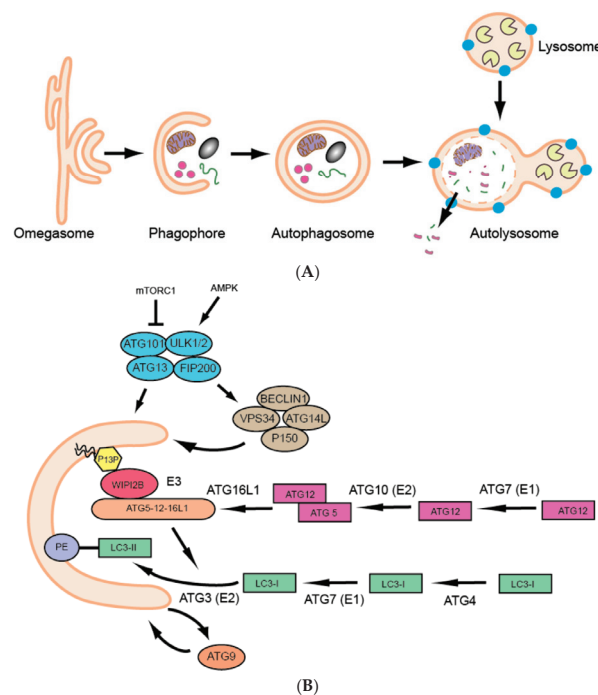
The molecular era of autophagy started with a series of genetic screens performed on unicellular yeast in the 1990s [2–5], which were followed by the identification of respective homologs in higher eukaryotes and resulted in the characterization of more than 30 AuTophagy-related (ATG) genes [6]. The ATG proteins essential for autophagosome formation are referred to as the ‘core’ autophagy machinery (Figure 1B) [7] and include: (1) the UNC-51-like kinase (ULK) complex composed of ULK1 or ULK2, ATG13, ATG101 and FIP200; (2) the class III phosphatidylinositol 3-kinase (PI3K) complex (PIK3C3), consisting of the catalytic subunit VPS34, as well as BECLIN1, p150 and ATG14L; (3) the two ubiquitin-like conjugation systems that lead to the conjugation of ATG12 to ATG5 and ATG8 to phosphatidylethanolamine (PE) in the phagophore membrane and finally (4) the transmembrane protein ATG9 [8,9]. Human ATG protein names are used here, see Table 1 for the respective zebrafish ATG orthologue names.

**Table 1.** Zebrafish (*Danio rerio*) orthologues of human autophagy genes, with amino acid percentage identity and allele availability at the Sanger ZMP. ULK: UNC-51-like kinase; ZMP: Zebrafish Mutation Project; PE: phosphatidylethanolamine, mTORC1: Mammalian Target of Rapamycin Complex 1, AMPK: Adenosine Mono-Phosphate Kinase; ER: Endoplasmic Reticulum; PtdIns3K: Phosphatidylinositol 3-Kinase; VPS34: Vacuolar Protein Sorting 34.

Core Autophagic Process	Mammalian Protein	Zebrafish Orthologue	Refseq Id of Zebrafish DNA/Protein	Ensemble Id of Zebrafish DNA/Protein	Amino Acid Identity	Role in Autophagy	Mutant Allele Availability at the Sanger ZMP
ULK1 complex	ULK1	<i>ulk1a</i>	NM_001130631, NP_001124103.1	ENSDART00000090534.4	50%	Phosphorylated by mTORC1 (negative) and AMPK (positive). Induces autophagy by phosphorylation of ATG13	ULK1a—Yes
		<i>ulk1b</i>	XM_005161121.3, XP_005161178.1	ENSDART00000112407.3			No
	ULK2	<i>ulk2</i>	XM_002664615.4, XP_002664661.3	ENSDART00000153726	74%		No
		ATG13/ KIAA0652	NM_200433, NP_956727	ENSDART00000052324.5	71%	Member of the ULK1 complex, phosphorylated by mTORC1 and ULK1	No
Nucleation step	Fip200/ RB1CC1	<i>rb1cc1</i>	XM_009302198.2, XP_009300473.1	ENSDART00000113014.3	59%	Scaffold for ULK1/2 and ATG13	Yes
		<i>atg101</i>	NM_001037239, NP_001032316	ENSDART00000063544.6	87%	Interacts with ATG13	No
	ATG14L	<i>atg14L/kinad0831</i>	NM_001024812, NP_001019983	ENSDART00000018683.10	67%	Autophagy-specific subunit of PIK3C3 complex I. ER binding motif	Yes
		PtdIns3K/ VPS34	NM_001328533, NP_001315462	ENSDART000000101265.4	87%	Catalytic subunit. Phosphorylates phosphatidylinositol to generated PI3-phosphate	No
Class III PI3-kinase complex (PIK3C3)	Beclin1	<i>beclin1</i>	NM_200872, NP_957166	ENSDART00000115237.3	79%	Subunit of PIK3C3. Regulatory function through binding to Bcl-2	Yes
		p150	XM_005158299.3, XP_001922676.1	ENSDART00000085228.5	82%	Adaptor protein for VPS34	No
	ATG12	<i>atg12</i>	NM_001246200, NP_001233129	ENSDART00000101304.4	71%	Ubiquitin like, conjugates to ATG5	Yes
		ATG7	XM_017358254.1, XP_017213743.1	ENSDART00000162152	77%	E1-like enzyme	Yes
Atg12 conjugation system	ATG10	<i>atg10</i>	NM_001037124, NP_001032201.1	ENSDART000000160159.1	50%	E2-like enzyme	No
		ATG5	<i>atg5/apg5L</i>	NM_205618, NP_991181	81%	Conjugated by ATG12	Yes
	ATG16L1	<i>atg16L1</i>	NM_001017854, NP_001017854	ENSDART000000161937.1	69%	Interacts with ATG5 to form the ATG12-5-16L1 complex, an E3 like ligase for Atg8 conjugation	No

Table 1. Cont.

Core Autophagic Process	Mammalian Protein	Zebrafish Orthologue	Refseq Id of Zebrafish DNA/Protein	Ensemble Id of Zebrafish DNA/Protein	Amino Acid Identity	Role in Autophagy	Mutant Allele Availability at the Sanger ZMP
Atg8 conjugation system	MAP1-Lc3A	<i>map1-lc3a</i>	NM_214739, NP_999904	ENSDART00000042322.3	96%	Ubiquitin like, conjugates to PE	No
	MAP1-Lc3B	<i>map1-lc3b</i>	NM_199604, NP_955898	ENSDART00000163508.1	93%		
	MAP1-Lc3C	<i>map1-lc3c</i>	NM_200298, NP_956592	ENSDART00000161846.2	72%		
	GABARAP	<i>gabaraipa</i>	NM_001013260, NP_001013278	ENSDART00000051547.3	98%	Ubiquitin like, conjugates to PE	No
	GABARAPL1	<i>gabaraapl1</i>	NM_001002707, NP_001002707	ENSDART00000060037.3	59%		
	GABARAPL2	<i>gabaraapl2</i>	NM_205723, NP_991286	ENSDART00000039485.6	97%		
	ATG4A	<i>atg4a</i>	NM_001024434, NP_001019605	ENSDART00000026666.10	70%	Atg8 C-terminal hydrolase, deconjugating enzyme	Yes
	ATG4B	<i>atg4b</i>	NM_001089352, NP_001082821	ENSDART00000121558.3	73%		
	ATG4C	<i>atg4c</i>	NM_001002103, NP_001002103	ENSDART00000051779.3	59%		
	ATG4D	<i>atg4da</i>	XM_009294436.2, XP_009292711.1	ENSDART00000152289.2	50%	E2-like enzyme	No
	ATG3	<i>atg4db</i>	NM_200022, NP_956316	ENSDART00000041304.7	82%		
		<i>atg3</i>					
	ATG2A	<i>atg2a</i>	XM_009307758.2, XP_009306033.1	ENSDART00000172444.1	55%	Proper closure of autophagosome	No
	ATG2B	<i>atg2b</i>	XP_001340508.3	ENSDART00000155615	42%		
	ATG9A	<i>atg9a</i>	NM_001083031, NP_001076500	ENSDART00000065411.6	71%		
Other core Atg proteins during autophagosome formation	ATG9B	<i>atg9b</i>	NM_001320078, NP_001307007	ENSDART00000147499.3	49%	Transmembrane protein on the autophagosome	No
	WIPI1	<i>wipi1</i>	NM_200391, NP_956685	ENSDART00000059533.4	71%		
	WIPI2	<i>wipi2</i>	NM_001327789, NP_001314718	ENSDART00000134026.2	82%		
	WIPI3 / WDR45B	<i>wipi3/tutr45b</i>	NM_200240, NP_956534	ENSDART00000152327.2	96%	Phosphatidyl-inositol 3-phosphate PI(3)P-binding proteins	Yes
	WDR45	<i>wipi4</i>	NM_200231, NP_956525	ENSDART00000130229.2	90%		
Autophagy receptor proteins	NCOA4	<i>ncoa4</i>	NM_201129, NP_957423	ENSDART00000017052.8	38%	Autophagy cargo receptor required during iron homeostasis	No
	SQSTM1 / p62	<i>sqstm1/p62</i>	NM_001312913, NP_001299842	ENSDART00000140061.2	44%		
	OPTN	<i>optn</i>	NM_001100066, NP_001093536	ENSDART00000014036.10	41%	Autophagy cargo receptor	No
	CALCOCO2 / NDP52	<i>calcoco2</i>	NM_001020741, NP_001018577	ENSDART00000152964.2	30%		
	NBR1	<i>nbr1</i>	NM_001305595, NP_001292524	ENSDART00000133048.2	38%	Autophagy cargo receptor during xenophagy and mitophagy	Yes
	TAX1BP1	<i>tax1bp1a</i>	NM_001346178, NP_001333107	ENSDART00000171664.1	44%		
		<i>tax1bp1b</i>	NM_212664, NP_997829	ENSDART00000040727.7	52%	Autophagy cargo receptor	Yes



