Sqstm1 knock-down causes a locomotor phenotype ameliorated by rapamycin in a zebrafish model of ALS/FTLD

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ABSTRACT

Mutations in SQSTM1, encoding for the protein SQSTM1/p62, have been recently reported in 1-3.5% of patients with amyotrophic lateral sclerosis and frontotemporal lobar degeneration (ALS/FTLD). Inclusions positive for SQSTM1/p62 have been detected in patients with neurodegenerative disorders, including ALS/FTLD. In order to investigate the pathogenic mechanisms induced by SQSTM1 mutations in ALS/FTLD, we developed a zebrafish model. Knock-down of the sqstm1 zebrafish ortholog, as well as impairing of its splicing, led to a specific phenotype, consisting of behavioral and axonal anomalies. Here, we report swimming deficits associated with shorter motor neuronal axons that could be rescued by the overexpression of wild type human SQSTM1. Interestingly, no rescue of the loss-of-function phenotype was observed when overexpressing human SQSTM1 constructs carrying ALS/FTLD-related mutations. Consistent with its role in autophagy regulation, we found increased mTOR levels upon knock-down of sqstm1. Furthermore, treatment of zebrafish embryos with rapamycin, a known inhibitor of the mTOR pathway, yielded an amelioration of the locomotor phenotype in the sqstm1 knock-down model. Our results suggest that loss-of-function of SQSTM1 causes phenotypic features characterized by locomotor deficits and motor neuron axonal defects that are associated with a misregulation of autophagic processes.
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are two neurological disorders characterized by the degeneration of neurons of motor cortex, brainstem and spinal cord and of the frontal and temporal lobes, respectively. ALS and FTLD are two extremes of a disease spectrum (ALS/FTLD) with clinical, pathological and genetic overlap. In fact, there is an increasing number of genetic factors that are found to be involved in both disorders, with \textit{C9orf72} being the most frequent (1,2). Patients with ALS/FTLD can show signs of other conditions, namely of Paget disease of bone (PDB) and inclusion body myopathy (IBM). The spectrum of phenotypes characterized by the concomitance of these clinical signs (called “multisystem proteinopathy”) has been associated with mutations in valosin containing protein (\textit{VCP}) (3), sequestosome 1 (\textit{SQSTM1}) (4) and heterogeneous nuclear ribonucleoproteins (\textit{hnRNPA2B1} and \textit{hnRNPA1}) (5).

Mutations in \textit{SQSTM1} (OMIM: 601530) were first identified in patients affected by Paget disease of bone (PDB), a chronic bone disease leading to deformations and fractures (6), accounting for 25–50\% of familial and 5–10\% of sporadic PDB patients (7). More recently, \textit{SQSTM1} mutations have also been detected in ALS (4) and FTLD disorders (8). A number of studies have confirmed the contribution of \textit{SQSTM1} mutations to 1-3.5\% of ALS/FTLD cases with or without familial history. About 40 different variants have been reported to date. Most of them are missense variants, involving highly conserved amino acidic residues, but a few truncating mutations have also been identified (4, 8-15). The SQSTM1/p62 protein, encoded by \textit{SQSTM1}, consists of 440-amino acids and different domains: a N-terminal PB1 domain, which allows p62 to polymerize; a TRAF6-binding sequence (TBS), which activates the NF-kB signal; a LC3-interaction region (LIR) and a C-terminal ubiquitin associated domain (UBA). SQSTM1/p62 is involved in different biological pathways, including the autophagy degradation pathway, where it plays a critical role. The SQSTM1/p62 protein binds
ubiquitinated targets, thanks to the UBA domain, and delivers them to the autophagosome, via the LIR. Furthermore, SQSTM1/p62 interacts with mTOR (mechanistic Target Of Rapamycin) pathway and regulates mTOR translocation to the lysosomes (16). Almost all the mutations causing PDB lie in the UBA domain. Mutations in ALS/FTLD are widespread in the gene, although their frequency is higher in the UBA and the LIR domains. The missense mutation p.P392L, in the UBA domain, is the most frequent in all the different clinical phenotypes.

