Accepted manuscript

Mitophagy and neurodegeneration: The zebrafish model system

Kim Wager[1,2] and Claire Russell[1]

[1]Department of Comparative Biomedical Sciences, The Royal Veterinary College, University of London, London, UK; [2]Department of Pharmacology, UCL School of Pharmacy, London, UK.

Competing financial interests and conflicts of interest:

The authors declare no competing financial interests or conflicts of interest.

Abstract:

Autophagy is responsible for the degradation of cytoplasmic components and organelles such as mitochondria. The selective degradation of damaged mitochondria by autophagy is termed mitophagy, and is an important quality control mechanism. Neurons, being highly specialized cells, are particularly susceptible to defects of autophagy. Impairments in mitochondrial function and their dynamics are present in many neurodegenerative diseases, and modulators of both mitochondrial physiology and autophagy present themselves as promising therapeutic targets. Zebrafish are now established as a valuable tool for disease modelling. A wide variety of genetic and molecular techniques can be employed to highlight pathogenic processes and dissect disease pathways. This review will explore the role that zebrafish have so far played in our understanding of mitophagy in neurodegeneration, and will discuss how they might be used to drive the wider mitophagy field forward.

Keywords: Mitophagy, autophagy, zebrafish, neurodegeneration, Parkinson disease

Abbreviations:

ATG, autophagy-related; BBB, blood brain barrier; CCCP, carbonylcyanide m-chlorophenylhydrazone; DA, dopaminergic; DPF, days post fertilization; GFP, green fluorescent protein; HPF, hours post fertilization; MOs, morpholinos; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyrdine; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; PD, Parkinson disease; ROS, reactive oxygen species; TALENs, transcription activator-like effector nucleases; TILLING, targeting induced local lesions in genomes; UPS, ubiquitin-proteasome system; ZFNs, zinc finger nucleases

1. Mitophagy is important for mitochondrial quality control.

Mitophagy is the process by which mitochondria are selectively degraded by the highly conserved autophagic machinery. It occurs during developmental processes in specialized cells such as erythrocytes, while in other cell types damaged mitochondria are removed in order to maintain a functional mitochondrial population. Mitochondria are membrane-bound organelles with several important roles in cellular function, including energy production by oxidative phosphorylation, calcium homeostasis, and the metabolism of fatty acids, amino acids and steroids. They are also the primary source of potentially damaging endogenous reactive oxygen species (ROS), which have been linked to neurodegeneration,[1] and can induce protein carbonyls, lipid peroxidation and DNA damage.[2] Importantly, release of cytochrome c from mitochondria triggers apoptosis, and so the clearance of damaged mitochondria is vital for cell survival. ROS are also able to increase the release of cytochrome c and induce the mitochondrial permeability transition pore (mPTP), both of which activate apoptosis.[3]

To prevent cellular damage from faulty mitochondria a number of protective mechanisms have evolved; for example, mitochondria have an endogenous proteolytic mechanism to degrade misfolded proteins,[4] proteins located on the inner and outer membranes can be degraded in the cytosol by the ubiquitin-proteasome system (UPS) and also specific mitochondrial components can be sequestered and directed to lysosomes for degradation by autophagy.[5] Furthermore, electron microscopy analysis has revealed that entire mitochondria are detectable in both yeast vacuoles and mammalian lysosomes.[6,7] It is this selective degradation of mitochondria by autophagy that is now termed mitophagy.

Autophagy is responsible for the degradation of cytoplasmic components and organelles. At least three distinct autophagic mechanisms have been identified that present substrates to the lysosome; macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is a tightly regulated process to ensure that cytosolic components are not inappropriately removed; it requires the formation of a double membrane called the phagophore, which expands and fuses to form a vesicle called an autophagosome that sequesters regions of cytosol containing proteins and organelles to be degraded.[8] Autophagosomes do not themselves contain degradative enzymes and so must fuse with lysosomes containing a range of acid hydrolases to form a structure referred to as an autolysosome. For many years macroautophagy was considered to be a nonselective bulk degradation process, but it now seems clear that there exist very selective subtypes of macroautophagy, which occur during nutrient-rich conditions to remove damaged organelles or toxic aggregates.[9] These selective pathways, including mitophagy, are mediated by autophagic adapter proteins.[10]

In this review, the focus is upon the study of mitophagy and its involvement in neurodegeneration using zebrafish as a model system. Defective autophagy has been associated with neurodegeneration,[11] and mutations in PTEN induced putative kinase 1 (*PINK1*),[12] the most common cause of recessive Parkinson disease (PD), have highlighted

the contribution of mitophagy to neurodegeneration. The study of mitophagy using *in vivo* models of neurodegeneration and relating them to normal mitochondrial biology will be essential to help understand disease mechanisms, and the advantageous features of the zebrafish position it as a tool to help move the mitophagy field forward.

2. What makes zebrafish a useful model to study the role of mitophagy in normal tissue and during neurodegeneration?

In recent years zebrafish have become a valuable tool for the study of vertebrate embryogenesis and disease modelling. Zebrafish develop rapidly, externally to the mother, in high numbers and are optically transparent, features not presented by mammalian systems. Neurogenesis commences approximately 10 h post fertilization (hpf) and is followed by synaptogenesis approximately 6 h later.[13] By 24 hpf brain morphogenesis is well advanced[14] and by 3 days post fertilization (dpf) the majority of morphogenesis is complete.[15] Characteristic body movements commence at around 17 hpf,[16] at 21 hpf (when manually removed from their chorions) they become responsive to touch,[16] at 52 hpf they hatch,[13] and at 4 dpf swim to pursue food particles.[17] Most zebrafish genes share 50-80% sequence identity[13] with human counterparts, their genome has been sequenced and, although not fully annotated, is available on bioinformatics databases. As demonstrated using PINK1 as an example, amino acid sequences are also well conserved and often more so in functional domains (**Fig. 1**).

The molecular mechanisms of autophagy are evolutionarily conserved from yeast to mammal[18] and genomic databases make it relatively easy to identify orthologous genes known to be involved in autophagy and, more specifically, mitophagy (Table 1).

Such a degree of conservation implies that these genes and processes are fundamental to the growth and maintenance of the organism. Manipulation of these genes is relatively easy in zebrafish compared to other vertebrates, therefore providing a good opportunity to

dissect these pathways. Their transparency makes them particularly amenable to targeted reporter transgene expression, including autophagy-related genes[19] or vital dyes such as LysoTracker, a stain that can be used to visualize lysosomes in live zebrafish.[20] Several neurodegenerative diseases have been modelled in zebrafish by expressing mutant forms of the human disease gene under the control of a zebrafish promoter. Examples of these genes include *MAPT,*[21,22] *SOD1*[23] and *HTT*.[24] Fusion of green fluorescent protein (GFP) to the disease-causing transgene can be used to monitor the clearance of protein aggregates,[24] and a similar approach of fluorescently tagging mitochondrial proteins can be used to monitor mitophagy in an intact organism under a variety of conditions. Highlighting this proposal, Plucinska *et al.* have recently developed a zebrafish line expressing fluorescently tagged mitochondria specifically in sensory neurons.[25] This model allows the researcher to follow mitochondrial flux in single axons using time-lapse microscopy. Given that expression of the fluorescent mitochondrial tag is GAL4 dependent, these so-called MitoFish can be crossed to any suitable GAL4-driver line,[26] thereby enabling expression in other cells and tissues. For information on GAL4-driver lines the ZFIN database provides a useful resource. A further advantage of the zebrafish is the fact that due to their rapid, external development, disease phenotypes tend to manifest in the larval stages (by 5 dpf) and so data can be obtained quickly. Typically the generation time for laboratory zebrafish is 3 months and a pair of zebrafish are able to produce up to 300 embryos weekly; this allows statistically significant sample sizes to be used at a substantially lower cost than mammalian systems.

2.1 How representative of humans is the zebrafish brain?

The zebrafish brain can be divided into the same regions as other vertebrates, consisting of the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon.[14] Furthermore, zebrafish neurons possess typical structural features, including the soma, dendrites and the axon, which can be myelinated or demyelinated. Astrocytes,[27]

oligodendrocytes[28] and microglia[29,30] have all been positively identified in the zebrafish brain. The identification of these cell types is important because their interactions with neurons and their involvement in disease processes is becoming increasingly relevant, and these *in vivo* interactions are something that cannot be easily replicated *in vitro*. The development of the blood brain barrier (BBB) in zebrafish has been shown by 3 dpf,[31] an important finding when considering the screening of potential therapeutics to modulate mitophagy and its involvement in neurodegeneration. Typically, upon development of the BBB, only lipophilic molecules with a diameter smaller than 500 daltons are able to pass through,[32] which severely restricts the accessibility of the brain to drugs. Permeability tests on the zebrafish BBB revealed similar properties to that of mammals,[31] confirming that zebrafish are a suitable model in which to test drugs destined for the brain or to test novel ways of escorting potential therapeutics to the brain.