**Figure 1.** (A) Schematic overview of the process of macroautophagy; (B) Schematic overview of the core autophagic proteins involved in autophagosome biogenesis.

In addition to these core autophagy proteins, the regulation and execution of the pathway is tightly controlled by a large number of proteins and lipids and we are only beginning to understand how their interconnections are regulated in time and space under various metabolic conditions and in different tissues. Dysfunctional autophagy is closely associated with tumorigenesis [10,11], immune disorders [12], neurodegeneration and aging [13], infectious diseases [14] and diabetes [15]. Thus, a detailed understanding of the molecular mechanisms involved in autophagy may open doors to various therapeutic approaches against diseases where autophagy plays an indispensable role.

Our understanding of how autophagy is regulated under different physiological and pathological conditions is largely based on research performed in different tractable animal model systems such as the fruit fly *Drosophila melanogaster* [16,17], nematode *Caenorhabditis elegans* [18], the mouse *Mus musculus* [19,20], oysters [21] and *Dictyostelium discoideum* [22]. Recently, there has been an exponential interest in using zebrafish (*Danio rerio*) for varied research owing to the immense advantages that it offers. The small size, high fecundity, external fertilization, transparent embryos, rapid development, and genetic tractability of zebrafish make it highly desirable for basic science and translational high throughput research [23].

We here review the current literature and the methods used to study autophagy in zebrafish, including DNA, RNA and protein-based methods. We also discuss different types of selective autophagy, with emphasis on mitophagy, xenophagy and aggrephagy and how these can be studied in zebrafish. Finally, we provide detailed information about different antibodies, chemical reagents and reporter lines that have been used to analyze autophagy in zebrafish and discuss how current methods could be improved to better understand autophagy in zebrafish.

## 2. Zebrafish Autophagy Genes

The identification of the zebrafish as a genetically tractable organism in the 1980s led to its immense usage in the 1990s, whereby a large number of mutations giving rise to specific phenotypes were discovered through large-scale mutagenesis screens [24]. However, this alone was insufficient to throw light on various rare and common human disorders as a high-quality zebrafish genome

sequence and complete annotation of zebrafish protein-coding genes with identification of their human orthologues was limited. The genome of the zebrafish has now been published as a well-annotated reference genome, providing key insights into the use of this vertebrate as a desirable model to mimic human disease states. In total, 84% of human disease-associated genes have at least one obvious zebrafish orthologue [25–27].

To be able to alter or modulate autophagy genetically in zebrafish, it is critical to delineate the representative ATG zebrafish orthologues from its yeast or mammalian counterparts. We searched for human ATG proteins from National Centre for Biotechnology Information (NCBI) and blasted their respective amino acid sequences against *Danio rerio*'s (taxid: 7955) reference proteins as a search set. The hit with highest query coverage and smallest E-value was selected to be an orthologue. We also compared the sequence with that annotated in the Ensemble genome browser. A detailed account of the core ATG proteins (mammalian) and their respective zebrafish orthologue with Refseq IDs and Ensemble IDs have been tabulated (Table 1). The overall amino acid identity between human and zebrafish core autophagy proteins range between 40 and 96% (Table 1).

### 3. Genome Editing Techniques

Genome editing, or the idea of introducing a desired change to the genomic DNA sequence, is currently driving a revolution in the medical field with the introduction of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR Associated Protein 9 (Cas9) technology [28–30]. An ideally desirable genome editing tool would edit any genomic locus with high efficiency, specificity and with little or no off-target effects. The basic process of nuclease-based genome editing is to create a specific double-strand break (DSB) in the genome and then allow the cell's own endogenous repair machinery to repair the break, by either non-homologous end-joining (NHEJ) or by homology-directed repair (HDR). The different techniques of genome editing used in zebrafish (CRISPR/Cas9, transcription activator-like effector nucleases (TALENs) and zinc finger nucleases) have been extensively reviewed elsewhere [31–37]. We will here discuss how genome editing could help drive the field of zebrafish autophagy.

#### 3.1. CRISPR/Cas9 Mutagenesis

The CRISPR/Cas9 technology has been widely adopted in the zebrafish community and has already come a long way from the first knock-out [38], to high-throughput mutagenesis screens [33], conditional knockout [39], multiplex knockout [40,41] and to targeted insertion of DNA elements [42]. It would be highly desirable to apply systematically all of these techniques into understanding the precise role of autophagy proteins in zebrafish development, physiology and pathology.

Briefly, CRISPR/Cas9-mediated genome editing in zebrafish is facilitated by the microinjection of a “short guide-RNA” (sgRNA) and Cas9 endonuclease protein into zebrafish embryo (at 1 cell stage), wherein the Cas endonuclease protein, forms a complex with the sgRNA molecule (now called the Cas9 holoendonuclease). Cas9 holoendonuclease or the corresponding RNAs (sgRNA + Cas9 messenger RNA (mRNA)) can be injected. The target DNA sequence, in addition to being complementary to the gRNA molecule, should also have a “protospacer-adjacent motif” (PAM), that is required for compatibility with the particular Cas protein being used. Once mobilized to the target DNA site, the Cas9 holoendonuclease generates a double-strand break (DSB), which can be used to create a knock-out or add a specific function to a gene (targeted knock-in). Autophagy can be manipulated by injecting sgRNA against the core autophagy genes (Table 1) together with either Cas9 mRNA or protein. It is very important to minimize or best, to negate, mutagenesis of an incorrect gene (off-target effect). Step-by-step protocols describing how to design an efficient sgRNA and the heuristic rules surrounding it, purifying Cas9 mRNA or using commercial Cas9 protein along with sgRNA have been reviewed previously [43,44]. The transparency of zebrafish larvae makes zebrafish highly desirable to use for generation of reporter lines. CRISPR/Cas9 can be used to tag core autophagy genes endogenously by “knocking-in” a reporter DNA element upstream/downstream of the autophagy

gene of interest, e.g., to generate a fusion protein at an endogenous locus. This is highly desirable in the study of autophagy, opening up the prospect of “double-tagging” an autophagy protein or a cargo of interest and following their degradation kinetics. “Double-tagging” is based on the principle of using tandem fluorescent tags, where one will be quenched (e.g., green fluorescent protein (GFP)) upon delivery to the acidic lysosome. CRISPR/Cas9-mediated genome editing can also be used to ablate a particular gene in a specific tissue or at a particular developmental time-point. As an example, *LoxP* sites can be “knocked-in” to flank an autophagy gene of interest and later by using the Cre recombinase, the gene can be inverted or excised, thereby creating a complete knock-out. This is suitable for genes whose knockout can be embryonically lethal.