From a pathological point of view, SQSTM1/p62 positive neuronal inclusions have been observed in several neurodegenerative diseases, including ALS and FTLD. In particular, basophilic inclusions positive for SQSTM1/p62 have been described in the anterior horn cells in ALS patients (17). In FTLD patients, SQSTM1/p62 co-localizes with TDP-43 and FUS in brain and spinal cord (18). Inclusions positive for SQSTM1/p62 are associated with glial TDP-43 positive inclusions in ALS/FTLD (19) and in TDP-43 negative inclusions in ALS/FTLD carriers of the C9orf72 expansion (20).

Mouse models have been developed specifically to study SQSTM1/p62 involvement in PDB. SQSTM1/p62 knock-out mice show reduced osteoclastogenesis and decreased IL-6 expression (21) and they develop insulin resistance, which leads to adult-onset obesity and diabetes (22). Interestingly, these mice develop an Alzheimer-like phenotype, consisting in cognitive impairment and anxiety, due to the presence of hyperphosphorylated protein tau and neurofibrillary tangles (23). Mice overexpressing a single point mutation (p.P394L, corresponding to the human p.P392L) have a PDB-like skeletal disorder (24,25) but the expression of the mutation on osteoclasts is not sufficient to induce the full PDB phenotype (24). Furthermore, spatial learning and long term memory deficits are present in mice overexpressing mutant SQSTM1/p62 (26).
Zebralish (Danio rerio) is largely used to study neurodegenerative disorders because of a series of technical reasons: these vertebrates produce large clutches of transparent eggs that develop externally and rapidly (27). Furthermore, zebralish and human genomes share a high homology and zebralish genes can be easily manipulated to test loss- or gain-of-function mechanisms. Transient loss- and/or gain-of-function zebralish models have been developed, by injection of antisense morpholino oligonucleotides (AMO) or mutant human RNA, respectively, for the major ALS causing genes, namely SOD1 (28), TARDBP (29), FUS (30, 31), C9orf72 (32). Zebralish embryos are also a good tool to assess in vivo the efficacy of new drugs: neuroprotective compounds have been tested on different ALS models (33-35).

We have generated a new zebralish model in order to determine whether the motor ALS phenotype induced by SQSTM1 mutations is due to a loss of function mechanism. Furthermore, considering the interaction of SQSTM1/p62 with mTOR, we have also evaluated the effect of the rapamycin treatment on phenotypic zebralish embryos.

RESULTS

sqstm1 is expressed throughout embryonic development in zebralish

The Danio rerio genome contains a single sqstm1 ortholog (ENSDARG00000075014), with two transcripts encoding for two different proteins. The percentage of identity between the human and zebralish sqstm1 proteins (ENSP00000374455 and ENSDARP00000117113, respectively) is overall (48%) with the C-terminal portion, encoded by exon 8 where the majority of pathogenic mutations lie, very highly conserved (80%) (Supplementary Fig. 1).

The expression of sqstm1 mRNA was verified by RT-PCR in zebralish embryos at various stages. sqstm1 mRNA was detected from 6hpf to 3 days post fertilization (dpf) (Fig. 1A).
Knockdown of sqstm1 in zebrafish, as well as its altered splicing, leads to a specific motor phenotype

Morpholino antisense oligos, designed to block the translation start site of the zebrafish sqstm1 ortholog (sqstm1-atg), were injected into wild-type zebrafish embryos to define whether sqstm1 knock-down led to a specific phenotype. Dose-dependent curves were first performed to assess the toxicity and determine the working concentration of the morpholino (data not shown). Following injection of sqstm1-atg AMO at the one cell embryonic stage, zebrafish embryos were tested for behavior at 48hpf. About 60% of sqstm1-atg embryos were phenotypic (Fig. 1B and Fig. 1C). The phenotype caused by knocking-down sqstm1 consisted in an abnormal motor behavior, specifically a reduced touch-evoked escape response (TEER). After stimulation by light touch to the tail, the individual swimming episodes were traced in order to quantify locomotor parameters (Fig. 2A). Total distance and velocity (average and maximum) were significantly reduced in embryos after sqstm1 knock-down (Fig. 2B, Fig. 2C and Fig. 2D). The morphology of the spinal motor neurons was analyzed by immunohistochemistry, using anti-Znp-1 antibody, which labels the synaptic protein synaptotagmin. We observed axonal deficits, namely disrupted arborization and shortening of the motor neuron axons, after sqstm1-atg injection (Fig. 3A and Fig. 3B). The specificity of the phenotype was confirmed by the injection of the same concentration of a control mismatch morpholino (sqstm1-mis), which did not cause any locomotor or axonal deficit (Fig. 1C, Fig. 2, Fig. 3 and Fig. 4A). The phenotypic features associated with abnormal axonal projections from motor neurons were specific as no deficits were detected upon immunolabeling of Mauthner cells, a pair of easily identifiable neurons with very long axons, upon knockdown of sqstm1 in zebrafish embryos (Supplementary Fig. 2A). We did not detect
any neuronal loss in sqstm1-\textit{atg} as compared to the mismatch control (Supplementary Fig. 2B).