Several regions of the zebrafish central nervous system, including the cerebellum, optic tracts and tectum, medulla, hypothalamus and cranial nerves, show structural similarity to the corresponding human structures.[**33**] Additionally, the main neurotransmitter systems involving acetylcholine, dopamine, GABA, glycine, glutamate, noradrenaline and serotonin are all present.[**34**] With reference to PD, a disorder in which mitophagy has been implicated, although dopaminergic (DA) neurons are identifiable by 18-20 hpf,[**35**] they are not present in the zebrafish midbrain; however, the observation of Parkinsonian traits in zebrafish treated with a DA neuron-selective toxin implies the existence of functionally equivalent circuitry. Axonal tracing shows axons ascending from the DA neurons of the ventral diencephalon to the striatum in the zebrafish, and these could be considered homologous to the nigrostriatal system.[**36**] The schematic presented in Figure 2 below highlights the zebrafish dopaminergic system, and for an excellent exploration of the zebrafish catecholaminergic system see Kastenhuber *et al.*[**37**]

2.2 Zebrafish are amenable to high throughput drug screens.

A considerable advantage of the zebrafish model system is as a tool to develop and screen candidate drugs and provide *in vivo* toxicity testing of new compounds, with the potential to treat human neurological disease. The ability to house larvae in 96-well plates facilitates high-throughput screening using definable, and in some cases automatable, phenotypic endpoints, such as locomotor behaviors or the monitoring of fluorescent reporters. These attributes of zebrafish are a huge advantage over the slow and expensive process used when testing compounds in mammals. Similarly, the application of pharmacological modulators of the autophagic pathway may be applied in a high-throughput manner by simply adding compounds to the fish water. Given that a cell-based drug screen does not provide adequate toxicity data and may produce hundreds of "hits" requiring validation, this provides a considerable advantage over cellular models. A further advantage of using an *in vivo* model over conventional target-based drug design is that small molecule screens can be carried out prior to understanding the molecular basis or pathways involved in disease. The use of zebrafish in the drug discovery process has been reviewed in Williams and Hong,[38] and Kaufman *et al.* have provided a protocol and review of zebrafish chemical screens.[39]

2.3 What molecular tools are available to manipulate the zebrafish genome?

The use of oligonucleotide-based reagents confers a genetic method by which to interrogate and disrupt the function of genes putatively involved in mitophagy. While this work has been performed widely in cells, similar studies have seldom been carried out *in vivo*, and utilizing zebrafish would be significantly faster and cheaper than mammals. Several antisense knockdown technologies have been explored,[40-43] but the primary method in zebrafish is through morpholino oligonucleotides (MOs). MOs are usually injected into the 1- to 4-cell embryo so that they become distributed throughout the animal as it develops. By designing MOs to span the start codon, translation can be blocked by inhibiting the procession of the

initiation complex.[44] Additionally, MOs can be designed to target intron/exon splice junctions to produce splice variants either lacking specific domains within proteins of interest or to introduce a frame-shift, producing an in-frame stop codon.[45] Splice-blocking MOs have the advantage that because they do not affect the maternal transcript, a target gene with maternal function can be examined.[46] To confirm MO specificity, it is prudent to perform a BLAST search of the MO sequence and to resequence the target of the experimental line to ensure that no single-nucleotide polymorphisms or sequence errors in the reference sequence are present.

When using MOs, it is important to demonstrate how effective knockdown has been. MOs targeted to the start codon provide a simple and effective approach; it can, however, be difficult to demonstrate that protein expression is reduced.[47] This can be achieved by whole-mount immunohistochemistry, but this relies on there being an antibody available to the protein of interest that is reactive to the zebrafish protein *in vivo*. Western blotting may also be used, but depending on antibody sensitivity a large number of animals may be required. In the absence of antibodies, GFP-tagged mRNA for the transcript of interest may be injected into an embryo along with the MO. If the MO successfully produces knockdown, then less fluorescence will be observed.[48] This technique does, however, assume that the endogenous mRNA is equally accessible to the MO. The advantage of splice-inhibiting MOs is the ability to test exactly what missplicing event has occurred by using RT-PCR and sequencing of the spliced product.

Injection of MOs at early developmental stages causes constitutive knockdown, but caution must be taken to ensure that the observed phenotype is not due to off-target effects. Suitable controls could be: i) injection of nonsense oligos, ii) a sense version of the experimental oligo, or iii) a mismatched oligo. Since none of these pseudo MOs will be able to bind to the target sequence, any observed effects can be assumed to be nonspecific. However, in our view, the ability to observe consistent phenotypes with multiple MOs (e.g.,

translation blocking, splice site) directed against a given target is a more valid control. Examples of nonspecific effects include neurodegeneration,[49] more widespread cell death[50] and epibolic failure.[51] As a further test of specificity, co-injection of a wild-type target mRNA can be used to see if the morphant phenotype can be rescued. The issues here are threefold: i) this mRNA will now also be a target for translation-blocking MO knockdown; ii) it is optimistic to assume that this injected mRNA will be correctly translated in the cells of interest; and iii) the protein translated from the injected mRNA is found in ectopic places and may cause a phenotype that hampers interpretation of the rescue experiment. To resolve the issue of knockdown of the injected mRNA by translation-blocking MOs, transcripts can be engineered to include mismatches, preventing the annealing of the MO. The design of these resistant transcripts can be aided by the use of software such as Gene Designer.[52] Alternatively, splice-site MOs can be used, which do not typically recognize spliced mRNAs.

A disadvantage of MO use is their dilution as the embryo grows, so they only act for a few days. To inhibit gene expression in later stages it is possible to use photoactivatable MOs[53,54] but these have only been available since June 2012 and so data concerning their use is limited.

While being a powerful technique, transient knockdown by MO may be insufficient in some cases, and the development of permanent genetic knockouts is desirable. Zinc finger nucleases (ZFNs)[55] may present a different approach to investigate putative genes involved in mitophagy. Perhaps the biggest advantage of these nucleases over MOs is their ability to confer robust germ line genetic alterations, with the ability to generate heterozygous carriers of a mutation in 6-8 months.[56] ZFNs are derived from a fusion of a Cys_2 Hys_2 zinc finger protein with the type IIS Fok1 endonuclease. Each finger recognizes a 4 base pair DNA sequence via an α-helical domain. Using molecular engineering, several fingers can be linked in tandem to allow site-specific recognition.[57] Cleavage is then initiated by the

endonuclease domain at the site determined by the zinc finger protein. Specificity is derived by the requirement for two ZFNs to bind the same locus in a specific orientation, to create a double-strand break. Eukaryotic cells may then implement double-strand break repair mechanisms. These include nonhomologous end joining and homology-directed repair.[58] Religation by nonhomologous end joining tends to result in the loss or gain of small amounts of sequence, typically resulting in a frameshift allele. Importantly, ZFNs have recently been used to introduce sequence-specific knockins in both rats and mice.[59] However, there are specificity issues and not all sequences can be targeted.

Recently a new tool for genome editing has been used with very promising results, consisting of a transcription activator-like effector fused to the Fok1 endonuclease, referred to as TALENs. Compared to ZFNs, TALENs are more predictable and specific.[60] TALENs are constructed to work in pairs, the specificity of which is encoded in their central consecutive repeat domain. Each repeat corresponds to one DNA base pair, and the di-amino acid motif repeats at the hyper-variable positions 12 and 13 determine sequence specificity.[60] TALENs cause targeted double-stranded DNA breaks with high efficiency[61] and can be designed to target any specific DNA sequence; moreover, they have been successfully tested in zebrafish.[61] TALENs provide a new way to not only knockout genes, but also show promise as a means to knockin disease genes at a specific locus, with the first successful modification of the zebrafish genome through homology-directed repair, including the insertion of a predefined donor sequence, being recently reported.[62] The TALEN technology is publicly available,[63] is affordable and can be constructed with relative ease in-house.

2.4 How can zebrafish mutagenesis screens be exploited?

Zebrafish mutagenesis screens have produced recessive mutations resulting in remarkable phenotypes, the research of which has added much to the field of developmental biology (detailed information on many of these mutants is available in a dedicated edition of

Development, 1996; 123). These forward genetic screens provide a way to observe the cellular and molecular events involved in normal development, physiology, behavior and disease. In order to find mutants with defects in mitophagy, interested laboratories may wish to use the tools referred to in this paper to identify mitophagy mutants and then, through linkage analysis, clone the causative gene (for a guide to positional cloning in zebrafish see Zhou and Zon).[64] It is also interesting to note that recent technological advances mean that mutation identification may be considerably sped up, in a cost-effective manner, by using whole genome sequencing and homozygosity mapping.[65,66] Information on zebrafish mutant lines and their availability is accessible through the Zebrafish International Resource Centre (ZIRC), the Zebrafish Mutation Project and the Tübingen zebrafish stock collection.

To speed up this process the reverse genetic technique, Targeting Induced Local Lesions IN Genomes (TILLING), may be chosen. This contrasts with forward genetic screens because, rather than finding a phenotype of interest and then seeking to discover in what gene the mutation lies, specific genes in lines generated by random mutagenesis are requested to be targeted for sequencing, irrespective of phenotype.[67,68] The advantage of TILLING lies in its ability to detect mutations in genes with subtle phenotypes that affect mitophagy and may therefore be undetectable by forward genetic screens. For further information on TILLING methods and its successes, readers are directed to an excellent review by D. Stemple.[69] Requests for genes to be screened by TILLING can be submitted to the FHCRC Zebrafish TILLING Project, while the Sanger Institute is now exome sequencing all their mutants and uploading new alleles to their website; requests to receive alerts on new alleles can be registered online. There are, however, limitations to the N-ethyl-N-nitrosourea (ENU) approach; for example, some genes are less likely to be mutagenized by random mutagenesis due to their small size, and some genes may be more or less likely to undergo mutation depending on their nucleotide composition[70] (Table 2).

3. Oxidative stress can damage mitochondria.

3.1 Oxidative stress can trigger the mitochondrial permeability transition.

A byproduct of energy production by oxidative phosphorylation in mitochondria is the production of ROS. Oxidation of DNA, proteins or lipids may cause cellular damage, and ROS accumulation in mitochondria risks mitochondrial DNA (mtDNA) mutation, lipid peroxidation and opening of the mPTP and inner membrane anion channel.[71] Cells may respond in a graded fashion to the opening of the mPTP, an event that leads to dissipation of the proton motive force, which results in the uncoupling of oxidative phosphorylation and reversal of the mitochondrial ATPsynthase.[72] Furthermore, the permeability transition causes severe mitochondrial swelling, culminating in the rupture of the outer mitochondrial membrane. If only a few mitochondria are affected then mitophagy is induced, whereas if higher numbers of mitochondria are involved then apoptosis is promoted[72] due to the mitochondrial release, upon rupture, of intermembrane proapoptotic factors such as cytochrome c, AIFM1 (apoptosis-inducing factor, mitochondrion-associated, 1) and DIABLO.[73] Mitophagy can therefore act to protect cells from apoptosis by removing damaged organelles that would otherwise activate a caspase-dependent cell death. Interestingly, PINK1, a protein implicated in PD, regulates the release of Ca^{2+} from the Na^{+}/K^{+} exchanger, with its loss leading to a lowered threshold for the opening of the mPTP, resulting in increased apoptosis.[74]

Animal models thus far used to help understand the permeability transition have been uninformative, highlighting the fact that in order to understand permeability transition, more selective inhibitors are required, and to meet this need zebrafish would be an ideal drug discovery tool.[75] On this basis, permeability transition in zebrafish has been characterised, and similar to the mammalian permeability transition, demonstrates Ca^{2+} dependency and

responds to the same modulators.[75] These findings therefore establish the zebrafish as a suitable model to screen inhibitors of the permeability transition that is relevant to human disease.