The use of CRISPR/Cas9-based targeted mutagenesis for deriving stable transgenic zebrafish or zebrafish knockout autophagy lines is in its initial phase. So far only one study has used this system to create mutant lines. CRISPR/Cas9-based mutagenesis in *spns1* and *atp6v0ca* genes induced premature autophagosome-lysosome fusion marked by insufficient acidity leading to developmental senescence and death [45]. *spns1* is thought to function as a lysosomal H<sup>+</sup>-carbohydrate symporter, which functions at a late and terminal stage of autophagy [46,47]. *atp6v0ca* encodes a sub-unit of the vacuolar-type H<sup>+</sup>-ATPase (v-ATPase) that counteracts *spns1* ablation effects in zebrafish. It is highly likely that we will soon see increasing use of CRISPR/Cas9 technology to modulate autophagy in zebrafish.

### 3.2. TALENs and ZFNs

Since the introduction of CRISPR/Cas9 for genome editing in zebrafish, the use of TALENs and ZFNs, which were used before for genome editing [36,37] have taken a back seat (for a review of these methods see references). The use of TALENs and ZFNs to study autophagy in zebrafish is limited. TALEN-mediated mutation of the nuclear hormone receptor *nr1d1* was shown to have a positive effect on autophagosome-autolysosome number and lead to upregulation of ATG genes. *nr1d1* mutants were also shown to affect the circadian clock by significantly upregulating the circadian clock genes, leading to the conclusion that the circadian clock regulates autophagy rhythms in zebrafish larvae [48].

### 3.3. Transient Gene Knockdown by Morpholino Oligonucleotides

Morpholino oligonucleotides or morpholinos, first developed by Dr. James Summerton, are oligomers of 25 morpholine bases that are targeted via complementary base pairing to the mRNA of interest. They silence the gene by either blocking the translational start site from the ribosomal machinery or by blocking the splice sites (donor/acceptor), thereby interfering with the binding of spliceosome components [49,50]. Morpholinos can be used to interrogate pathways and associate genes with a phenotype and this can be done easily by just injecting an optimal volume of the morpholino solution into the yolk sac of a zebrafish embryo at the 1–4 cell stage. Morpholinos provide precise spatial targeting of multiple gene products [51] and are extremely useful for silencing and analyzing maternal gene expression [52]. However, a drawback of morpholinos is the relatively frequent off-target effects. Off-target effects are often caused by the induction of p53 that leads to apoptosis, but can also be p53-independent [53,54]. Inconsistencies between morphant and CRISPR mutant phenotypes have been seen in some studies [54], whereas others have shown that such inconsistencies can be explained by a compensating gene that is upregulated in the mutants, but not in the morphants [55]. Recent reports point out off-target single nucleotide variations (SNVs) in CRISPR-repaired mice, fished out via whole genome sequencing (WGS) [56]. Therefore, if used with the appropriate controls, morpholinos remain a useful tool [57].

Morpholinos have been employed vigorously to analyze autophagy in zebrafish and have provided valuable insight into the role of autophagy in development and disease. Knockdown of Atg5, Atg7 and Beclin1 [58,59], Atg4da [60], Ambra1a and Ambra1b [61,62] all show an important role of autophagy during embryogenesis. One of the common phenotypes seen consistently among these studies is a cardiac defect, indicating a very specific role of autophagy in cardiac

morphogenesis/function, in alignment with previous studies on rodents [63]. Moreover, knockdown of optineurin, an ubiquitin-binding autophagy-receptor protein, was shown to cause motor axonopathy due to defective autophagic clearance of accumulated SOD1-G93A aggregates [64], defective vesicle trafficking in the axons [65], and increased susceptibility to *Salmonella enterica* infection [66]. Morpholino-mediated depletion of Spns1, a lysosomal transporter, was found to upregulate embryonic cellular senescence [46] and this was counteracted by the depletion of the lysosomal v-ATPase, which together suppresses developmental senescence and increases life-span [45]. Transient depletion of p62/sqstm1, another ubiquitin-binding autophagy receptor protein, in zebrafish embryos was shown to increase susceptibility to *Shigella flexneri* and *Mycobacterium marinum* in the host, indicating the role of autophagy against bacterial infection [67,68]. In another study involving the knockdown of p62/sqstm1 in zebrafish, it was seen that the ablation caused a specific locomotor phenotype characterized by a specific axonopathy of descending motor neuron projections [69]. Sorting nexin 14 knockdown in zebrafish larvae led to neuronal cell death (neurodegeneration) associated with defective autophagic degradation, ultimately resulting in cerebellar ataxias [70].

Several reports have indicated an indirect escalation or enervation of autophagy in zebrafish models of gene ablation by morpholinos. Zebrafish embryos depleted of the phosphatidylinositol 3-phosphatase *MTMR14* (better known as Jumpy) showed an increase in autophagy at 1 day post fertilization (dpf) [71], consistent with previous results in mammalian cells, showing that MTMR14 dephosphorylates PI(3)P in the early autophagic membranes, thereby inhibiting autophagy [72]. We recently found that the PX domain protein *Hs1bp3* also regulates the formation of autophagosomes by a novel negative-feedback mechanism on membrane lipids. Morpholino-mediated depletion of *Hs1bp3* in zebrafish embryos caused an increase in GFP-Lc3 puncta, which was rescued by co-injection of mRNA encoding the human HS1BP3 protein, thereby validating the conserved role of *Hs1bp3* as negative regulator of autophagy in vivo [73] (Figure 3).

In another study, depletion of collagen VI (*COLVI*), a protein crucial for structural integrity, cellular adhesion, migration and survival, resulted in reduced lipidation of Lc3 and reduced expression of Beclin1, suggesting an overall inhibition of autophagy in these morphants, ultimately leading to muscle dysfunction [74]. The role of autophagy in survival of hematopoietic cells was observed in a disrupted ribosome biogenesis model of zebrafish where *rps19* was ablated using translation morpholino [75,76]. A detailed list of all morpholinos used to analyze autophagy in zebrafish has been included in another review [77].

### 3.4. Mutations

In a major effort to generate mutant zebrafish lines, Christiane Nüsslein-Volhard and Wolfgang Driever orchestrated two of the largest mutagenesis screens ever performed in zebrafish [24,78]. These studies brought forth about 1500 mutations in more than 400 genes, but neither these original screens nor any later screens have revealed a mutant allele of a core autophagy gene. One possible reason for this could be that such mutations would be early embryonic lethal or it might be explained by the late onset of autophagy-related phenotypes in zebrafish.

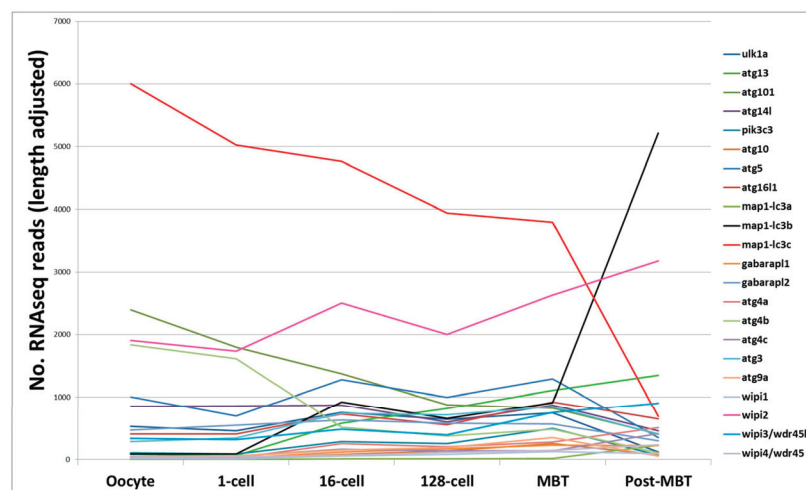
A high quality sequence assembly of the zebrafish genome was initiated by the Sanger Institute (UK) in 2001 and completed in 2013 [25]. The Sanger Institute also initiated a systematic effort called the Zebrafish Mutation Project (ZMP) [26], which has created mutant alleles in over 16,000 protein-coding genes, including a number of core autophagy genes (Table 1). Using such autophagy mutant lines would provide valuable insight into the role of autophagy in physiological processes.