To test the functionality of the well-conserved C-terminal region of the zebrafish SQSTM1/p62, we designed a splice blocking morpholino (sqstm1-\textit{spl}), which caused an alternative splicing of the sqstm1 transcript by impairing the splicing of intron 7- exon 8 (Fig. 4A). The phenotype observed after the injection of the sqstm1-\textit{spl} was similar to the one observed after the injection of the translation blocking sqstm1-\textit{atg} (Fig. 4B). As for sqstm1-\textit{atg}, embryos injected with sqstm1-\textit{spl} had a deficient touch-evoked escape response (Fig. 4B), shorter and more disorganized motor neuronal axons (Fig. 4C) and statistically reduced total distance, average velocity and maximum velocity (Fig. 2B, Fig. 2C and Fig. 2D). Reconstituted trajectories of individual swimming episodes (Fig. 4D) show the markedly reduced mobility of sqstm1-\textit{spl} injected zebrafish.

These results demonstrate that sqstm1 knockdown leads to a specific loss of function motor phenotype in a zebrafish model.

\textbf{Wild type human SQSTM1 RNA, but not mutant RNA, rescues the motor phenotype}

To test the functional conservation between human and zebrafish \textit{SQSTM1}, we co-injected human SQSTM1 RNA together with the zebrafish-specific morpholinos, sqstm1-\textit{atg} or sqstm1-\textit{spl}, after verifying that after injection of RNAs alone zebrafish embryos were completely normal (data not shown). The locomotor (swimming distance, average and maximum velocity) and the motor axon projection phenotypes caused by the knock-down of zebrafish \textit{sqstm1} were significantly rescued by the introduction of the human wt SQSTM1 (Fig. 1C, Fig. 2, Fig. 3 and Fig. 4), suggesting a conserved functional identity between the two orthologues. Contrary to the wild-type RNA, co-injection of the human mutant SQSTM1
RNA, expressing the p.P392L mutation (mutRNA), did not rescue the phenotype caused by knock-down of the zebrafish sqstm1. Indeed, the percentages of phenotypic fish (Fig. 1C and Fig. 4B), the swimming parameters (Fig. 2 and Fig. 4D) as well as the motor axon length (Fig. 3) were not improved in the mutant SQSTM1 RNA rescue condition. Similar results were obtained with a second mutant SQSTM1 RNA, bearing a truncating mutation (D391X), which leads to the skipping of the last exon and to the impairing of the UBA domain (Supplementary Fig. 3A). No locomotor phenotype was appreciated after the injections of high concentrations of wild type and mutant human RNAs alone (Supplementary Fig. 3B and 3C).