3.2 Oxidative stress can induce mitophagy.

Conditions of oxidative stress induce the expression of BNIP3L/NIX, due to the action of HIF1A [hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)].[76] BNIP3L then binds BCL2 and BCL2L1 at the outer mitochondrial membrane, which causes the release of BECN1, to which they were previously bound to inhibit autophagy.; the release of BECN1 then activates the autophagic machinery.[76] Although PINK1 and PARK2/PARKIN are necessary to tag mitochondria for recognition by the autophagic machinery, they do not induce autophagy itself; the latter is dependent on BNIP3L.[77] Furthermore, carbonylcyanide m-chlorophenylhydrazone (CCCP)-induced production of ROS and consequent mitophagy only occurs in the presence of BNIP3L, and PARK2, ubiquitin and SQSTM1/p62 mitochondrial translocation are also BNIP3L dependent.[77] The implications of these findings are that BNIP3L, being instrumental for CCCP-induced depolarization, can, in some circumstances, act upstream of the PINK1-PARK2 pathway.[77] *BNIP3L* is conserved in zebrafish and therefore presents a good target for knockdown studies (Table 1).

3.3 Antioxidant trials in zebrafish.

In neurodegenerative disease, ROS increase. Antioxidants have therefore been considered as potential treatments, yet zebrafish research indicates that using antioxidants as a treatment for neurodegeneration may in some instances be flawed. In zebrafish models of Huntington disease, antioxidants exacerbate the disease phenotype.[78] Although reducing ROS, some antioxidants inhibit both basal and induced autophagy, thus increasing the levels of protein

aggregates. The potential benefits of ROS reduction must therefore be balanced against the possibility of increased aggregate formation. Given the fact that the role of protein aggregates in pathology is uncertain, zebrafish may be an ideal organism in which to study this further.

4. How can zebrafish contribute to studies on the role of mitophagy in neurodegeneration?

4.1 The mechanisms of mitophagy are conserved in zebrafish.

Thus far, three models of mitophagy have been proposed; that identified in yeast,[79] mitochondrial removal during mammalian red blood cell development[80] and that mediated by the PINK1-PARK2 pathway, identified by studies of Parkinson disease. [81-85] Mitophagy is also used to regulate the number of mitochondria according to metabolic requirements and as a method of quality control.

In yeast, the different steps required for autophagy are mediated by autophagy-related (Atg) proteins. Thus far, 33 *ATG* genes have been identified as being involved in autophagy,[86] many of which are also involved in mitophagy, and 5 further genes, which are specific to mitophagy. What has become apparent is that only approximately 14 of these autophagy genes, and none of the mitophagy-specific genes, have a clear human ortholog.[87] To increase our understanding of autophagic processes and mitochondrial quality control, it will be important to either identify orthologs of the yeast proteins using novel methods, or study autophagy in higher eukaryotes such as zebrafish.

Those proteins involved in the core machinery of autophagy are conserved in mammals and most are essential for mitophagy.[88] The fact that the bulk of these proteins are common to both autophagy and mitophagy raises the question of how the autophagosome is directed selectively to mitochondria. The majority of these proteins are required for both nonselective and selective autophagy, but in some cases specific proteins are required that

allow specific cargos to be identified for sequestration into autophagosomes. For example, it has recently been shown in yeast that the protein Atg32 is required to selectively tag mitochondria for autophagy, and that this protein is not required either for other types of selective or nonselective autophagy.[88] Furthermore, Atg32 interacts with Atg8 and the scaffold protein Atg11, which recruit the mitochondria for delivery to the vacuole.[88] Atg32 expression is increased under oxidative stress, which implies it has a role in quality control; however, *ATG32* null yeast appear to have no mitochondrial defects.[89] According to the Ensembl genome browser, zebrafish possess eight homologs of *ATG8*: *gabarapa, gabarap, gabarapl2, zgc92606, map1lc3c, map1lc3a, cr847510.1 and map1lc3b* in decreasing order of homology. Unfortunately *ATG32* and *ATG11* do not have any easily identifiable homologs in higher organisms, although it is possible that proteins with similar roles do exist.

In development, removal of healthy mitochondria is necessary in certain instances. For example, mammalian red blood cells, the eye lens and mature sperm lack mitochondria due to their removal during the differentiation process. A protein identified as being required for mitophagy during development is BNIP3L, which was introduced above. This is a binding partner of the ubiquitin-like modifier proteins from the microtubule-associated protein 1 light chain 3 (MAP1LC3) and GABA receptor associated protein (GABARAP) family of *ATG8* homologs, and ablation of this interaction abolishes mitochondrial clearance in murine reticulocytes.[90-93] *MAP1LC3* (known as *map1lc3a, b and c* in zebrafish) are homologs of the yeast gene *ATG8*, and one form of this protein, MAP1LC3-II (phosphatidylethanolamine modified MAP1LC3), is used to monitor autophagy because it localizes to autophagosomal membranes.[94] GFP-MAP1LC3 is frequently used to assess autophagy and, if GFP-*map1lc3* transgenic zebrafish were to be treated with a fluorescent mitochondrial stain such as MitoTracker, could be used to investigate mitophagy in live zebrafish. Two transgenic zebrafish lines expressing GFP-tagged versions of zebrafish Map1lc3b and Gabarapa have been developed and, as expected, accumulate in lysosomes on

drug challenge.[19] This supports the use of zebrafish to monitor autophagy *in vivo*. Additionally, in zebrafish, *gabarapa* knockdown results in microcephaly,[95] whereas the equivalent knockout mouse is phenotypically normal.[96] This highlights the fact that zebrafish may be able to impart mechanistic detail unavailable in other systems.

It has been shown in zebrafish by monitoring the expression levels of Map1lc3b, that autophagosome synthesis is increased by the application of rapamycin and other autophagy inducers. Furthermore, this expression can be increased using drugs to inhibit lysosomal function, such as pepstatin A, E64d and ammonium chloride.[19] In this same study, GFP tagged Map1lc3b and Gabarapa distribution was monitored, demonstrating the validity of the technique. Through the generation of transgenic mitochondrial reporter lines, perhaps crossed to *map1lc3b* or *gabarapa* knockout lines, mitochondrial dynamics could be observed during pharmacological or genetic manipulation, to shed further light on the role of mitophagy in neurodegeneration.

4.2 Abnormal autophagy can lead to neurodegeneration.

Macroautophagy may play a protective role in neurodegenerative diseases where proteins misfold and accumulate, such as Alzheimer[97] and Huntington diseases,[24,78,98,99] amyotrophic lateral sclerosis[100] and PD.[101,102] These diseases and others have been successfully modelled in zebrafish, and therefore these models represent an excellent opportunity in which to explore whether autophagy is defective and to modulate its activity in order to determine if this results in phenotypic improvement (Table 3).

On the basis that the upregulation of autophagy might be protective, zebrafish provide an ideal high-throughput model system for candidate drug screening. The drug lithium[103] for example, is a potentially interesting autophagic upregulator. Despite its

potentially harmful off-target effects, it is an approved drug and is prescribed when alternatives are not effective. Lithium use in zebrafish has been successfully demonstrated to reduce TAU phosphorylation[104] in a TAU transgenic zebrafish model, and so is worthy of investigation as an *in vivo* modulator of autophagy.

4.3 Mutations in mitochondrial fusion and fission proteins cause neurodegeneration.

Originally thought to be static structures, it is now clear that mitochondria are actually highly dynamic organelles that can move throughout the cell, undergo repeated cycles of fusion (whereby two mitochondria combine to form a single organelle) and fission (in which long tubular mitochondria split into two or more smaller fragments) and are selectively degraded.[105] This dynamism allows mitochondrial networks to distribute mitochondria throughout the cell, respond to changes in the cellular environment and protect against mitochondrial dysfunction. Importantly, mitochondrial dynamics and mitophagy are closely related,[106,107] with a shift in the balance from fusion to fission promoting mitochondrial clearance.

Fusion is associated with cell survival and aids in the process of maintaining mitochondrial protein quality control, mtDNA integrity and the redistribution of metabolites.[1] For some years it has been known that fusion is mediated by both the outer mitochondrial membrane proteins mitofusin 1 and 2 (MFN1 and MFN2), which are dynamin-like GTPases, and the inner mitochondrial membrane protein, optic atrophy 1 (OPA1).[108] Mitochondrial depolarization promotes fragmentation due to the loss of OPA1[109] and MFN1/2.[110,111] Evidence of the importance of mitochondrial dynamics is the fact that mutations in MFN2[112] result in Charcot-Marie-Tooth disease type IIA, and OPA1 mutations cause dominant optic atrophy.[113] *mfn2* knockdown in zebrafish causes profound degeneration of motoneuron axons, but no alterations in mitochondrial morphology are observed.[67] It is

possible that incomplete knockdown is insufficient to cause mitochondrial defects, but, if so, the implication is that the neuronal death is not being caused by mitochondrial defect. Given that Charcot-Marie-Tooth disease type IIA is a dominant disorder, it may be that haploinsufficiency (which would be modelled by partial knockdown) is not the cause, and instead mutant MFN2 causes a gain of function. Further study of this model may provide understanding of the role of MFN2.

Mutations in a number of genes related to fusion and fission cause human neuropathies, and neurons with long axons such as sensory and motor neurons are particularly susceptible. Mitochondrial fission in mammals requires the large cytoplasmic GTPase DNM1L (dynamin 1-like) and FIS1 [fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)].[114] The formation of synapses demands large amounts of ATP, and, as shown in Drosophila, a reduction in mitochondrial fission due to DNM1L (known as Drp1 in Drosophila) mutation results in elongated mitochondria, with a reduction of synapse formation and synaptic dysfunction.[115]

Recently it has been shown that overexpression in neuroblastoma cells of wild-type PARK7/DJ-1, a putative sensor of oxidative stress (mutations in which cause autosomal recessive PD), results in mitochondrial elongation, whereas PD-associated mutants display increased levels of DNM1L and consequently fragmented mitochondria.[116] Similarly, studies of protein expression from Alzheimer disease brain reveal a reduction in the amount of DNM1L, OPA1, MFN1 and MFN2 with increased levels of FIS1.[117] Mimicking these expressional changes in neuronal cell culture results in decreased mitochondrial density in neuronal processes that could be rescued by DNM1L overexpression. Conversely, excessive DNM1L-mediated fission increases apoptosis due to increased cytochrome c release.[118] Zebrafish possess homologs of all five of the genes encoding these proteins and so again are an excellent system in which to study their function more closely *in vivo* (Table 1).