## 4. RNA-Based Analysis

Autophagy is known to be tightly regulated by posttranslational modifications of autophagy proteins (e.g., phosphorylation of ULK1 by mTORC1 and AMPK oppositely regulate the activity of the ULK1 complex) and by regulation of protein levels. But in order to obtain a real estimation of autophagy it is necessary to also monitor their mRNA levels [79]. It is however important to note

that increased mRNA levels of autophagic genes should not be interpreted as increased autophagy, as it can be a compensatory mechanism. A detailed list of primers used to assess the expression of autophagy-related genes by quantitative real-time PCR (qRT-PCR) in zebrafish has been reviewed recently [77]. Zebrafish embryos and larvae are also very suited for whole mount in situ hybridization (WISH), which provides information about the spatial expression of a particular gene in the whole organism. This does not aid much in answering questions on autophagy activity, but still could help analyze the spatial arrangement of autophagy genes under certain conditions. WISH expression patterns are systematically catalogued in the zebrafish information network (ZFIN) database (zfin.org).

mRNA sequencing is a sensitive and accurate method for analyzing the transcriptomes of disease states and/or of biological processes. Prior to the activation of the zebrafish embryo genome, maternally-derived mRNA regulate early development in zebrafish [80,81]. This occurs at the 10th cell division (~3.5 h post-fertilization) when the zebrafish zygotic genome gets activated, also known as the mid-blastula transition (MBT) [82]. Mathavan and colleagues applied mRNA deep sequencing (mRNA-seq) to gain a comprehensive understanding of all transcriptional processes occurring from the unfertilized egg to early gastrulation [83]. We procured the raw data and fished for “core autophagy genes” in the data (available in the Gene Expression Omnibus (GEO) database, accession number GSE22830). Almost all of the core autophagy genes are expressed maternally at quite low levels, except for *map1-lc3c* which is expressed at high level from the oocyte to MBT. Interestingly, while the expression of *map1-lc3c* tapers off post MBT, there is a correspondingly strong increase in *map1-lc3b* expression levels at MBT, suggesting that *map1-lc3c* plays an important role during the early embryonic cell divisions, with *map1-lc3b* being more important later. *Wipi2* is consistently highly expressed across the early cell divisions to gastrulation (Figure 2). Several other mRNA-seq datasets are publicly available in the GEO database, also covering other later development stages. For example, in a developmental time series from 1 to 6 days post fertilization it was shown that the autophagy modulator gene *dram1* is upregulated during *Mycobacterium marinum* infection [84].



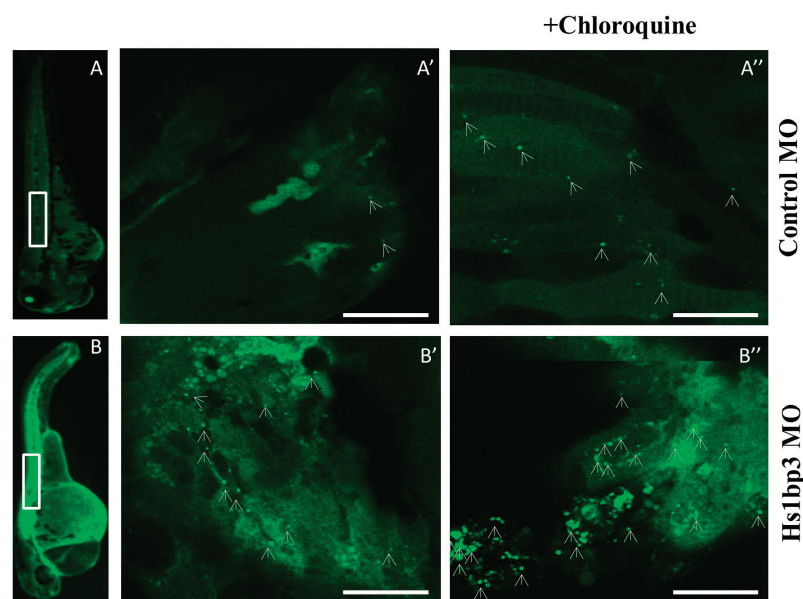
**Figure 2.** Messenger RNA sequence (mRNA-seq) analysis. Line plot of core autophagy gene transcripts analyzed by mRNA-seq in zebrafish embryos from the oocyte stage to post-mid blastula stage transition.

## 5. Protein-based analysis

### 5.1. Fluorescence Microscopy

The most widely used marker to study autophagy is Atg8/Lc3, as this protein becomes conjugated to PE in the autophagic membrane upon induction of autophagy and remains bound throughout the pathway [85]. The lipidated form of Lc3 (called Lc3-II) can be visualized as cytoplasmic puncta by immunofluorescence microscopy or by a shift in molecular weight when analyzed by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (described below). To analyze Lc3 lipidation *in vivo*, it is common to measure the increase in Lc3 puncta, using models where the N-terminus of Lc3 is tagged to a fluorescent reporter protein such as GFP. In zebrafish, GFP-Lc3 can be visualized *in vivo* during development due to the transparency of zebrafish embryos (Figure 3). Transgenic GFP-Lc3 and GFP-Gabarap fish have been generated [86] and are described in more detail below. Zebrafish larvae beyond 2 dpf develop pigments, which would be a hindrance for normal fluorescent microscopy. However, larvae can still be visualized for cellular activities in transgenic reporter lines by supplementing the media with 1-phenyl-2-thiourea (PTU), which inhibits melanogenesis or by using zebrafish strains that have mutations affecting pigment production [87]. Fusion of autophagosomes with lysosomes can be readily detected *in vivo* by the addition of LysoTracker Red to fish media prior to visualization [86].



**Figure 3.** Confocal imaging of Tg(CMV:GFP-Lc3). Representative confocal images of GFP-Lc3 puncta (autophagosomes) in the trunk area of GFP-Lc3 transgenic zebrafish embryos injected with control morpholino or Hs1bp3 translational-blocking morpholino and imaged at 2 days post fertilization (dpf) with or without pre-treatment with chloroquine (10 mM) for 6 h. Scale bars, 10  $\mu$ M for the confocal images. Panel A, B shows the whole zebrafish larvae at 2 days post fertilization highlighting the trunk area chosen for confocal imaging; Panel A', A'', B', B'' shows respective confocal images.

Fluorescence methods (reporter lines or immunofluorescence (IF)) are more sensitive and quantitative as compared to molecular techniques like western blotting. Increased autophagic activity is usually marked by a significant change in the number of fluorescent puncta. However, it is very important to note here that an increase in GFP-Lc3 puncta can be caused by an increased flux or by impairment of autolysosome formation [79]. Therefore, proper flux experiments must be done (e.g., stimuli in the absence or presence of lysosomal inhibitors) to be able to conclude. While quantifying live *in vivo* images from a reporter line like the GFP-Lc3 line, it has to be done on a 'per-cell area' basis rather than simply using the total number (or percentage) of cells displaying puncta. This point is important as in zebrafish larvae which have a constant supply of nutrients from the yolk-sac, there could be some cells displaying a basal level of GFP-Lc3 puncta under "fed" conditions. In situations where endogenous Lc3 could be stained with a specific antibody, it is important to counterstain the nuclei with DAPI or Hoechst and then quantify puncta on a 'per-cell' basis. Another important caveat to be noted when using GFP-Lc3 is its tendency to bind to aggregates, especially when working with protein-aggregation models or when overexpressed [88]. Interpretation

of autophagy in these models should be done by negating off the background fluorescence by having an untagged internal GFP control [79] or by the use of a C-terminal glycine mutant GFP-LC3 that is defective in ubiquitin-like conjugation with phosphatidylethanolamine, (GFP-LC3<sup>G120A</sup> as a negative control) [89] or by another fluorescent protein tandemly fused to GFP, e.g., red fluorescent protein (RFP) (GFP-RFP-Lc3). Using lysosomal dyes (e.g., LysoTracker Red) in tandem with GFP-Lc3 is another useful approach [46,86,90]. Here the colocalization of GFP-Lc3 and LysoTracker can be used as an indicator of autophagy.

The use of transgenic zebrafish models to study autophagy was kick-started by Klionsky and co-workers who developed the Tg(CMV:GFP-Lc3) and Tg(CMV:GFP-Gabarap) transgenic lines [86]. The Tg(CMV:GFP-Lc3) line has been used in various studies giving important insights into the functional significance of autophagy and autophagy modulators in vivo [46,58,67,68,73,91–94]. Tg(fabp10:EGFP-Lc3) and Tg(TαCP:GFP-Lc3) were used recently for looking into autophagy in the liver [95] and in photoreceptors [90], respectively.