**Rapamycin rescues the motor phenotype induced by sqstm1 knockdown and splice alteration**

Considering the role of SQSTM1/p62 in the regulation of mTOR activity, we measured mTOR expression levels after sqstm1 knock-down. We detected increased levels of MTOR (Fig. 5A) by quantitative PCR. To further determine if an increased mTOR activity contributed to the phenotypic features observed upon sqstm1 knockdown, we tested the effect of rapamycin, a well-known mTOR inhibitor. Treatment with rapamycin of 48hpf wild-type and sqstm1-mis zebrafish embryos had no effect on the swimming parameters measured over a period of 2 days. We incubated phenotypic sqstm1-atg as well as sqstm1-spl injected embryos with rapamycin for 24 hours and we analyzed the spontaneous swimming for 2 days after the treatment. The swimming response was improved in larvae treated with rapamycin, compared to the untreated ones: both the total distance swum and the total duration were increased (Fig. 5B and Fig. 5C).
These results suggest that inhibition of mTOR activity by rapamycin can ameliorate the motor phenotype induced by knock-down of sqstm1 in zebrafish and can be considered for further analysis as a modifier of the pathogenicity of SQSTM1 mutations.

DISCUSSION

Mutations in SQSTM1 have been recently reported in patients with ALS/FTLD with a frequency of 1-3.5%. Furthermore, inclusions positive for SQSTM1/p62 were found in ALS/FTLD patients (with and without SQSTM1 mutations) (18). The detection of mutations in patients with different genetic backgrounds and the presence of pathological SQSTM1/p62 positive inclusions argue in favor of considering SQSTM1 as an ALS/FTLD associated genes. The involvement of SQSTM1 in the pathogenic mechanism has not been yet clarified.

Recently, SQSTM1 mutations have been associated with neuronal and glial phospho-TDP-43 pathology in patients’ brains (15). Increased levels of SQSTM1/p62 were described in the spinal cord of ALS patients carrying SQSTM1 mutations, supporting the hypothesis of a gain-of-function mechanism (12). However, the deletion of SQSTM1 in mice induced neurodegeneration and led to an Alzheimer-like phenotype (23), arguing for a loss-of-function mechanism.

In this study, we developed a zebrafish model to address the loss-of-function hypothesis.

Knockdown of the zebrafish sqstm1, by targeting the ATG, as well as alteration of its splicing, by impairing the splicing of intron 7- exon 8, caused a specific locomotor phenotype. This phenotype consisted of behavioral deficits, namely a reduced response after stimulation with a consequent reduction of swimming parameters, and a specific axonopathy of descending motor neuron projections. We could confirm that the phenotype was specifically due to the alteration of sqstm1 by using a splice blocking morpholino as well as a control
morpholino. Furthermore, we could rescue the swimming deficits and axonal abnormalities
upon the co-injection of the morpholino with human wild type SQSTM1 RNA, indicating a
good functional conservation between the zebrafish and human SQSTM1. Also, in
concordance with loss of function properties, two SQSTM1 mutations were unable to rescue
the motor phenotypic features when co-injected with the morpholino but they did not cause
toxicity if injected alone. Our results led to the hypothesis that the prevalent mechanism
through which SQSTM1 mutations induce ALS/FTLD is a loss-of-function, although the
possibility of a gain-of-function cannot be excluded. Recently, a novel stop mutation has been
described in a patient with FTLD, arguing for loss-of-function being a possible mechanism in
ALS/FTLD caused by SQSTM1 mutations (36). Our zebrafish model confirms that partial
loss-of-function of the SQSTM1 gene can lead to motor defects. While the strategy employed
here cannot directly explain the pathogenicity of dominantly inherited heterozygous missense
mutations, we can speculate that such genetic modifications could lead to a partial loss-of-
function of the protein, sufficient to induce the late-onset disorder. Recent evidence from
other ALS models demonstrated that, in some cases, a combination of gain- and loss-of-
function defines the phenotype, as reported for TDP-43 (29, 42), FUS (31, 43) and VAPB (44,
45).