5. Disease-induced mitophagic defects—PINK1 and PARK2.

Recent studies have shown that the PINK1-PARK2 pathway is a governor of mitochondrial quality control. The finding that mutations in either *PINK1* or *PARK2* cause autosomal recessive PD with mitochondrial defects has highlighted the role of mitochondria in the pathogenesis of common neurological disorders, and is providing insight into the mechanisms of mitophagy. *PINK1* encodes a serine/threonine kinase that is constitutively synthesized and localized to mitochondria where it is normally cleaved and degraded.[83] PINK1 accumulates on the outer mitochondrial membrane in response to depolarization and recruits PARK2, an E3 ubiquitin ligase that under normal conditions is cytoplasmically localized. PARK2 mediates ubiquitination of itself and other targets[119] resulting in mitochondrial clustering. It has also now been shown that the receptor protein SQSTM1 is recruited to clustered mitochondria and acts as a targeting signal for mitophagy, a role requiring ubiquitination by PARK2.[85] Additionally, VDAC1 (voltage dependent anion channel 1) has been identified as a target of PARK2-mediated ubiquitination. Polyubiquitin chains on VDAC1 do not attract the proteasomal machinery, but instead attract SQSTM1 (Fig. 3a). siRNA-mediated knockdown of VDAC1 in HeLa cells results in significantly reduced mitochondrial clearance in response to CCCP treatment, demonstrating that VDAC1 is required for mitophagy.[85]

Drosophila *Pink1* mutants exhibit grossly enlarged mitochondria[120] with fragmented cristae, sensitivity to oxidative stress[121] and a slightly reduced number of dopaminergic neurons. Moreover, ATP levels are dramatically reduced indicating abnormal mitochondrial function.[121] The phenotypes of *Pink1* and *Park* (the *Drosophila PARK2* ortholog) mutants are markedly similar,[121-123] and epistasis experiments[120,124] indicate that *Pink1* acts upstream of *Park* in a common pathway that maintains mitochondrial integrity. Pink1 localizes to mitochondria, with its kinase domain facing the cytoplasm. This is required to recruit Park to damaged mitochondria to promote their clearance. Several studies have shown that another

substrate for ubiquitination by Park is Marf (the Drosophila ortholog of MFN2),[110,111] which is then removed from the mitochondrial membrane by the AAA-ATPase VCP/p97 (valosin containing protein) and degraded by the UPS (Fig. 3b).[125] In SH-SY5Y cells, the loss of MFN2 renders mitochondria unable to undergo fusion and so they are subsequently removed by mitophagy, a phenomenon that can be rescued by inactivation of DNM1L.[126] It is therefore suggested that in PD, mutant PARK2 contributes by failing to trigger the removal of dysfunctional mitochondria by both mitophagy and the UPS (Fig. 3).

The fact that *PARK2* encodes an E3 ubiquitin ligase, an enzyme responsible for tagging proteins destined for degradation by the UPS, implies that toxicity may be related to the accumulation of poorly degraded proteins. This may also explain the failure of *PARK2* mutant patients to form Lewy bodies. Assuming that Lewy bodies are protective, their absence may accelerate the disease course.

Fusing enhanced GFP with the mitochondrial localization signal of cytochrome c oxidase subunit VIII has allowed the development of a transgenic zebrafish expressing GFP-targeted mitochondria.[127] Using this line, several apoptosis-inducing agents have been tested and mitochondrial fragmentation subsequently observed using confocal imaging in real-time and *in vivo*. Should these transgenic fish be crossed with other transgenics, such as that expressing human *PARK2* (which also expresses dsRed)[128] for example, then a powerful tool for the observation of mitophagy in PD pathogenesis will have been developed. Similarly, the generation of a line containing GFP-tagged mitochondria crossed with a line containing a fluorescent lysosomal transgenic reporter[19] may prove useful for the study of mitophagy. The advantage of a fluorescent mitochondrial zebrafish line over the use of dyes is that these dyes are reliant on mitochondrial membrane potential for their accumulation and obviously require extra experimental steps. However, it is also the case that relative fluorescence levels of these stains have been used successfully as a readout of mitochondrial membrane potential[129] and so the correct tool must be chosen as required.

In zebrafish studies of PD, mitochondrial membrane potential has been tested in isolated mitochondria,[128,130] but no study of mitochondrial dynamics or mitophagy was carried out prior to their isolation, and so it is suggested that further experiments may prove informative. Gross morphological analysis has been carried out on mitochondria in some zebrafish models of PD,[128,131] but these did not show any abnormalities. It is possible that analysis in morphants is too early to observe gross morphological defects in mitochondria, but that these arise over a longer time course as a result of compromised mitochondrial function. In order to determine if this is indeed the case, stable knockout lines of both *pink1* and *park2* are required. Bandmann *et al.*[132] have reported the discovery of a *pink1* mutant by TILLING. This mutant has a premature stop codon in exon 7 (Y431X). This mutant does not display any locomotor problems, although at 5 dpf a significant reduction in the number of tyrosine hydroxylase (TH)-positive cells and a reduction in mitochondrial activity have been shown. To our knowledge mitophagy has not been investigated in this mutant.

5.1 Manipulations of *park2* in zebrafish.

The zebrafish Park2 protein shares 62% identity to the human protein, rising to 78% identity in functionally relevant regions.[131] The gene structure of zebrafish *park2* is identical to that of human *PARK2*, consisting of 12 exons. In 2009, Flinn *et al.*[131] developed a zebrafish *park2* knockdown model by MO, which produced very interesting results (Table 3). The MO resulted in a 51 amino acid deletion disrupting the in-between ring domain required for ubiquitination of some proteins. Other domains, including the ubiquitin ligase domain and two RING domains, remained intact and so the protein may have some residual enzymatic function.[131] It was shown that by 3 dpf, zebrafish *park2* morphants display a reduction in dopaminergic neurons of ~20% in the diencephalon.[131] These zebrafish also display an ~45% reduction in the activity of mitochondrial respiratory chain complex I; this corroborates well with the view that complex I function is specifically lost in human *PARK2*-related PD and

sporadic PD.[133] Treatment of *park2* knockdown zebrafish with MPP+ (1-methyl-4-phenylpyridinium) results in ~50% reduction in diencephalic dopaminergic neuronal loss.[131] Unfortunately, however, these zebrafish do not display significant locomotor impairment as measured by observation of swimming behavior at 5 dpf. As with human patients, it may be that a threshold of dopaminergic neuron loss must be reached before locomotor problems manifest. *Drosophila Park* mutants display muscle cell apoptosis, as well as swollen mitochondria and disordered cristae in energy sensitive tissues, including the male germ-line and adult flight muscle.[134] These features are not, however, found in the mitochondria of striatal neurons in mouse knockout models,[135] and similarly transmission electron microscopy of mitochondria from the fast muscles of *park2* knockdown zebrafish embryos show no such features, but do display electron dense material in the T-tubules.[131] These T-tubules are rich in L-type Ca^{2+} channels similar to that of the dopaminergic neurons of the substantia nigra pars compacta, which utilize high Ca^{2+} currents for pacemaking. Both the increased need to pump Ca^{2+} back out of the neuron, and for mitochondria to buffer excess Ca^{2+}, may cause mitochondrial stress.[131] It is possible that swimming behavior and mitochondrial morphology are not altered until later in the disease process, which due to the transient nature of MO activity cannot be tested in this model. Importantly, it may also be the case that these data point to the fact that Park2 has additional roles to those relating to mitochondrial function, or that mitochondrial dysfunction is not causative but an epiphenomenon of advanced disease. It will therefore be important to generate a stable *park2* knockout zebrafish line, which may present the first successful vertebrate model of *PARK2* mutant patients. Should this be combined with other fluorescent lines, then zebrafish will provide a way of studying autophagy and mitochondria in ways not possible in other systems.

PARK2 is protective against cellular stress and is upregulated as a consequence. To clarify whether this is also the case in zebrafish, Fett *et al.*[128] treated a zebrafish cell line

called Pac2 with rotenone. RT-PCR revealed a two-fold expression increase of *park2* mRNA. Furthermore, SH-SY5Y cells transiently expressing zebrafish Park2 are protected against kainite-induced excitotoxicity.[128] Antisense gripNA™ targeting the exon-intron junction of *park2* exon 2 was used by the same group to knock down *park2,* resulting in a 53% reduction in Park2 protein.[128] No morphological or behavioral alterations were detected and the number of dopaminergic neurons was also unaffected; considering the fact that in human, *PARK2* mutations are recessive, it is likely that a 50% reduction in PARK2 is not deleterious, and so this result is not unexpected.

To confirm if the neuroprotective capacity of Park2 demonstrated *in vitro* was evident *in vivo*, a human PARK2-overexpressing transgenic zebrafish model was created using a GAL4-VP16/UAS *PARK2*-dsRed bidirectional expression system[128] (Table 3). Under normal developmental conditions, quantification of apoptotic cells in the transgenic did not differ from that of controls, whereas *park2* knockdowns showed a slight increase in apoptotic cells.[128] A heat shock approach whereby 2 dpf embryos were incubated at 39°C for 1 h was used to determine if a proteotoxic stress might confirm a protective effect of PARK2. Apoptosis is increased in the *park2* knockdown zebrafish compared to wild-type controls, whereas in the transgenic a significant reduction in apoptosis was shown.[128] This study again exemplifies the advantage of zebrafish over mammalian models, in that it is possible to watch the cells expressing the protein of interest and, in combination with the use of vital stains, observe subcellular processes active in those particular cell types.

5.2 Manipulations of *pink1* in zebrafish.

Zebrafish *pink1* consists of 8 exons, identical to that of human, and encodes a 574 amino acid protein with 54% identity to human. Functional protein sequence prediction shows an N-terminal mitochondrial targeting sequence and a C-terminal serine-threonine kinase domain, confirming its similarity to the human protein.