The introduction of the tandem fluorescent tagged Lc3 in mammalian cell lines [96] opened up for the possibility of making similar tandem tagged (e.g., RFP/mCherry-GFP) reporter lines in model organisms. As briefly mentioned above, the underlying principle of using a tandem tag to study autophagy is based on the pH sensitivity of GFP, with the GFP signal being quenched when the tagged protein reaches the acidic environment of the lysosome, while the red signal (RFP/mCherry) is stable. Thus, the ratio between yellow (autophagosomes) and red (autolysosomes) signal can readily be used to quantify autophagic flux. A recent study used a transgenic zebrafish line expressing the tandem tag for Lc3 under the control of a photoreceptor promoter, Tg(TαCP:mCherry-GFP-map1lc3b) [97]. The tandem-tag principle can be exploited to generate other zebrafish reporter lines (by tandem-tagging selective autophagy cargo or autophagic receptors) that would contribute to our understanding of autophagy and the mechanisms underlying its role in zebrafish development and physiology.

As mentioned earlier, one should be cautious when interpreting Lc3 data. An increase in Lc3 levels should be validated by estimating the total autophagic flux, by e.g., treating samples with and without lysosomal inhibitors, such as Bafilomycin A1 or chloroquine (Figure 3). There are however reports that lysosomal inhibitors could inhibit mTORC1 and induce “unwanted” autophagy [98–100]. Taking these loopholes into consideration, the Mizushima group recently constructed a novel probe, GFP-Lc3-RFP-Lc3ΔG, which they tested in zebrafish as well [101]. The transgenic zebrafish line that ubiquitously expresses Tg(GFP-Lc3-RFP-Lc3ΔG) aided robust assessment of autophagic flux by the measurement of the GFP/RFP ratio. The underlying principle here is that the reporter probe will be cleaved by endogenous Atg4 proteases into equimolar amounts of GFP-Lc3 and RFP-Lc3ΔG, a mutant unable to become conjugated to the autophagy membrane. Thus, while GFP-Lc3 becomes lipidated and degraded by autophagy, the RFP-Lc3ΔG remains in the cytosol, serving as an internal control. Autophagic flux can then be estimated by calculating the GFP/RFP signal ratio.

Fluorescent reporter lines of other autophagy core components or probes to detect autophagic membranes would also be desirable. The zebrafish transgenic reporter lines Tg(TαCP:YFP-2XFYVE) and Tg(TαCP:tRFP-t-2XFYVE) are examples of the latter. The FYVE domain is a conserved protein motif characterized by its ability to bind with high specificity to phosphatidylinositol 3-phosphate (PI(3)P), a phosphoinositide highly enriched in early endosomes, but also detected in early autophagic structures and found to be important for autophagy [102]. These zebrafish transgenic reporter lines (Tg(TαCP:YFP-2XFYVE) and Tg(TαCP:tRFP-t-2XFYVE)) were recently used to characterize endolysosomal trafficking events upon ablation of the polyphosphoinositide phosphatase, Synaptojanin1 (*synj1*) in cone photoreceptors [97]. A summary of the zebrafish autophagy reporter lines used in zebrafish can be found in Table 2.

**Table 2.** Constitutive and transient reporter constructs used to study autophagy in zebrafish.

Reporter	Expression	Reference
Tg(CMV:GFP-Lc3)	Ubiquitous	[86]
Tg(CMV:GFP-Gabarap)	Ubiquitous	[86]
Tg(pT2-mCherry-Sqstm1)	Ubiquitous	[45]
Tg(pT2-Lamp1-mCherry)	Ubiquitous	[45]
Tg(TαCP:mCherry-GFP-Map1lc3b)	Cone photoreceptors	[97]
Tg(TαCP:GFP-Map1lc3b)	Cone photoreceptors	[97]
Tg(TαCP:YFP-2XFYVE)	Cone photoreceptors	[97]
Tg(CMV:EGFP-Map1lc3b; CMV:mCherry-Map1lc3b)	Ubiquitous	[46]
Tg(CMV:EGFP-Gabarapa; CMV:mCherry-Map1lc3b)	Ubiquitous	[46]
Tg(fabp10: EGFP-Map1lc3b)	Liver	[95]
Tg(TαCP:GFP-Map1lc3b)	Cone photoreceptors	[90]
pEGFP-Map1lc3b	Transient (embryonic cells)	[103]
mCherry-Lc3 mRNA	Transient	[104,105]
pDest(CMV:RFP:GFP:Lc3) mRNA	Transient	[105]
GFP-Lc3-RFP-Lc3ΔG mRNA	Transient	[101]
mCherry-Map1lc3b	Transient	[106]
hsp70l:RFP-Map1lc3b	Transient	[61]

In cases where there are no stable reporter lines available and one wants to investigate autophagy during embryonic development (up to 5 dpf or depending upon the half-life of the transcribed mRNA), it is possible to inject in vitro transcribed mRNA for a reporter tagged to Lc3 or any other autophagy marker protein, such as mCherry-Lc3 mRNA in vitro transcribed from the vector pDest(CMV:RFP-GFP-Lc3) [104–106].

## 5.2. Western Blotting

The most widely used method for analyzing autophagy is by measuring the levels of lipidated or membrane bound form of Atg8/Lc3B (Atg8-PE/Lc3B-II), as it runs at a different molecular weight than the cytosolic form of Lc3 (Lc3-I) by SDS-PAGE [79,107]. This method has been used to measure levels of autophagy in some zebrafish autophagy studies [46,58,86,95,104,106,108,109]. Again, it cannot be concluded that a mere increase in Lc3-II levels corresponds to increased autophagy, as this can also be due to autolysosomal formation defects, and it is therefore important to do proper autophagic flux experiments (as described above) to conclude about increased/reduced autophagy.

It is very critical to differentiate between the lipidated Lc3-II and the unlipidated Lc3-I when immunoblotting for Lc3. As these two bands lie pretty close to each other (approximately 14 and 16 kDa), one can be masked by the other and this problem is intensified if the zebrafish embryo is not devolged prior to preparing the lysate. The yolk sac is enriched with the protein Vitellogenin and this can cause overloading effects while blotting, if not removed by a devolging buffer such as Ringer's solution [110]. It is also critical to use gels that give a good separation in the 15 kDa area.

Reproduction of Lc3 blots can be a major hindrance, primarily attributed to changes in experimental setups. The lysis buffer used, the incubation times for blocking, and the primary and secondary antibodies as well as washing periods should be optimized. The type of membrane used for blotting also makes a difference, as Lc3-II binds more effectively to the polyvinylidene fluoride (PVDF) membrane whereas nitrocellulose has a higher affinity for Lc3-I. It is also beneficial to dry the membrane for a short time after transfer to potentially stabilize the binding of Lc3 to the membranes. The following should also be taken into consideration while blotting for Lc3: sensitivity issues of Lc3-I to freeze-thawing (lysates should be run right after boiling), and comparison of Lc3-II levels to a housekeeping protein (e.g., actin or tubulin) rather than comparing them to Lc3-I, as Lc3-I levels can vary (e.g., upon cellular stress and from tissue to tissue) and not necessarily represent autophagy levels. Finally, it is necessary to also monitor the *lc3B* mRNA levels and to compare the correlation between protein Lc3B and mRNA *lc3B* [79].

Even though Lc3 remains the primary target to reveal levels of autophagy, other core autophagy proteins have also been studied. Knockdown of Atg5, Atg7 and Beclin1 in zebrafish were validated via Western blotting in a study aimed at investigating a possible role of autophagy during zebrafish embryogenesis [58]. Beclin1 levels were also examined in ambra-1 knockdown embryos [62]. A detailed list of autophagy related antibodies successfully used for Western blotting and immunofluorescence in zebrafish is shown in Table 3.

**Table 3.** List of antibodies ever used to detect autophagy-related proteins in zebrafish. (Catalogue numbers listed in *italics* have been used for immunostaining too).