Over the last few years a number of transient and stable transgenic zebrafish models have
been developed to define genetic causes of ALS and related neurodegenerative diseases (27).
Certain limitations do arise from the use of zebrafish as a neurodevelopmental model to study
pathogenic mechanisms leading to ALS and FTLD, which have mid-life onset in patients
affected by these neurodegenerative disorders. However, it is important to note that altering
expression of several genes related to early-onset motor neuron degeneration, such as
knockdown of SMN1 (38) and ALS2 (39), as well as genes mainly involved in later-onset
ALS and FTLD, such as overexpression of mutant SOD1 (28) and overexpression and
knockdown of TDP-43 or FUS (29-31), cause similar phenotypic features described as abnormal axonal projections from motor neurons and motor deficits at the level of spontaneous and evoked swimming. Furthermore, stable transgenic models for these neurological disorders have recently been generated and characterized, including deficits in SMN1 deletion mutant zebrafish larvae (40) as well as motor abnormalities in adult mutant SOD1 transgenic zebrafish (41). Indeed, these stable transgenic models also display similar defects at the neuromuscular junction as transient transgenics, though with a later onset of these neuropathological features (37). This accumulating evidence suggests that common developmental and possibly shared degenerative pathways could be affected in ALS and FTLD patients, including those harbouring SQSTM1 mutations. Indeed, the zebrafish genetic model for SQSTM1 mutations described here suggests the preferential targeting of motor neurons by SQSTM1 loss of function, consistent with other ALS-causing mutant proteins as well as depleted expression of several of the ALS/FTLD causing genes, and indicates a role for SQSTM1 expression in motor neuron degeneration. Thus, it will be important to develop deletion mutants for the SQSTM1 orthologous gene, and to follow these transgenic zebrafish throughout their lifespan for any motor or morphological deficits.

The role of SQSTM1/p62 in the regulation of autophagic processes has already been described (46). Here we show that knock-down of sqstm1 resulted in an increase in mTOR levels in our zebrafish model. Consistent with an involvement of the mTOR pathway in the sqstm1 knock-down phenotype, treatment with rapamycin, a well-known inhibitor of mTOR, was able to ameliorate spontaneous swimming defects caused by sqstm1 alteration. The partial rescue of motor deficits resulting from knockdown of sqstm1 obtained by rapamycin indicates that mTOR-activated autophagic degradation is involved in the phenotypic features described in this zebrafish model. Nevertheless, it is not possible to exclude at this point that they could be in part or fully due to overexpression of mTOR as a consequence of sqstm1
knockdown. Unfortunately, we were not able to assess alterations of SQSTM1/p62 and other autophagy markers at the protein level: commercial antibodies available are often unable to properly recognize the zebrafish proteins, as previously reviewed (47).

Since inhibition of autophagy was described in TDP-43-depleted cells (48), rapamycin and other autophagy activators were tested in FTLD mouse models with TDP-43 proteinopathies. By inducing autophagy, these compounds were able to rescue the motor dysfunctions in mice (49, 50) as well as to reduce TDP-43 levels, by enhancing its turnover. Compounds stimulating autophagy also improve the survival of primary murine neurons and human iPSC-derived neurons and astrocytes harboring TDP-43 mutations (51). Furthermore, TDP-43 aggregates, increased when autophagy is inhibited, co-localize with SQSTM1/p62, indicating a role for SQSTM1/p62 in the clearance of TDP-43 (52). It has been recently shown that SQSTM1/p62 physiologically binds to TDP-43 and is likely involved in its degradation (53).

All these data and the amelioration of the motor phenotype in our sqstm1 knock-down zebrafish treated with rapamycin could suggest that induction of autophagy should be potentially considered as a good candidate for treatment of ALS/FTLD caused by SQSTM1 mutations. More largely, it could represent a common therapeutic avenue in neurodegenerative diseases with prevalent TDP-43 aggregation.

In this perspective, it will be important to define if SQSTM1/p62 interacts functionally or genetically with TDP-43, FUS and/or C9orf72. At this time, SQSTM1 and TARDBP alterations have never been described together; on the contrary, three patients have been described carrying SQSTM1 mutations and concomitant C9orf72 expansion (15). Moreover, patients carrying the C9orf72 expansion have a peculiar neuropathological pattern. While SQSTM1/p62 usually co-localizes with TDP-43 and FUS in brain and spinal cord of ALS/FTLD patients (18), C9orf72 carriers present neuronal cytoplasmic and intranuclear inclusions that are positive for ubiquitin and SQSTM1/p62 and negative for TDP-43 (20, 54).
The zebrafish model described here is pertinent for multigenic analysis (31) and to test epistatic interactions amongst \textit{TARDBP}, \textit{C9orf72} and \textit{SQSTM1}.