To further elucidate the role of PINK1 in PD, a zebrafish MO knockdown model has been generated[130] (Table 3). In this study, the morphants showed a developmental abnormality, which leads to neurodegeneration. Anti-acetylated tubulin immunohistochemistry revealed less prominent commissures, and disorganization of the paramedial descending axonal tract in the hindbrain and spinal cord was also observed.[130] With relevance to the human PD phenotype, TH-positive dopaminergic neurons in the diencephalon are significantly reduced in number, whereas serotonergic neurons are unaffected. In the same study, *in situ* hybridization probes for *park2* and *reelin* (a general marker of neurons) demonstrate reduced expression, while that of *fezl* and *neurogenin 1* is upregulated. *Fezl* is required for the development of dopaminergic neurons and acts upstream of *neurogenin 1*. This therefore suggests an attempted compensatory action. Importantly, motor defects are observed by loss of escape response at 72 hpf. Further confirmation that *pink1* is homologous to human was provided by the observation that the injection of human wild-type *PINK1* mRNA partially rescues the MO phenotype, including reversal of the lost escape response. Injection of human *PINK1* mRNA with either the PD mutations A168P or W437X fails to rescue the MO phenotype.[130] These mRNA rescue experiments highlight the utility of the zebrafish model to validate homologous genes using simple micro-injection techniques, again offering an advantage over mammalian systems.

Interestingly, Xi *et al.*[136] were unable to replicate a significant reduction in dopaminergic, ventral diencephalic neurons in a *pink1* knockdown zebrafish model (Table 3). This model did, however, display altered patterning and projection of these neurons with a concomitant locomotor defect.

Due to the fact that *pink1* morphants show a short tail phenotype similar to that of the *wnt* mutant *pipe tail*, it was hypothesized that beta catenin levels would be depleted and that glycogen synthase kinase 3 beta (Gsk3b) would be upregulated, both consequences of *wnt* inhibition.[130] Treatment of the morphants with a nonspecific Gsk3b inhibitor, or the specific

inhibitor SB216763, rescues ~20% of morphants with the short tail phenotype and causes a rise in the levels of beta catenin, thus confirming the involvement of the *wnt* pathway. These findings may converge with the possible role of LRRK2 in WNT signalling[137] and therefore indicate a common route to the neurodegeneration characteristic of PD. LiCl treatment does not, however, improve the loss of dopaminergic neurons in the morphant brain. This suggests that Gsk3b may play a role in the peripheral phenotypes observed, but that other factors are involved in the dopamine neuron loss. Further study of Wnt signalling, the known *pink1* mutant[132] and generation of another *pink1* knockout model is therefore recommended.

The observed rise in Gsk3b levels also appears to play a role in the increased level of apoptosis present throughout the morphants, as shown by acridine orange staining. Again, LiCl treatment reduces the activity of Casp3, an activator of the effector caspases of the apoptotic cascade. Mitochondria isolated from morphants at 24 hpf incubated with 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a dye used to detect mitochondrial membrane potential, showed a reduction in membrane potential and an increase in ROS. LiCl treatment did not rescue the loss of membrane potential, but did reduce the ROS levels.[130] These data collectively suggest the possibility that Gsk3b inhibitors and antioxidants may present as possible therapeutic agents that could be successfully tested in zebrafish models.

The PINK1-PARK2 pathway has also been linked to mitochondrial dynamics, a factor of particular importance in neurons. In rat neurons, mitochondrial depolarization results in PINK1-PARK2 interaction with a Rho-GTPase called RHOT1 (alias Miro1), a protein that anchors kinesins to mitochondria.[138] PINK1 first phosphorylates RHOT1, which is then tagged with ubiquitin by PARK2 prior to its degradation. The loss of RHOT1 prevents the movement of mitochondria, thus segregating them for removal.[139] Whether or not RHOT1 is essential for mitophagy has yet to be elucidated. To take these experiments further in order to try and link the PINK1-PARK2 pathway to mitochondrial dynamics and

mitophagy, live imaging of fluorescently-tagged mitochondria and, for example, their trafficking along microtubules using a stain such as Tubulin Tracker Green, could be performed.

6. Defects of the respiratory chain produce 'Parkinsonism' and mitochondrial fragmentation.

The mitochondrial respiratory chain comprises five protein complexes, and defects of this pathway can potentially cause disease.[140] Mitochondria have been associated with neurodegenerative disease for some time, the research of which has focussed on the role of complex I in PD. The inhibitor of complex I, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), induces Parkinsonian-like clinical and neuropathological signs in humans and nonhuman primates.[141] MPTP is converted to MPP+ by the enzyme MAOB (monoamine oxidase B). It is this metabolite that is taken up by SLC6A3/DAT [solute carrier family 6 (neurotransmitter transporter, dopamine), member 5/dopamine active transporter] and is neurotoxic. Incubation of larval zebrafish in MPTP or application of MPP+ directly, induces progressive dopamine neuron loss in the posterior tuberculum, and a paucity of reflex response to touch with reduced escape velocity and swimming distance[142] (Table 3). This toxicity is mediated by the same mechanisms as in humans, as shown by the observation that L-deprenyl (an inhibitor of MAOB), the SLC6A3 inhibitor nomifensine, and MO knockdown of the SLC6A3 all protect neurons from MPTP damage.[143] It has also been shown that rotenone, another complex I inhibitor, produces dopaminergic cell loss, and complex I deficiency is reduced in cells derived from PD patients.[144] Complex I inhibition is also associated with mitochondrial fragmentation that can be rescued in SH-SY5Y cells either by inhibition of DNM1L or overexpression of MFN1.[145]

An important consequence of complex I inhibition is the proportional increase in production of ROS[146] due to restricted electron transfer. Interestingly, zebrafish are often

incubated in methylene blue, a drug used to inhibit fungal growth during development. Methylene blue is an alternative electron carrier that can bypass complex I blockade; in rats it has been shown to attenuate rotenone-induced mitochondrial dysfunction.[147] If zebrafish mitochondrial research is being conducted it may be that methylene blue use is best eliminated to avoid confounding results.

Methylene blue has been tested on a zebrafish transgenic model of *TAU*-P301L, a mutation causative of frontotemporal dementia with Parkinsonism[148] (Table 3). However, neither abnormal TAU phosphorylation nor misfolding of TAU is significantly different to that of the non-drug treated group. In this same study, methylene blue failed to rescue neuronal cell death, improve swimming behavior or rescue the axonal outgrowth of motoneurons. The implications of these findings are that methylene blue does not inhibit aberrant kinase activity and that the abnormal phosphorylation of TAU is likely to cause its misfolding. Although in a phase 2 clinical trial in Alzheimer patients[148] a significant improvement in cognition was observed, it seems the mechanism of action is unlikely to be through the inhibition of the proposed TAU aggregation. It may be the case that the drug acts through its effect on mitochondria or that it is a regulator of autophagy; both of these possibilities could be explored further in zebrafish by, for example, assessing the levels of transcripts from genes involved in autophagy, or by following the process with vital stains.

Given these negative results it is possible that methylene blue (or any other compound with a negative result) may not be well absorbed by the zebrafish. This would appear not to be the case with methylene blue due to the fact that in a further study, zebrafish expressing mutant human TARDBP and FUS, causative of some types of amyotrophic lateral sclerosis, methylene blue was shown to be beneficial[149] (Table 3). In this study, motor activity was partially rescued, as was the abnormal motoneuron morphology. In addition, it was shown that methylene blue was protective of oxidative stress, and so highlights the use

of zebrafish to test disease-modifying compounds and illustrates the promise of methylene blue as a neuroprotective agent in specific cases.

7. Conclusion.

Mitophagy has now been established as a mechanism to regulate mitochondrial health, and mitochondrial dysfunction is involved in a number of neurodegenerative diseases. The molecular machinery of autophagy and mitophagy is becoming better understood, largely through yeast studies, but there remains a need to identify more of the proteins involved and to understand the process more fully in higher organisms. Zebrafish are a well-established tool of the developmental biology field, but their potential has yet to be fully realized as a human disease model. Although the use of MOs to knock down genes of interest is a very powerful tool, fundamental to the success of zebrafish models will be the development of more transgenic lines, particularly human disease gene knockins. It is hoped that through these models, a greater understanding of the underlying pathological processes, such as that of altered mitochondrial dynamics, can be elucidated. Additionally, zebrafish are an excellent organism in which to validate potential modulators of mitophagy *in vivo,* and bring the use of new therapeutic compounds closer to the bedside faster and more cheaply than has previously been possible.

References

1. Santos D, Cardoso SM. Mitochondrial dynamics and neuronal fate in Parkinson's disease. Mitochondrion 2012; 12:428–37

2. Murphy MP, Holmgren A, Larsson N-G, Halliwell B, Chang CJ, Kalyanaraman B, et al. Unraveling the biological roles of reactive oxygen species. Cell Metab 2011; 13:361–6

3. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417:1-

13

4. Langer T, Käser M, Klanner C, Leonhard K. AAA proteases of mitochondria: quality control of membrane proteins and regulatory functions during mitochondrial biogenesis. Biochem Soc Trans 2001; 29:431–6

5. Ashrafi G, Schwarz TL. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ 2012; 20:31-42

6. Clark SL. Cellular differentiation in the kidneys of newborn mice studies with the electron microscope. J Biophys Biochem Cytol 1957; 3:349–62

7. Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol 1992; 119:301–11

8. Tanida I. Autophagy basics. Microbiol and Immunol 2010; 55:1–11

9. Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol 2011; 12:9–14

10. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy 2011; 7:279–96

11. Ravikumar B, Sarkar S, Davies JE, Futter M, García-Arencíbia M, Green-Thompson ZW, et al. Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev 2010; 90:1383–435

12. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MMK, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004; 304:1158–160

13. Kabashi E, Brustein E, Champagne N, Drapeau P. Zebrafish models for the functional genomics of neurogenetic disorders. Biochim Biophys Acta 2011; 1812:335–45

14. Kimmel CB. Patterning the brain of the zebrafish embryo. Annu Rev Neurosci 1993; 16:707–32

15. Kimmel C, Ballard W, Kimmel S. Stages of embryonic development of the zebrafish.

Am J Dev Dyn1995; 203:253–310

16. Saint-Amant L, Drapeau P. Time course of the development of motor behaviors in the zebrafish embryo. J Neurobiol 1998; 37:622–32