Antibody	Company	Catalogue No.	Reference
LC3	Novus biologicals	NB100-2220	[93,108,111–116]
	Novus biologicals	NB100-2331	[86,94,117]
	Proteintech	12135-1-AP	[118]
		4108	[45,109]
	Cell Signaling	Not indicated	[74,104]
		2775	[62,114]
		Not indicated	[119]
	MBL	PD014	[95]
		PM036	[115]
	Sigma	L7543	[59]
	Abcam	<i>ab51520</i>	[106]
	Thermo Scientific	<i>PA1-46286</i>	[68]
Gabarap	Non-commercial		[86]
SQSTM1/p62	Abnova	H00008878-M01	[111]
	Cell Signaling	5114	[94,112]
	Abcam	ab109012	[117]
		<i>ab31545</i>	[68]
	MBL Japan Cliniscience	Not indicated PM045	[119,120] [67]
mTOR	Cell Signalling	2983	[116]
Phospho-mTOR, Ser2448	Cell Signaling	2971	[121]
Akt	Cell Signaling	Not indicated	[74]
Phospho-Akt, Ser473	Cell Signaling	9271	[74,121]
Phospho-S6K, Thr389	Cell Signaling	9205	[121]
Phospho-S6K	Cell Signaling	Not indicated	[104]
S6k	Cell Signaling	2708	[121]
Beclin1	R&D systems	Not indicated	[120]
	Abcam	Not indicated	[104]
	Santa Cruz	H-300 11427	[58,62]
Lamp-2A	Abcam	ab18528	[121]
Atg5	Novus biologicals	NB110-53818	[59,93]
	Abcam	Not indicated <i>ab540333</i>	[108] [59]
	Abgent	AP1812a, AP1812b	[59]
Actin (loading control)	Sigma	Not indicated	[108]
$\alpha$ -Tubulin (loading control)	Sigma	T5168	[73]
GAPDH (loading control)	Millipore	Not indicated	[108]

### 5.3. Transmission Electron Microscopy (TEM)

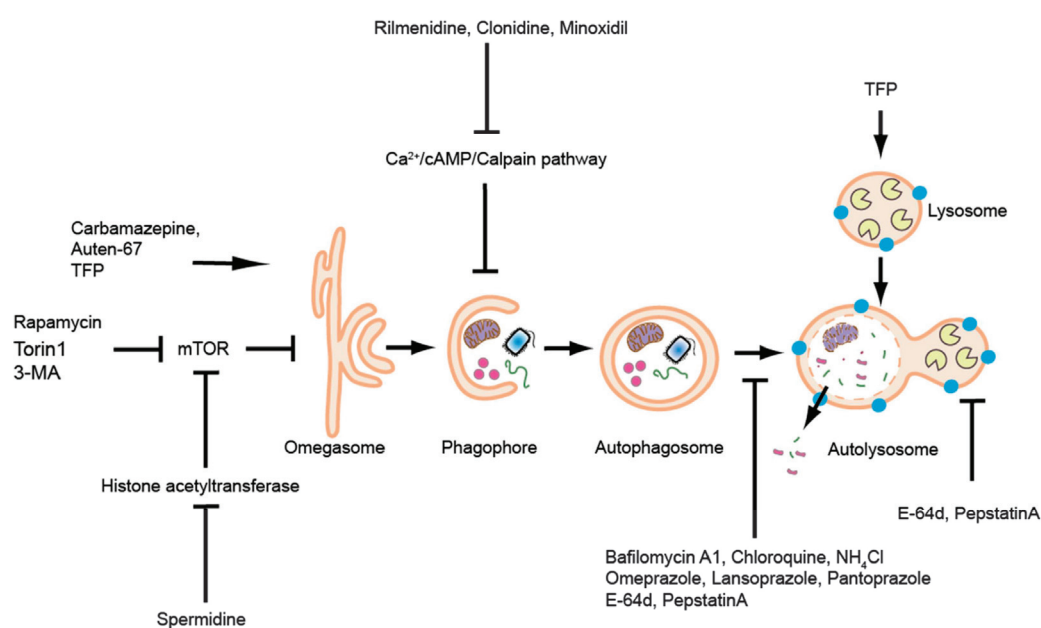
Autophagy was first discovered in the 1950s using transmission electron microscopy (TEM) [122]. TEM is a classical and widely used method to observe autophagic structures. If properly sampled, TEM

provides superlative ultrastructural images with much higher resolution than any light microscope or super-resolution microscope. It gives details of cellular coats, cellular components and bodies in their natural environment [79,123].

TEM has been used to a limited extent in zebrafish autophagy research, owing to the difficulty in sampling and instrument availability. TEM has been used to demonstrate the presence of autophagosomes during zebrafish embryogenesis [58], during caudal fin [91] and muscle regeneration [94] and a variety of other contexts. For example, TEM revealed an increased number of autophagosomes and autolysosomes in the intestinal epithelial cells of zebrafish harboring a mutation in a ribosomal RNA processing gene, *pwp2h* [106]. Here increased autophagy enhanced survival of this zebrafish ribosomopathy model. In contrast, aberrant autophagy was observed in a zebrafish motor dysfunction model [71], in Atrogin1-deficient zebrafish [117] and in a variety of zebrafish bacterial infection models [67,68,92]. The *Salmonella* plasmid virulence gene, *spvB*, was shown to enhance bacterial virulence by inhibiting autophagy [120].

## 6. Chemical/Pharmacological Modulations

Zebrafish embryos are easily treatable by waterborne exposure. Drugs that can modulate autophagic activity by either inducing it, decreasing it or blocking autophagosome-lysosome fusion have been well-used in zebrafish [124]. A detailed list of reagents used to interfere with autophagic activity in zebrafish (until 2014) has been reviewed previously [124]. We here present a list of autophagic modulators used in papers published after 2014 (Table 4, Figure 4).



**Figure 4.** Schematic overview of the autophagic pathway and a partial list of reagents (reagents used beyond 2014, Table 2) that modulate autophagy in zebrafish are indicated.

**Table 4.** List of reagents used to modulate autophagic activity in zebrafish (post-2014).

Reagent	Conc.	Observed Effect	Reference
<b>Reagents increasing autophagy</b>			
Rapamycin	400 nM	Inhibited mTOR, activated autophagy; ameliorated kidney cysts and preserved kidney function	[112]
	1 $\mu$ M	Increased autophagy dependent release of Tumor necrosis factor $\alpha$ and Interleukin-8 (TNF $\alpha$ and IL-8) in mycobacterium-infected zebrafish larvae	[105]
	10 $\mu$ M	Enhanced clearance of protein aggregates in FLNC <sup>W2710X</sup> mutants	[113]
	30 $\mu$ M	Enhanced the clearance of A152T-tau, reduced hyperphosphorylated tau	[108]
Torin1	0.4 $\mu$ M	ATP-competitive mTOR inhibitor; increased Lc3-I and Lc3-II levels; increased resistance of zebrafish embryos to <i>Salmonella</i> Typhimurium infection	[119]
Rilmenidine	50 $\mu$ M	Imidazoline-1 receptor agonist, reduced cyclic adenosine monophosphate (cAMP) levels; enhanced the clearance of A152T-tau	[108]
Clonidine	30 $\mu$ M	Imidazoline-1 receptor agonist, reduced cAMP levels; enhanced the clearance of A152T-tau	[108]
Carbamazepine	20 $\mu$ M	mTOR-independent autophagy activator; attenuated kidney cysts	[112]
	50 $\mu$ M	Increased autophagy-dependent cytokine release	[105]
	0.5 mM	Enhanced clearance of protein aggregates in FLNC <sup>W2710X</sup> mutants	[113]
Minoxidil	400 nM	Inhibited L-type Ca <sup>2+</sup> channel currents, thereby activating autophagy via a cyclical mTOR independent pathway; attenuated kidney cysts	[112]
Auten-67	50 $\mu$ M	Upregulated autophagy by inhibiting phosphatase activity of MTMR14, which is a negative regulator of autophagic membrane formation.	[125]
Spermidine	5 mM	Inhibited acetyl-transferases; enhanced clearance of protein aggregates in FLNC <sup>W2710X</sup> mutants	[113]
Trifluoperazine (TFP)	1 mM	Activated Transcription Factor EB (TFEB) which is a master regulator of autophagy pathway, activated autophagy	[111]
<b>Reagents blocking autophagosome-lysosome fusion</b>			
Bafilomycin A1 (BafA1)	20 nM	Autophagosome-lysosome fusion inhibitor; slight increase in Lc3-II	[117]
	25 nM	Significant increase in Lc3-II	[104]
	167 nM	Showed defects in autophagy flux	[112]
	200 nM	Zebrafish larvae recapitulated atp6v0ca morphant, reduced yolk opacity and senescence phenotypes	[45]
Chloroquine	10 $\mu$ M	Autophagosome-lysosome fusion inhibitor; blocked autophagy and increased GFP-Lc3 punctae	[73]
	2 mM	Reduced muscle regeneration on blocking autophagy	[94]
	100 $\mu$ M	Decreased Lc3 accumulation, defective autophagy	[113]
	5 $\mu$ M	Increased Lc3 accumulation in Kri1 <sup>cas002</sup> mutant	[104]
	2.5 $\mu$ M	Significant accumulation of autophagosomes in zebrafish larvae infected with mycobacterium	[105]
	50 $\mu$ M	Accumulation of Lc3-II and p62; no effect on zebrafish infection with <i>Salmonella</i> Typhimurium	[119]
Omeprazole	100 $\mu$ M	Late-stage autophagy inhibitor; rescued senescence phenotype	[45]
Lansoprazole	100 $\mu$ M	Late-stage autophagy inhibitor; rescued senescence phenotype	[45]
Pantoprazole	100 $\mu$ M	Late-stage autophagy inhibitor; rescued senescence phenotype	[45]
Pepstatin A	5 $\mu$ g/mL	Prevented autolysosomal maturation and turnover	[45]
E-64d	5 $\mu$ g/mL	Prevented autolysosomal maturation and turnover	[45]
Ammonium chloride	100 mM	Prevented autolysosome maturation; blocked autophagy and increased GFP-Lc3 punctae	[113]
	100 mM	Significant increase in Lc3-II	[117]
<b>Early autophagy inhibitor</b>			
3-MA	10 mM	Inhibited PIK3C3 activity; significant reduction of autophagy visualized by Lc3-II puncta	[104]

