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Novel aspects of TDP-43's interaction with ALS-related autophagy genes

Raphaël Munoz-Ruiz

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Raphaël Munoz-Ruiz. Novel aspects of TDP-43's interaction with ALS-related autophagy genes. Neurobiology. Sorbonne Université, 2019. English. NNT : 2019SORUS268 . tel-03140331

HAL Id: tel-03140331

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PhD Thesis – Sorbonne Université, Paris

Doctoral School – « Cerveau Cognition et Comportement »

Novel aspects of TDP-43's interaction with ALS-related autophagy genes

Raphael Munoz-Ruiz

Under the supervision of Dr. Egor Kabashi

U1163 “Translational Research for Neurological Diseases”, Institut Imagine, Paris


INSTITUT DES MALADIES GÉNÉTIQUES

Defended on the 1st of October 2019

Composition of the jury:

Pr. Claire Legay – rapporteur

Dr. Caroline Rouaux – rapporteur

Pr. Hervé Tostivint – examiner and representative of Sorbonne Université

Dr. François Giudicelli – examiner

Dr. Egor Kabashi – thesis supervisor

Remerciements :

Je tiens à remercier chaleureusement le Dr. Edor Kabashi pour son aide. Avec le Dr. Sorana Ciura, vous m'avez permis de réaliser mon projet de thèse dans d'excellentes conditions. Cela inclut bien évidemment la composition de cette équipe formidable. Des plus anciens jusqu'aux nouveaux arrivants, je n'aurais pas pu espérer un entourage plus convivial, amical et bienveillant. Je crains fortement que ma vision de l'environnement professionnel soit biaisée pour le reste de ma jeune carrière.

Merci donc à Solène, Grégoire, Mélissa, Claire et Chloé. Vous avez collectivement créé une atmosphère amicale et d'entraide que j'ai plaisir à retrouver chaque matin (pas trop tôt quand même). Merci à Letizia, Hortense et Anca, ma bien-aimée famille de laboratoire. En y ajoutant Doris, la machine à rendre les gens meilleurs, et Corinne, qui a toute mon affection, je peux véritablement dire que, dès mon arrivée dans ce laboratoire, j'ai été particulièrement chanceux. Je suis profondément convaincu que vous m'avez fait grandir.

Annis-Rayan Bourefis, mon fidèle compagnon de bataille. Ça serait ridicule de résumer notre amitié à cette thèse. Je te remercie simplement d'être Annis et d'être mon ami depuis mon exil à Paris. C'est maintenant à toi de passer ta thèse... qu'est-ce que tu veux que je te dise...je te souhaite du courage et espère que je vais pouvoir t'accompagner comme tu l'as fait pour moi. J'espère aussi que nous pourrons livrer d'autres batailles ensemble.

Je voudrais remercier tous les membres du jury ainsi que les personnes qui m'ont aidé par leurs conseils et expertise bienveillante lors de ma thèse, le Pr. Claire Legay, le Dr. Caroline Rouaux, le Dr. François Giudicelli, le Pr. Hervé Tostivint et le Dr. Pierre-François Pradat.

Enfin, un grand merci à toute ma famille, que j'aime de tout mon cœur. Maman, Papa, Juliette et Cécile, merci pour votre soutien. Même à distance, j'ai toujours ressenti votre présence à mes côtés. Merci également à tous mes amis d'avoir été à mes côtés. Merci Clément, Julien, Thomas, Sonia, Antoine et Pierre.

Raphaël

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Résumé - Abstract

La Sclérose Latérale Amyotrophique (SLA) est une maladie dégénérative et incurable des neurones moteurs conduisant à une paralysie musculaire et une perte quasi-totale des commandes motrices. Cela conduit au décès du patient en l'espace de 2 à 5 ans après l'apparition des premiers symptômes. La présentation clinique de la maladie est assez hétérogène. Cependant, la signature histopathologique de la SLA est la présence d'inclusions cytoplasmiques ubiquitinées insolubles dans les neurones atteints, ce qui indique une défaillance de l'homéostasie protéique. Dans la grande majorité des cas, la protéine de liaison aux ARNs TDP-43 est retrouvée en agrégats dans ces inclusions.

Le profil génétique de la maladie est complexe et de nombreux gènes mutés ont été identifiés dans la pathologie, chacun ne représentant qu'une portion relative faible de la totalité des cas. Pourtant, deux groupes de gènes ressortent : un constitué de gènes impliqués dans le métabolisme des ARNs et un autre de gènes de l'homéostasie protéique, plus particulièrement de l'autophagie, un processus de dégradation des protéines. De manière logique aux vues de l'histopathologie et de la génétique, les perturbations du métabolisme des ARNs ainsi que de l'autophagie sont particulièrement étudiées dans la SLA. De plus, ces deux processus sont intimement liés dans l'homéostasie globale de la cellule.

Pour mieux comprendre les interactions pouvant exister entre ces deux mécanismes impliqués dans la maladie, j'ai cherché à identifier de nouveaux rôles que pourrait jouer TDP-43, acteur majeur du métabolisme des ARNs, dans l'autophagie. De plus, la relation entre TDP-43 et les récepteurs de l'autophagie p62/SQSTM1 et OPTN ainsi que de leur activateur TBK1, tous identifiés mutés chez des patients, est peu connue.