17. Granato M, van Eeden FJ, Schach U, Trowe T, Brand M, Furutani-Seiki M, et al. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 1996; 123:399–413

18. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ 2005; 12:1542–552

19. He C, Bartholomew CR, Zhou W, Klionsky DJ. Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos. Autophagy 2009; 5:520–6

20. He C, Klionsky DJ. Analyzing autophagy in zebrafish. Autophagy 2010; 6:642-4

21. Bai Q, Garver J, Hukriede N, Burton E. Generation of a transgenic zebrafish model of Tauopathy using a novel promoter element derived from the zebrafish *eno2* gene. Nucleic Acids Res 2007; 35:6501–16

22. Tomasiewicz H, Flaherty D, Soria J, Wood J. Transgenic zebrafish model of neurodegeneration. J Neurosci Res 2002; 70:734–45

23. Ramesh T, Lyon AN, Pineda RH, Wang C, Janssen PML, Canan BD, et al. A genetic model of amyotrophic lateral sclerosis in zebrafish displays phenotypic hallmarks of motoneuron disease. Dis model mech 2010; 3:652–62

24. Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol 2008; 4:295–305

25. Plucinska G, Paquet D, Hruscha A, Godinho L, Haass C, Schmid B, et al. In vivo imaging of disease-related mitochondrial dynamics in a vertebrate model system. J Neurosci 2012; 32:16203–212

26. Halpern ME, Rhee J, Goll MG, Akitake CM, Parsons M, Leach SD. Gal4/UAS

transgenic tools and their application to zebrafish. Zebrafish 2008; 5:97–110

27. Grupp L, Wolburg H, Mack AF. Astroglial structures in the zebrafish brain. J Comp Neurol 2010; 518:4277–87

28. Yoshida M, Macklin WB. Oligodendrocyte development and myelination in GFP-transgenic zebrafish. J Neurosci Res 2005; 81:1–8

29. Svahn AJ, Graeber MB, Ellett F, Lieschke GJ, Rinkwitz S, Bennett MR, et al. Development of ramified microglia from early macrophages in the zebrafish optic tectum. Dev Neurobiol 2012; 73:60-71

30. Cuoghi B, Mola L. Microglia of teleosts: facing a challenge in neurobiology. Eur J Histochem 2009; 51:231–40

31. Jeong J-Y, Jeong J-Y, Kwon H-B, Kwon H-B, Ahn J-C, Ahn J-C, et al. Functional and developmental analysis of the blood–brain barrier in zebrafish. Brain Res Bull 2008; 75:619–628

32. Miller G. Breaking down barriers. Science 2002; 297:1116–18

33. Sager JJ, Bai Q, Burton EA. Transgenic zebrafish models of neurodegenerative diseases. Brain Struct Funct 2010; 214:285–302

34. Best JD, Alderton WK. Zebrafish: An in vivo model for the study of neurological diseases. Neuropsychiatr Dis Treat 2008; 4:567–76

35. Holzschuh J, Ryu S, Aberger F, Driever W. Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. Mech Dev 2001; 101:237–43

36. Rink E, Wullimann M. The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum)q. Brain Res 2001; 889:316–30

37. Kastenhuber E, Kratochwil CF, Ryu S, Schweitzer J, Driever W. Genetic dissection of dopaminergic and noradrenergic contributions to catecholaminergic tracts in early larval

zebrafish. J Comp Neurol 2010; 518:439–58

38. Williams CH, Hong CC. Multi-step usage of in vivo models during rational drug design and discovery. Int J Mol Sci 2011; 12:2262–74

39. Kaufman CK, White RM, Zon L. Chemical genetic screening in the zebrafish embryo. Nat Protoc 2009; 4:1422–32

40. Zhao Z, Cao Y, Li M, Meng A. Double-stranded RNA injection produces nonspecific defects in zebrafish. Dev Biol 2001; 229:215–23

41. Dong M, Fu Y-F, Du T-T, Jing C-B, Fu C-T, Chen Y, et al. Heritable and lineage-specific gene knockdown in zebrafish embryo. PLoS One 2009; 4:e6125

42. De Rienzo G, Gutzman JH, Sive H. Efficient shRNA-mediated inhibition of gene expression in zebrafish. Zebrafish 2012; 9:97–107

43. Urtishak KA, Choob M, Tian X, Sternheim N, Talbot WS, Wickstrom E, et al. Targeted gene knockdown in zebrafish using negatively charged peptide nucleic acid mimics. Dev Dyn 2003; 228:405–13

44. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. Zebrafish 2009; 6:69–77

45. Draper BW, Morcos PA, Kimmel CB. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis 2001; 30:154–6

46. Bennett JT, Stickney HL, Choi W-Y, Ciruna B, Talbot WS, Schier AF. Maternal nodal and zebrafish embryogenesis. Nature 2007; 450:E1–2, discussion E2–4

47. Eisen JS, Smith JC. Controlling morpholino experiments: don't stop making antisense. Development 2008; 135:1735–43

48. Yang Z, Liu N, Lin S. A zebrafish forebrain-specific zinc finger gene can induce ectopic dlx2 and dlx6 expression. Dev Biol 2001; 231:138–48

49. Nasevicius A, Ekker SC. Effective targeted gene ‘knockdown’ in zebrafish. Nat Genet 2000; 26:216–20

50. Lele Z, Bakkers J, Hammerschmidt M. Morpholino phenocopies of the swirl, snailhouse, somitabun, minifin, silberblick, and pipetail mutations. Genesis 2001; 30:190–4

51. Imai Y, Talbot WS. Morpholino phenocopies of the bmp2b/swirl and bmp7/snailhouse mutations. Genesis 2001; 30:160–3

52. Villalobos A, Ness J, Gustafsson C, Minshull J, Govindranjan S. Gene Designer: a synthetic biology tool for constructing artificial DNA segments. BMC Bioinformatics 2006; 7:285

53. Shestopalov IA, Sinha S, Chen JK. Light-controlled gene silencing in zebrafish embryos. Nat Chem Biol 2007; 3:650–1

54. Tomasini AJ, Schuler AD, Zebala JA, Mayer AN. PhotoMorphs™: A novel light activated reagent for controlling gene expression in zebrafish. Genesis 2009; 47:736–43

55. Ekker SC. Zinc finger-based knockout punches for zebrafish genes. Zebrafish 2008; 5:121–3

56. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol 2008; 26:695–701

57. Urnov FD, Miller JC, Lee Y-L, Beausejour CM, Rock JM, Augustus S, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 2005; 435:646–51

58. Doyon Y, Doyon Y, McCammon JM, McCammon JM, Miller JC, Miller JC, et al. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol 2008; 26:702–8

59. Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ. Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 2010; 29:64–7

60. Boch J, Bonas U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol 2010; 48:419–36

61. Sander J, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, et al. Targeted gene

disruption in somatic zebrafish cells using engineered TALENs. Nature 2011; 29:697-8

62. Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG II, et al. In vivo genome editing using a high-efficiency TALEN system. Nature 2012; 1–7

63. DeFrancesco L. Move over ZFNs. Nat. Biotechnol. 2011; 29:681–4

64. Zhou Y, Zon LI. The Zon Llaboratory guide to positional cloning in zebrafish. Methods Cell Biol 2011; 104:287–309

65. Voz ML, Coppieters W, Manfroid I, Baudhuin A, Berg Von V, Charlier C, et al. Fast homozygosity mapping and identification of a zebrafish ENU-induced mutation by whole-genome sequencing. PLoS One 2012; 7:e34671

66. Obholzer N, Swinburne IA, Schwab E, Nechiporuk AV, Nicolson T, Megason SG. Rapid positional cloning of zebrafish mutations by linkage and homozygosity mapping using whole-genome sequencing. Development 2012; 139:4280–90

67. Vettori A, Bergamin G, Moro E, Vazza G, Polo G, Tiso N, et al. Developmental defects and neuromuscular alterations due to mitofusin 2 gene (MFN2) silencing in zebrafish: a new model for Charcot-Marie-Tooth type 2A neuropathy. Neuromuscular Disord 2011; 21:58–67

68. Moens CB, Donn TM, Wolf-Saxon ER, Ma TP. Reverse genetics in zebrafish by TILLING. Brief Funct Genomic Proteomic 2008; 7:454–9

69. Stemple DL. TILLING—a high-throughput harvest for functional genomics. Nat Rev Genet 2004; 5:145–50

70. Acevedo-Arozena A, Wells S, Potter P, Kelly M, Cox RD, Brown SDM. ENU mutagenesis, a way forward to understand gene function. Annu Rev Genomics Hum Genet 2008; 9:49–69

71. Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 2007; 17:422–7

72. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, et al. The mitochondrial permeability transition in cell death: a common mechanism in necrosis,

apoptosis and autophagy. Biochim Biophys Acta 1998; 1366:177–96

73. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 2007; 462:245–53

74. Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, et al. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Mol Cell 2009; 33:627–38

75. Azzolin L, Basso E, Argenton F, Bernardi P. Mitochondrial Ca2+ transport and permeability transition in zebrafish (Danio rerio). Biochim Biophys Acta 2010; 1797:1775–79

76. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 2009; 29:2570–81

77. Ding WX, Ni HM, Li M, Liao Y, Chen X, Stolz DB, et al. Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and parkin-ubiquitin-p62-mediated mitochondrial priming. J Biol Chem 2010; 285:27879–890

78. Underwood BR, Imarisio S, Fleming A, Rose C, Krishna G, Heard P, et al. Antioxidants can inhibit basal autophagy and enhance neurodegeneration in models of polyglutamine disease. Hum Mol Genet 2010; 19:3413–29

79. Tal R, Winter G, Ecker N, Klionsky DJ, Abeliovich H. Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. J Biol Chem 2007; 282:5617–24

80. Aerbajinai W. The proapoptotic factor Nix is coexpressed with Bcl-xL during terminal erythroid differentiation. Blood 2003; 102:712–17

81. Narendra D, Tanaka A, Suen D-F, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008; 183:795–803

82. Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RLA, Kim J, et al. PINK1-dependent

recruitment of Parkin to mitochondria in mitophagy. Proc Natl Acad Sci USA 2010; 107:378–83

83. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 2010; 8:e1000298

84. Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol 2010; 189:211–21

85. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 2010; 12:119–31

86. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle 2007; 6:1837–49

87. Deas E, Wood NW, Plun-Favreau H. Mitophagy and Parkinson's disease: the PINK1-parkin link. Biochim Biophys Acta 2011; 1813:623–33

88. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev Cell 2009; 17:98–109

89. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev Cell 2009; 17:87–97

90. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci USA 2007; 104:19500–05

91. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, et al. Essential role for Nix in autophagic maturation of erythroid cells. Nature 2008; 454:232–5

92. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, et al. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep 2009; 11:45–51

93. Kanki T, Klionsky DJ. The molecular mechanism of mitochondria autophagy in yeast.

Mol Microbiol 2010; 75:795–800

94. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012; 8:445–544

95. Komoike Y, Shimojima K, Liang J-S, Fujii H, Maegaki Y, Osawa M, et al. A functional analysis of GABARAP on 17p13.1 by knockdown zebrafish. J Hum Genet 2010; 55:155–62

96. O'Sullivan GA, Kneussel M, Elazar Z, Betz H. GABARAP is not essential for $GABA_A$ receptor targeting to the synapse. Eur J Neurosci 2005; 22:2644–8

97. Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci 2008; 28:6926–37

98. Martinez-Vicente M, Tallóczy Z, Wong E, Tang G, Koga H, Kaushik S, et al. Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. Nat Neurosci 2010; 13:567–76

99. Sarkar S, Korolchuk VI, Renna M, Imarisio S, Fleming A, Williams A, et al. Complex inhibitory effects of nitric oxide on autophagy. Mol Cell 2011; 43:19–32

100. Maday S, Wallace KE, Holzbaur ELF. Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. J Cell Biol 2012; 196:407–17

101. Ebrahimi-Fakhari D, Wahlster L, McLean PJ. Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol 2012; 124:153–72

102. Friedman LG, Lachenmayer ML, Wang J, He L, Poulose SM, Komatsu M, et al. Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of α-synuclein and LRRK2 in the brain. J Neurosci 2012; 32:7585–93

103. García-Arencíbia M, Hochfeld WE, Toh PPC, Rubinsztein DC. Autophagy, a guardian against neurodegeneration. Sem Cell Dev Biol 2010; 21:691–8

104. Paquet D, Bhat R, Sydow A, Mandelkow E-M, Berg S, Hellberg S, et al. A zebrafish model of tauopathy allows in vivo imaging of neuronal cell death and drug evaluation. J Clin Invest 2009; 119:1382–95

105. Knott AB, Perkins G, Schwarzenbacher R, Bossy-Wetzel E. Mitochondrial fragmentation in neurodegeneration. Nat Rev Neurosci 2008; 9:505–18

106. Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 2008; 27:433–46

107. Kanki T, Klionsky DJ. Mitophagy in yeast occurs through a selective mechanism. J Biol Chem 2008; 283:32386–93

108. Cerveny KL, Tamura Y, Zhang Z, Jensen RE, Sesaki H. Regulation of mitochondrial fusion and division. Trends Cell Biol 2007; 17:563–69

109. Ishihara N, Fujita Y, Oka T, Mihara K. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J 2006; 25:2966–77

110. Ziviani E, Tao RN, Whitworth AJ. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. Proc Natl Acad Sci USA 2010; 107:5018–23

111. Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One 2010; 5:e10054

112. Züchner S, Mersiyanova IV, Muglia M, Bissar-Tadmouri N, Rochelle J, Dadali EL, et al. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat Genet 2004; 36:449–51

113. Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, et al. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat Genet 2000; 26:207–10

114. Smirnova E, Griparic L, Shurland DL, van der Bliek AM. Dynamin-related protein

Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell 2001; 12:2245–56

115. Verstreken P, Ly CV, Venken KJT, Koh T-W, Zhou Y, Bellen HJ. Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. Neuron 2005; 47:365–78

116. Wang X, Petrie TG, Liu Y, Liu J, Fujioka H, Zhu X. Parkinson's disease-associated DJ-1 mutations impair mitochondrial dynamics and cause mitochondrial dysfunction. J Neurochem 2012; 121:830–39

117. Wang X, Su B, Lee H-G, Li X, Perry G, Smith MA, et al. Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci 2009; 29:9090–103

118. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, et al. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 2001; 1:515–25

119. Kahle PJ, Haass C. How does parkin ligate ubiquitin to Parkinson's disease? EMBO Rep 2004; 5:681–85

120. Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature 2006; 441:1157–61

121. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 2006; 441:1162–66

122. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci USA 2003; 100:4078–83

123. Whitworth AJ, Theodore DA, Greene JC, Benes H, Wes PD, Pallanck LJ. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a Drosophila model of Parkinson's disease. Proc Natl Acad Sci USA 2005; 102:8024–29

124. Yang Y, Gehrke S, Imai Y, Huang Z, Ouyang Y, Wang J-W, et al. Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci USA 2006; 103:10793–98

125. Tanaka A, Cleland MM, Xu S, Narendra DP, Suen D-F, Karbowski M, et al. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol 2010; 191:1367–80

126. Lutz AK, Exner N, Fett ME, Schlehe JS, Kloos K, Lämmermann K, et al. Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. J Biol Chem 2009; 284:22938–51

127. Jung Kim M, Ho Kang K, Kim C-H, Choi S-Y. Real-time imaging of mitochondria in transgenic zebrafish expressing mitochondrially targeted GFP. Biotech. 2008; 45:331–4

128. Fett ME, Pilsl A, Paquet D, van Bebber F, Haass C, Tatzelt J, et al. Parkin is protective against proteotoxic stress in a transgenic zebrafish model. PLoS One 2010; 5:e11783

129. Pendergrass W, Wolf N, Poot M. Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. Cytometry 2004; 61:162–9

130. Anichtchik O, Diekmann H, Fleming A, Roach A, Goldsmith P, Rubinsztein DC. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. J Neurosci 2008; 28:8199–207

131. Flinn L, Mortiboys H, Volkmann K, Köster RW, Ingham PW, Bandmann O. Complex I deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (*Danio rerio*). Brain 2009; 132:1613–23

132. Bandmann O, Flinn L, Mortiboys H. POMD08 Zebrafish models for early onset Parkinson's disease. J Neurol Neurosurg Psychiatry 2010; 81:e59–e59

133. Xi Y, Noble S, Ekker M. Modeling neurodegeneration in zebrafish. Curr Neurol Neurosci Rep 2011; 11:274–82

134. Dodson MW, Guo M. Pink1, Parkin, DJ-1 and mitochondrial dysfunction in Parkinson's disease. Curr Opin Neurobiol 2007; 17:331–7

135. Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 2004; 279:18614–22

136. Xi Y, Ryan J, Noble S, Yu M, Yilbas AE, Ekker M. Impaired dopaminergic neuron development and locomotor function in zebrafish with loss of pink1 function. Eur J Neurosci 2010; 31:623–33

137. Berwick DC, Harvey K. LRRK2 functions as a Wnt signaling scaffold, bridging cytosolic proteins and membrane-localized LRP6. Hum Mol Genet 2012; 21:4966-79

138. Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 2011; 147:893–906

139. Liu S, Sawada T, Lee S, Yu W, Silverio G, Alapatt P, et al. Parkinson's disease-associated kinase PINK1 regulates Miro protein level and axonal transport of mitochondria. PLoS Genet 2012; 8:e1002537

140. Perier C, Vila M. Mitochondrial biology and Parkinson's disease. Cold Spring Harb Perspect Med 2012; 2:a009332

141. Vila M, Przedborski S. Genetic clues to the pathogenesis of Parkinson's disease. Nat Med 2004; 10:S58–62

142. Lam CS, Korzh V, Strahle U. Zebrafish embryos are susceptible to the dopaminergic neurotoxin MPTP. Eur J Neurosci 2005; 21:1758–62

143. McKinley ET, Baranowski TC, Blavo DO, Cato C, Doan TN, Rubinstein AL. Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. Brain Res Mol Brain Res 2005; 141:128–37

144. Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial

complex I deficiency in Parkinson's disease. J Neurochem 1990; 54:823–7

145. Gomez-Lazaro M, Bonekamp NA, Galindo MF, Jordán J, Schrader M. 6-Hydroxydopamine (6-OHDA) induces Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells. Free Radic Biol and Med 2008; 44:1960–9

146. Hasegawa E, Takeshige K, Oishi T, Murai Y, Minakami S. 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem Biophys Res Commun 1990; 170:1049–55

147. Wen Y, Li W, Poteet EC, Xie L, Tan C, Yan L-J, et al. Alternative mitochondrial electron transfer as a novel strategy for neuroprotection. J Biol Chem 2011; 286:16504–15

148. van Bebber F, Paquet D, Hruscha A, Schmid B, Haass C. Methylene blue fails to inhibit Tau and polyglutamine protein dependent toxicity in zebrafish. Neurobiol Dis 2010; 39:265–71

149. Vaccaro A, Patten SA, Ciura S, Maios C, Therrien M, Drapeau P, et al. Methylene blue protects against TDP-43 and FUS neuronal toxicity in *C. elegans* and *D. rerio*. PLoS One 2012; 7:e42117

Figure 1. Alignment of human PINK1 protein with zebrafish Pink1 protein. It can be seen that amino acid conservation is higher in the kinase domain (higher density of black bars) containing important interaction sites (purple arrows). Secondary structure prediction also shows high similarity of motifs. PKc – protein kinase domain. It should be noted that the conservation of amino acid residues is higher in functional domains as compared to the total protein. For example, MUSCLE alignment of the human PINK1 protein against zebrafish

Pink1 produces an identity of 54%; however, aligning just the kinase domain results in an alignment of 71%.

Figure 2. Schematic of the zebrafish dopaminergic system in 3 dpf old larvae. ac, anterior commissure; act, anterior catecholamine tract; AP, area postrema; CH, caudal hypothalamus; DC, diencephalic cluster; eht, endohypothalimc tract; hhp, hypothalamic-hypophyseal projections; lcp, lateral catecholaminergic projections; mlct, medial longitudinal catecholaminergic tract; MO, medulla oblongata; OB, olfactory bulb; obla, olfactory bulb local arbors; pc, posterior commissure; PO, preoptic region; poht, posterior hypothalamic tract; Pr, pretectum; prp, pretectal projections; Prtep, pretectal tectoprojections; nos. 1-6 ventral diencephalic cluster. Adapted from Kastenhuber *et al.*[37]

Figure 3. The PINK1-PARK2 pathway to mitophagy. (**A**) PARK2 ubiquitinates VDAC1, which attracts SQSTM1, a targeting signal for mitophagy. (**B**) Upon mitochondrial depolarization, PINK1 accumulates on the outer mitochondrial membrane to recruit PARK2. PARK2 ubiquitinates MFN1/2, which are removed by VCP and degraded by the UPS. Loss of MFN1/2 inhibits mitochondrial fusion, so that mitochondria are removed by mitophagy.