In conclusion, we have generated the first zebrafish model of \textit{SQSTM1} and demonstrated that its loss-of-function induces a motor phenotype characterized by markedly reduced motor behavior associated with motor neuron deficits, mimicking ALS. Furthermore, these phenotypic features induced by \textit{sqstm1} knock-down were rescued by treating zebrafish with rapamycin, thus suggesting that autophagy stimulation could represent a therapeutic perspective in ALS/FTLD. Further studies are needed to define genetic interactions and to understand the mechanisms underlying the pathophysiology of SQSTM1/p62 in ALS/FTLD in order to develop therapeutic strategies.

**MATERIALS AND METHODS**

**Zebralsh studies**

Adult and larval zebrafish (\textit{Danio rerio}) were maintained at the ICM (Institut du Cerveau et de la Moelle épinière, Paris) fish facility and bred according to the National and European Guidelines for Animal Welfare. Experiments were performed on wild-type embryos from AB and TL strains. NGN:GFP and TUB:GFP transgenic lines were used as neuronal markers. All procedures were approved by the Institutional Ethics Committee at the Research Center of the ICM.

**Reverse Transcription PCR**

Total RNA from embryos at 48 hours post fertilization (hpf) was extracted using the TRIzol reagent (Invitrogen) and treated with DNase (Ambion) to remove residual contaminating DNA. RNA was quantified using the Nanodrop 8000 (Thermo Scientific) and its quality was
checked using the 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using Transcriptor Universal cDNA Master Mix (Roche). A PCR was performed to assess the expression pattern of sqstm1 using primer pairs amplifying the sequence between exon 7 and 9. Primers specific for β-actin were used as a positive control. Primer sequences are available upon request.

**Microinjections**

Morpholino antisense oligonucleotides (AMO) were designed complementary to the zebrafish SQSTM1 orthologue, sqstm1 (NC_007125.5), and synthesized from GeneTools. A morpholino (sqstm1-atg), binding to the ATG of the shortest transcript, was used to knockdown the gene (5’-ATGAAGAGACGGAAAGTGTCATCCT-3’). A control AMO (sqstm1-mis), containing five mismatch nucleotides and not binding anywhere in the zebrafish genome, was used to assess the specificity of the observed phenotype (5’-ATCAACAGACGGAAACTCTCATCCT-3’). A splice-blocking morpholino (sqstm1-spl) was used to target the intron 7- exon 8 junction (5’-GGTCTTTCTGCCATTTTGAAACAGA-3’). Human wild type and mutant (p.P392L) SQSTM1 RNAs were cloned into a pCS2+ vector (at the ICM Vectorology Platform) and a truncated SQSTM1 RNA, missing the last exon (D391X), was cloned into a pCR4-TOPO vector (Invitrogen). They were transcribed from Snabl-linearized pCS2+ and NotI-linearized pCR4-TOPO using SP6 and T3 polymerase, respectively, with the mMESSAGE Machine SP6 Kit (Ambion) and purified. Injections were performed in 1–2 cell stage zebrafish eggs, using a Picospritzer pressure ejector, as previously described (31). Dose-dependent curves of morpholinos and RNAs toxicity were established. Microinjections were performed at 0.6 mM (sqstm1-atg and sqstm1-mis) and 0.8 mM (sqstm1-spl). Wild-type and mutant SQSTM1 RNAs were microinjected at a concentration of 100ng/µl. Embryos were maintained at 28°C and manually dechorionated using fine forceps at 24hpf.
**Touch-evoked escape response (TEER)**

At 48hpf zebrafish embryos were tested for behavior under a stereomicroscope (Zeiss, Germany). They were touched lightly at the level of the tail with a tip and scored as previously described (29). Their responses were recorded with a Grasshopper digital camera (Point Grey) at a rate of 30 frame/sec and quantified using ImageJ.

**Immunohistochemistry**

Embryos were fixed at 48hpf and whole-mount immunohistochemical analysis was performed, as previously described (31). Mouse monoclonal anti-Znp-1 and anti-3A10 antibodies (Hybridoma Bank, Iowa, USA) were used for the staining of primary motor neuron axons and of Mauthner neurons, respectively. Whole-mount embryos were imaged on an Olympus IX83 microscope (Olympus). Axonal length was quantified using Image J.