## 7. Selective Autophagy

While induction of autophagy upon nutrient deprivation or other forms of stress is believed to be an unselective process when it comes to the types of cargo being sequestered and degraded to supply cells with essential building-blocks to survive the period of stress until cellular homeostasis is restored. Autophagy can however also be a highly selective process, with different cargo-specific sub-types, including lipophagy (autophagy of lipid droplets), ferritinophagy (autophagy of iron bound ferritin), lysophagy (autophagy of lysosomes), reticulophagy (autophagy of ER), ribophagy (autophagy of ribosomes), xenophagy (autophagy of pathogens), aggrephagy (autophagy of protein aggregates) and mitophagy (autophagy of damaged mitochondria). Specific cargo binding proteins that also interact with Lc3/GABARAP proteins (so-called autophagy receptors) have been identified and found to facilitate selective autophagy by connecting cargo to the autophagy membrane. Selective autophagy plays an important house-keeping function under basal nutrient-rich conditions to mediate the removal of superfluous or damaged organelles and protein aggregates that otherwise could be toxic. Zebrafish has been used to study degradation of mitochondria and protein aggregates in different neurodegenerative disorder models and to investigate the role of autophagy in protection against

pathogens, as reviewed below. For most other types of selective autophagy zebrafish have either been not used at all or very scarcely used.

### 7.1. Mitophagy

Selective removal of mitochondria is termed as mitophagy. The degradation of mitochondria by autophagy was already reported in the late 1950s when Clark and Novikoff observed mitochondria within membrane-bound compartments called “dense-bodies”, which were later shown to contain lysosomal enzymes [126,127]. The term mitophagy was coined by Lemasters and colleagues when they observed the engulfment of mitochondria into vesicular structures coated with Lc3 [128]. Mitophagy is also seen in yeast and this has helped dissect the molecular machinery required for the process [129,130]. Some of the proteins required for yeast mitophagy do not have a mammalian orthologue (e.g., Atg32, Atg11), but have functional homologues, e.g., the outer mitochondrial membrane protein NIX acts both like Atg32 and Atg11 [131]. Mitophagy has also been found to be important during key developmental processes, such as the maturation of reticulocytes, after which the matured red blood cells lack mitochondria [132,133].

The E3 ubiquitin ligase Parkin is a major player in mitophagy [134]. Mitochondrial recruitment of Parkin is mediated by the accumulation of PTEN-induced putative kinase protein 1 (PINK1) on depolarized mitochondria [135–137]. NIX has also been shown to promote Parkin translocation and thereby promote mitophagy in mouse embryonic fibroblasts [138]. Loss of function mutations in the gene encoding Parkin (*park2*) have been linked to Parkinson’s disease (PD) with loss of dopaminergic neurons in the substantia nigra, a region in the mid brain that is responsible for motor function [139]. Parkinsonian syndrome has also been shown in zebrafish morphants lacking *pink1* [140] and *park2* [141], with dopaminergic cell loss. A TILLING (targeting-induced local lesions in genomes) mutant for *pink1* also shows significant reduction in the number of tyrosine hydroxylase (TH)<sup>+</sup> cells and a reduction in mitochondrial complex I activity [142]. Thus, mitophagy dysfunction or an inability to degrade damaged mitochondria leading to accumulation of mitochondrial damage is a likely cause of PD.

Proteins involved in mammalian mitophagy are well conserved in zebrafish [143], which makes zebrafish a good model to further delineate the functional significance of mitophagy in vivo. There have not yet been many mitophagy studies in zebrafish, but several tools exist to study mitochondrial dynamics. One study tried to observe sites for mitophagy in Rohon Beard neurons of zebrafish where *UAS:LC3.GFP* was coinjected with *UAS:mitoTagRFP-T* into the *Isl2b:Gal4* transgenic line. Lc3 was found to colocalize with mitochondria, but proper mitophagy assays were not performed [144]. It would be highly interesting to see if Lc3 disappeared over time from these contact points. As mentioned earlier, a tandem-based approach to tagging mitochondrial proteins would help in observing their degradation kinetics via autophagy. It is a highly exciting time for zebrafish mitophagy studies. One major problem is however the lack of antibodies for zebrafish mitochondrial proteins, but with larger research interest churning up for mitochondrial studies, this scenario is likely to diminish fast.

### 7.2. Aggrephagy

Several neurodegenerative disorders and prion diseases are characterized by neuronal protein aggregates and inclusion body formation. Aggregates are formed due the accumulation of misfolded proteins [145]. Misfolded proteins can either be degraded by the ubiquitin–proteasome system (UPS), through chaperone-mediated autophagy (CMA) or by macroautophagy. Almost all soluble proteins (except for the long lived proteins) are turned over by the UPS, but as large protein-aggregates are difficult to degrade by the UPS, they are degraded by autophagy [146].

The zebrafish is a well-known model for the study of neurodegenerative disorders. Pharmacological modulation of autophagy in such zebrafish models of neurodegeneration has shown promising results. The first study to mention autophagy in zebrafish used a zebrafish Huntington’s disease (HD) model expressing EGFP-HDQ71 aggregates, where autophagy was found to be upregulated by reagents such as calpastatin, calpeptin, 2’5’DDA and clonidine (Table 2), resulting

in a decrease in EGFP-HDQ71 aggregates [147]. HD is caused by glutamine expansions (polyQ) in the gene encoding the Huntingtin protein that make it prone to misfold and aggregate. In another study using the zebrafish HD model expressing EGFP-HDQ71 it was demonstrated that autophagy inducers like rapamycin and clonidine cleared the aggregate in the retina [148]. A zebrafish model of Alzheimer's disease (AD) is characterized by neuronal tau aggregates and was found to have reduced aggregate clearance and decreased Lc3-II levels upon overexpression of phosphatidylinositol binding clathrin assembly protein (Picalm) [114]. PICALMs are known to interact with and thereby regulate the endocytosis of Soluble NSF Attachment Protein Receptor proteins (SNAREs), such as VAMP2, VAMP3 and VAMP8 [149]. In a recent study, it was seen that clonidine, rilmenidine and rapamycin had positive effects on the clearance of aggregated A152T-tau. It was also observed that transient overexpression of Atg5 upregulated autophagy in zebrafish larvae by 2 dpf, evident by an increase in lipidated Lc3-II and a reduction in hyperphosphorylated tau—one that causes aggregation of tau [108]. BAG3 is a key component of the chaperone-assisted selective autophagy (CASA) pathway [150]. It was recently found that in a transgenic zebrafish model of myofibrillar myopathy (induced by expression of a mutant of filamin C (FLNC<sup>W2710X</sup>-eGFP)) the BAG3-mediated CASA pathway is impaired and insufficient in clearing the FLNC<sup>W2710X</sup> aggregates, and that autophagy promoting compounds like rapamycin or carbamazepine facilitated aggregate reduction [113].