Table 1. Zebrafish orthologs of genes involved in mitophagy.

Human protein	Function	Action	Zebrafish orthologous gene	Protein identity (%)

				(ClustalW)
BCL2	Pro-survival apoptosis regulator	Inhibits autophagy	*bcl-2*	46.0
BCL2L1	Pro-survival apoptosis regulator	Inhibits autophagy; regulates VDAC	*bcl2l-1*	51.7
BNIP3L	Putative mitophagy receptor	Interaction with MAP1LC3/GABARAP; recruits autophagic machinery	*bnip3la* *bnip3*	46.4 69.5
DNM1L	Outer membrane fission	Regulates mitochondrial fission	*dnml-1*	*90.0*
FIS1	Outer membrane fission	Regulates mitochondrial fission	*fis1*	67.8
MFN 1 MFN2	Outer membrane fusion	Ubiquitinated by PARK2; MFN1/2 Ub-dependent degradation precedes mitophagy induction	*mfn1* *mfn2*	68.4 83.0
SMCR7L	Outer membrane fusion	Recruits DNM1L to outer membrane; promotes fusion	*smcr7a*	69.9
OPA1	Inner membrane fusion	Regulates mitochondrial fusion	*opa1*	82.6

PARK2	E3 ubiquitin ligase	Ubiquitinates MFN1/2, VDAC1	*park2*	62.0
PINK1	Serine/threonine protein kinase	Phosphorylates PARK2 and recruits it to mitochondria	*pink1*	53.7
SQSTM1	Receptor protein	Interacts with ubiquitinated proteins to recruit autophagic machinery	*sqstm1*	35.9
VDAC1	Voltage dependent anion channel, outer mitochondrial membrane	Ubiquitination by PARK2 induces recruitment of autophagic machinery	*vdac1*	85.5

Table 2. Useful resources for sourcing mutant zebrafish lines and understanding anatomy and gene expression.

Resource	Web link	Information available
Zebrafish Information Resource Centre	http://zebrafish.org/zirc/fish/lineAll.php	Genotype/phenotype information and line availability.
Zebrafish Mutation Project	http://www.sanger.ac.uk/Projects/D_rerio/zmp/	Information on all knockout lines. Possible to search by human ortholog.
Tübingen zebrafish stock collection	http://www.eb.tuebingen.mpg.de/research/departments/genetics/zebrafish-stock-collection.html	Information on all available mutant lines available. Will also perform IVF on request.
FHCRC Zebrafish TILLING Project	http://www.zfishtilling.org/fhcrc/	Information on all mutants so far found. Request for genes to be screened by TILLING can be submitted.
ZF-Models	http://www.sanger.ac.uk/Projects/D_rerio/zf-models.shtml	List of all knockout lines and request genes to be screened by TILLING.
Zebrafish Brain Atlas	http://www.ucl.ac.uk/zebrafish-group/zebrafishbrain/index.php	Multimedia anatomical resource. Further links are provided to other useful resources including imaging

		protocols.
ZFIN	http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg	Zebrafish model organism database. Comprehensive information available on all aspects of zebrafish research.

Table 3. Overview of the key findings attained from zebrafish models of neurodegenerative disease.

Disease	Type of model	Protein	Outcome summary	Reference
Tauopathy	Transgenic	4R TAU-GFP fusion	Neuronal expression. TAU accumulations, tangles. TAU phosphorylation. No stable line.	Tomasiewicz *et al.* 2002
		4R/0N TAU	Neuronal expression. TAU accumulations, tangles.	Bai *et al.* 2007
FTDP-17	Transgenic	TAU P301L	Motor axonal outgrowth delay. Loss of escape response. Apoptosis in spinal cord. TAU phosphorylation. TAU aggregation.	Paquet *et al.* 2009
			Methylene blue treatment of this	vanBebber *et al.* 2010

			model failed to reduce any of the phenotypes.	
Alzheimer	Morphant	Appa/b	Defective conversion-extension movements. Not rescued by APPswe.	Joshi *et al.* 2009
Alzheimer	Transgenic	Amyloid beta 42	Melanophore expression resulted in aberrant phenotype at 16 months.	Newman *et al.* 2010
Huntington	Transient over-expression	HTT Q102-GFP	Protein aggregation. Apoptotic cells lacked inclusions. Increased embryonic lethality. Hsp40 and Hsp70 suppressed polyQ aggregation and toxicity. Two	Schiffer *et al.* 2007

			compounds of the N-benzylidene-benzohydrazide class inhibited aggregation.	
	Transient over-expression	HTT Q102-GFP	Methylene blue treatment reduced aggregates but failed to protect against toxicity.	vanBebber *et al.* 2010
	Transgenic	HTT Q71-GFP	Mutant huntingtin expressed in rod photoreceptors. Forms aggregates and reduced rhodopsin expression. Several compounds tested that reduce aggregates and increase rhodopsin expression.	Williams *et al.* 2008

	Transgenic	HTT Q71-GFP	Nitric oxide, an inhibitor of autophagy was inhibition by L-NAME successfully reducing huntingtin aggregates.	Sarkar *et al.* 2011
	Morphant	Htt	Variety of developmental defects. Most notably hypochromic blood. Suggested role of Htt in utilization of endocytosed iron.	Lumsden *et al.* 2007
	Transgenic	HTT Q71-GFP	Antioxidants increased the number of mutant huntingtin aggregates.	Underwood *et al.* 2010

ALS	Transient over-expression	SOD1	No phenotype.	Lemmens *et al.* 2007
	Transient over-expression	SOD1 A4V SOD1G93A SOD1G37R	Abnormal length and branching of motor neurons. Rescued by upregulation of Vegf and exacerbated by MO of Vegf.	Lemmens *et al.* 2007
	Transgenic	Sod1 G93R	Larval NMJ defect, late stage muscle atrophy, decreased endurance, paralysis and premature death.	Ramesh *et al.* 2010
	Transgenic	Sod1	No phenotype	Ramesh *et al.* 2010
ALS	Transient over-expression	TARDBP G348C TARDBP A315T	Abnormal length and branching of motor neurons.	Kabashi *et al.* 2010

			Motor deficit.	
	Transient over-expression	TARDBP A382T	Abnormal motor axon length. Motor deficit.	Kabashi *et al.* 2010
	Morphant	Tardbp	Abnormal length and branching of motor neurons. Motor deficit. Rescued by WT TARDPB but not mutant forms.	Kabashi *et al.* 2010
	Transgenic	TARDBP G348C	Motor deficit. Abnormally shortened and branched motor neuron axonal processes. Rescued by methylene blue.	Vaccaro *et al.* 2012
	Transgenic	FUS R521H	Motor deficit. Abnormally shortened and	Vaccaro *et al.* 2012

			branched motor neuron axonal processes. Rescued by methylene blue.	
ALS	Morphant	Alsin	Swimming deficits, motor neuron perturbation.	Gros-Louis *et al.* 2008
Spinal Muscular Atrophy	Morphant TILLING mutant	Smn1 Smn1	Truncated spinal motor neurons with increased axonal branching. Reduction in levels of synaptic vesicle protein SV2 at the NMJ. Rescued by human SMN1.	McWhorter *et al.* 2003 Boon *et al.* 2009
Hereditary Spastic Paraplegia	Morphant	Spastin	Much reduced motor axonal projections. Aberrant migration of some motor neuron populations.	Wood *et al.* 2006

			Apoptosis of spinal motor neurons. Impaired swimming. Disordered microtubules.	
Parkinson Disease	Morphant	Park2	Complex I activity reduced. Dopaminergic cell loss. Increased sensitivity to MPP^{+}. Abnormal T-tubules. No locomotor defect.	Flinn *et al.* 2009
	Antisense GT-gripNA™	Park2	50% knockdown. No developmental phenotype. Increased susceptibility to cellular stress.	Fett *et al.* 2010
	Transgenic	PARK2	Increased resistance to cellular stress.	Fett *et al.* 2010

	Morphant	Pink1	Decreased numbers of dopaminergic neurons. Altered mitochondrial function. Elevation of Gsk3b activity. Loss of escape response.	Anichtchik *et al.* 2008
	Morphant	Pink1	Reduction in Th expression. Increased sensitivity to MPTP. Locomotor deficit.	Sallinen *et al.* 2010
	Morphant	Pink1	Minimal alteration in number of dopaminergic neurons. Altered patterning and projection of dopaminergic	Xi *et al.* 2010

			neurons in ventral diencephalon. Locomotor dysfunction.	
	TILLING mutant	Pink1	Reduced Th positive cells. Reduction in mitochondrial complex I activity.	Bandmann *et al.* 2010
Parkinson Disease	Morphant	Park7	Loss of dopaminergic neurons after exposure to hydrogen peroxide and the proteasome inhibitor MG132. Increased *tp53* and *Bax* expression prior to toxin exposure.	Bretaud *et al.* 2007
Parkinson Disease	Morphant	Lrrk2	Significant loss of dopaminergic neurons in the ventral	Sheng *et al.* 2010

			diencephalon. Reduced and disorganized axon tracts in the midbrain. Locomotor defect.	
Parkinson Disease	Pharmacological	N/A	MPTP or 6-OHDA injection reduces dopamine concentration in the brain. No significant reduction in dopaminergic neuron number. No increase in apoptotic cells. Marked locomotor deficits.	Anichtchik *et al.* 2004
	Pharmacological	N/A	Transgenic line developed expressing GFP in monoaminergic neurons. MPTP	Wen *et al.* 2008

			exposure reduces number of Th positive neurons in posterior tuberculum of ventral diencephalon.	
	Pharmacological	N/A	Zebrafish larvae subjected to either MPTP or MPP+ demonstrated swimming defects and perturbed development of SLC6A3-positive diencephalic cells.	Lam *et al.* 2005