**Rapamycin treatment**

After TEER analysis, 48hpf embryos were incubated overnight in 96-well plates. Rapamycin was added to the embryo’s water at the final concentration of 1 μM. Spontaneous swimming between 6 and 48 hours after the treatment was captured using a Zebralab system (ViewPoint, France) and analyzed using the Fast Data Monitor software (ViewPoint, France). Embryos were recorded always at the same time and the experiment was repeated 3 different times.

**Quantitative PCR**

Quantitative real-time PCR was performed on LightCycler® 480 Real-Time PCR System (Roche), using SYBR Green reagents. Expression of mTOR was compared to GAPDH using custom probes (QuantiTect Primer Assay Dr_mtor_1_AG and Dr_gapdh_1_SG, Qiagen). Relative expression was obtained according to the $2^{-\Delta\Delta CT}$ method and statistical significance was analyzed using the t-test.
Statistics

Data were plotted and analyzed using the Sigma Plot software (Systat Software Inc., San Jose, CA, USA) and statistical significance was determined using one-way ANOVA test. Significance was established at p<0.05.

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Conflict of Interest Statement

None declared.
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Legends to Figures

Figure 1. *sqstm1* is expressed in zebrafish embryos and its knock-down leads to motor phenotype. (A) RT-PCR shows that *sqstm1* is expressed in zebrafish from 6 hpf to 3 dpf. (B) Phenotypes of 48 hpf non-injected embryos and *sqstm1-atg* injected embryos. (C) Bar graph of the distribution of phenotypes after the TEER analysis at 48 hpf. Percentages of zebrafish with motor deficits ("motor") are increased after *sqstm1* knock-down, rescued after the co-injection of the morpholino with the wild type human SQSTM1 RNA but not with the mutant one. Percentages in non-injected and embryos injected with *sqstm1-mis* are comparable.

Figure 2. Locomotor parameters are affected by *sqstm1* knock-down. (A) Tracing of the swimming trajectories of 48 hpf larvae following light touch: 10 traces were used per condition. (B-D) Quantification of the TEER analysis: (B) total distance, (C) average velocity, (D) maximum velocity. *p<0.05 when compared to mismatch, #p<0.05 when compared to the respective morpholino.

Figure 3. *sqstm1* knock-down causes axonopathy in zebrafish. (A) Quantification of motor neuronal axonal length (*p<0.05 when compared to mismatch). A significant decrease in axonal length is present at 48 hpf after *sqstm1* knock-down. (B) Immunohistochemistry on motor neurons using anti-Znp1 antibody.

Figure 4. Alteration of *sqstm1* splicing leads to motor and axonal defects. (A) *sqstm1* expression is altered after injection of *sqstm1-spl* but not of *sqstm1-mis*. (B) Distribution of phenotypes after the TEER analysis at 48 hpf. Bar graph indicating that percentages of
zebrafish with a motor phenotype are increased after injection of sqstm1-spl. (C)

Quantification of motor neuronal axonal length after immunohistochemistry with anti-Znp1 antibody. (*p<0.05 when compared to non-injected). (D) Tracing of the swimming paths of 48hpf larvae following light touch: 10 traces were used per condition.

**Figure 5. Knockdown of sqstm1 leads to autophagy alterations.** (A) Relative quantification showing that mTOR transcript levels are increased in zebrafish embryos after sqstm1 knockdown (compared to GAPDH levels) *p < 0.05. (B-C) Quantification of spontaneous swimming parameters obtained using a ViewPoint system: total distance (B) and total duration (C) in embryos injected with sqstm1-atg and sqstm1-spl treated with rapamycin and untreated controls (*p<0.05). Swimming parameters have been captured between 6 and 48 hours post treatment (hpt).

**Abbreviations**

ALS/FTLD: amyotrophic lateral sclerosis/ frontotemporal lobar degeneration

SQSTM1: sequestosome 1

sqstm1-atg: sqstm1 ATG blocking morpholino

sqstm1-mis: sqstm1 mismatch morpholino

sqstm1-spl: sqstm1 splice blocking morpholino