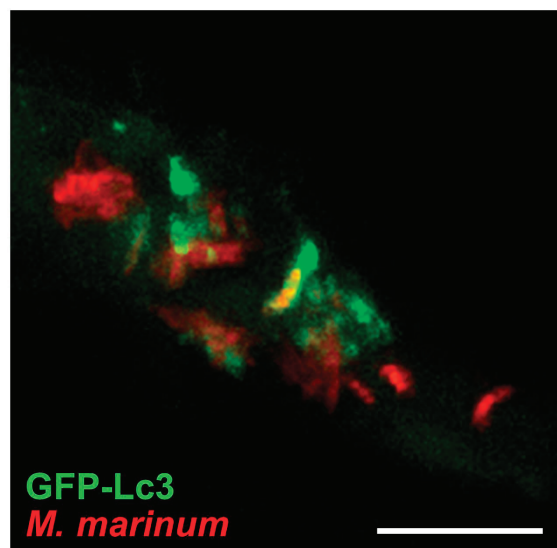
The zebrafish as a model is proving to be essential for understanding disease mechanisms of several neurodegenerative disorders characterized by insoluble protein aggregates. There are a plethora of studies showing reduction of protein aggregates by the induction of autophagy. So far, molecular studies on the sequestration of aggregates into autophagosomes have not elucidated the role of different autophagy proteins during autophagy in zebrafish. This is very likely to change in the near future with the advent of CRISPR/Cas9 technology and the availability of antibodies for protein studies. The transparency of the zebrafish and its amenability to different drugs makes them an excellent model for neurodegenerative research.

### 7.3. Xenophagy

The role of autophagy as an anti-microbial mechanism was first demonstrated in studies by Yoshimori and co-workers, who showed that *Streptococcus pyogenes* multiplied in Atg5-deficient cells and by Deretic and co-workers, who showed that intracellular survival of *Mycobacterium tuberculosis* could be limited by starvation-induced or rapamycin-induced autophagy [151,152]. Since then autophagy has become recognized as a crucial defense mechanism against bacterial, viral, fungal, and protozoan pathogens [14]. Once internalized by host cells, microbial invaders often escape from phagosomes into the cytosol, where they become targets for xenophagy. Cytoplasmic microbes or damaged membranes of phagosomes are marked by molecular tags such as ubiquitin and galectins, which are the substrates for recognition by selective autophagy receptors that are also involved in mitophagy [153]. The autophagy receptors p62 and optineurin have been shown to protect against bacterial infections in zebrafish models [66,67]. Autophagy-related mechanisms distinct from xenophagy also play a role in host defense. In particular, Lc3 can be recruited directly to phagosomes in a process named Lc3-associated phagocytosis (LAP) [154]. Pathogens have evolved various strategies to evade xenophagy or LAP [155,156]. Zebrafish models for *Salmonella typhimurium* and *Mycobacterium marinum* infection have been used to study some of the virulence mechanisms that pathogens use to counteract the host autophagy response [119,120,157]. In addition, it has been shown that pharmacological stimulation of autophagy can improve the zebrafish host defense against *Mycobacterium marinum* infection [105,118].

GFP-Lc3 transgenic zebrafish have been used to study bacterial infections with *Shigella flexneri* and *M. marinum* [67,77,102,158]. Both these pathogens have the ability to escape from phagosomes and replicate inside the cytosol. A proportion of cytosolic *Shigella* bacteria are trapped inside cage-like structures formed by septins, which are cytoskeletal components that prevent *Shigella* from actin tail formation and cell-to-cell spreading [159]. In vitro studies have shown that septin-caged

*Shigella* are targeted to autophagy [159]. In agreement, in vivo imaging of zebrafish embryos demonstrated recruitment of GFP-Lc3 and the presence of bacteria in autophagosomes was confirmed by ultrastructural analysis [67]. Septin-caged *M. marinum* bacteria were also observed in zebrafish embryos, but the significance of septin caging in relation to autophagic targeting remains to be investigated [67]. Entrapment of *M. marinum* bacteria by GFP-Lc3 vesicles could be visualized by confocal time lapse imaging of infected zebrafish [92]. This study also revealed that GFP-Lc3 vesicles frequently appeared as puncta in close vicinity of single bacteria or bacterial clusters (Figure 5). Correlative light and electron microscopy confirmed that these vesicles represent autophagosomes, which might contribute to the delivery of ubiquitinated antimicrobial peptides to the *M. marinum*-containing compartments [92,160].



**Figure 5.** Confocal imaging of Tg(CMV:EGFP-Mapllc3b) on infection. GFP-Lc3 signal around clusters of *M. marinum* bacteria in 4-day-old zebrafish larva at 3 days post infection. Scale bars, 10  $\mu$ M.

Dram1 is an autophagy modulator that is induced during infection of zebrafish by the MYD88-NF $\kappa$ B-dependent signaling pathway, which occurs downstream of pathogen recognition by Toll-like receptors [68]. Expression of zebrafish Dram1 can also be induced by the p53-stabilizing agent roscovitin, in agreement with the identification of human DRAM1 as a p53 target gene [68,161]. Overexpression of Dram1 by mRNA injection was found to result in increased lysosomal acidification of *M. marinum* containing compartments and to improve resistance of zebrafish embryos to the infection [68]. In addition, Dram1 overexpression enhances GFP-Lc3 recruitment to *M. marinum* and this function requires the cytosolic DNA sensor Sting and the ubiquitin receptor p62. In agreement, morpholino knockdown of Dram1 reduced GFP-Lc3 recruitment to *M. marinum* and impaired host defense [68]. Dram1 is a member of a conserved family of transmembrane proteins and localizes predominantly to lysosomes [161]. Its precise mechanism of action is currently unknown, but a recent study on a human family member, DRAM2, suggests an interaction with the Beclin1-Vps34-UVRAG complex, which leads to displacement of the inhibitor Rubicon and thereby enhanced PI3K activity [162]. Since *M. marinum* infection in zebrafish mimics aspects of human tuberculosis, further research into the Dram1-mediated selective autophagy pathway could help to develop novel strategies for host-directed anti-tuberculosis therapy [160].

Recently, the zebrafish has also been used to study the host autophagic response to a viral infection [109]. Zebrafish can be infected with spring viremia of carp virus (SVCV), a member of the rhabdovirus family. Infection with this virus induces the production of Tnf $\alpha$ , a potent pro-inflammatory cytokine that normally serves a host-protective function, but is exploited by certain viruses to their

benefit. GFP-Lc3 imaging and Western blot analysis showed that depletion of  $Tnf\alpha$  increased autophagy in SVCV-infected larvae. Since depletion of  $Tnf\alpha$  also improved resistance to SVCV infection, the authors concluded that inhibition of autophagy is the mechanism behind the deleterious effect of  $Tnf\alpha$  on viral clearance [109]. A wide variety of other zebrafish infection models provide excellent tools to further advance our understanding of the role of autophagy in host-pathogen interactions [163].

## 8. Future Perspective

The zebrafish is fast becoming one of the best vertebrate models for studying disease states and conditions. Owing to the various advantages that they pose and the ease at which the present advancements in genome editing technology can be applied, zebrafish hold unparalleled potential for all basic and translational research. Existing studies of autophagy in zebrafish have presented invaluable insight into the role of autophagy in development, disease progression and drug discovery. There is still however a need for antibodies that recognize specific zebrafish autophagy proteins, and their modifications (at present a limitation). The contribution of CRISPR/Cas9 to scientific research has been immense, but the overall technology depends upon efficient sgRNAs and thus having a database system to maintain and expand the existing sgRNAs is a must. Autophagy research has been expanding and the vitality of autophagy as a degradation system has been acknowledged worldwide with Yoshinori Ohsumi receiving the Nobel Prize in Medicine or Physiology in 2016. Autophagy research using the zebrafish as a model system looks promising for many more breakthroughs and new therapeutics against many diseases.

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