J'ai développé des modèles de modulation de l'expression de *TARDBP*, le gène codant pour TDP-43. Chez l'embryon de poisson zèbre, nous avons déjà rapporté que la sous-expression de *tardbp* conduisait à un déficit moteur ainsi qu'à une axonopathie. Nous associons maintenant cela avec une régulation négative de *tbk1*. De manière intéressante, de l'ARN de *TBK1* humain permet un sauvetage partiel de ce phénotype. La sous-expression de *tardbp* s'accompagne aussi par une altération du profil d'expression de gènes clés de l'autophagie, qui est en contradiction avec ce que nous, et d'autres équipes, obtenons sur des modèles cellulaires humains. Toutefois, le modèle de cellules de neuroblastome SH-SY5Y permet l'existence de tels profils antagonistes après déplétion de TDP-43, selon que nous modulons l'activité autophagique via de la Torine 1, un puissant activateur de l'initiation de l'autophagie. De manière concomitante, la Torine 1 inhibe la liaison de TDP-43 aux ARNs de *RAPTOR* et *OPTN* mais promeut la liaison à de nouveaux partenaires, les ARNs de *TBK1* et *p62/SQSTM1*. De plus, nous rapportons l'observation d'effets antagonistes sur *p62/SQSTM1* lors de la surexpression progressive de *TARDBP* dans ces cellules. Plus généralement, ces modulations de l'expression de *TARDBP* se sont traduites par l'apparition de certains phénomènes pathologiques qui accompagnent l'agrégation de TDP-43 chez les patients SLA : la déplétion nucléaire de TDP-43 lors de la sous-expression ; son clivage et sa localisation anormale dans le cytoplasme pour la surexpression. Comme la fonction de liaison aux ARNs de TDP-43 semble capitale dans ces mécanismes, nous avons développé de nouvelles techniques de visualisation des ARNs *in vivo* et *in vitro*.

Cette thèse renforce donc les liens entre métabolisme des ARNs et autophagie dans le contexte de la SLA. De plus, nous mettons la lumière sur la faculté de TDP-43 de s'adapter au contexte cellulaire et d'influer différemment sur l'autophagie.

Amyotrophic Lateral Sclerosis (ALS) is a late-onset incurable degenerative disease of the motor neurons which leads to muscle paralysis and loss of nearly all motor commands. Patients die within 2-5 years after appearance of first symptoms. The clinical presentation of the disease is quite heterogeneous. However, the histological hallmark of the disease is the presence of insoluble ubiquitin-positive inclusions in the cytoplasm of affected neurons. Such a phenomenon is a sign of protein homeostasis failure. In the vast majority of cases, the inclusions are positive for TDP-43, an RNA-binding protein which is found aberrantly aggregated.

The genetic etiology of ALS is complex and numerous mutated genes have been identified in patients and each account for a rather small percentage of cases. However, two important groups stand out: a group of genes involved in RNA metabolism and another involved in protein homeostasis and more particularly in a key degradative process called autophagy. Logically, based on histopathologic and genetic evidence, defects in these two processes are widely investigated in ALS pathogenesis. Moreover, RNA metabolism and autophagy are interestingly intertwined in the global maintenance of cellular homeostasis.

In order to understand the potential interactions between these two ALS-relevant processes, I sought to define novel roles that TDP-43, a key actor of RNA-metabolism, could play in the autophagy pathway. Of note, TDP-43 interaction with ALS-associated p62/SQSTM1 and OPTN, both identified as autophagy receptors, as well as TBK1, their upstream activator, is poorly known.

Here, I developed models to modulate *TARDBP*, the gene that encodes for TDP-43. In the zebrafish embryo, we report that an already established transient knockdown of *tardbp* that leads to motor deficits and axonopathy, is associated with a downregulation of *tbk1*. Interestingly, human *TBK1* RNA was able to ameliorate these motor deficits and axonopathy which indicate an epistatic interaction between *tardbp* and *tbk1*. The knockdown of *tardbp* was also accompanied with changes in the expression profile of key autophagy genes that were in opposition with what we, and other teams before, report when depleting TDP-43 levels in human cell lines. However, the SY-SH5Y neuroblastomal cellular model also revealed that such antagonist effects on autophagy obtained upon TDP-43-depletion could be observed depending on the initial modulation of autophagy, notably through treatment with Torin 1, a potent activator of autophagy initiation. Concomitantly, Torin 1 inhibited binding to known RNA targets *RAPTOR* and *OPTN* but also promoted binding to novel targets *p62/SQSTM1* and *TBK1*. Furthermore, antagonist effects on *p62/SQSTM1* were also obtained upon gradual overexpression of *TARDBP* in cells. Overall, both modulation of *TARDBP* triggered the appearance of certain pathological traits of TDP-43 proteinopathy that are found in patients along with the aggregation of the protein: nuclear depletion for the knockdown; truncation and mislocalization in the cytoplasm for overexpression. As TDP-43's RNA-binding function appeared deeply involved in these mechanisms, we developed promising RNA-tracking techniques for *in vivo* and *in vitro* applications.

Therefore, this thesis strengthens the link between RNA metabolism and autophagy in the context of ALS and unveils the TDP-43's ability to adapt to cellular context and influence autophagy in different manners.

Introduction

I) Amyotrophic Lateral Sclerosis

A) History and definition

In the 1860's, French neurologist Jean-Martin Charcot (1825-1893; **Fig i 1**) was the first to describe a correlation of clinical features namely progressive muscle atrophy, muscle spasticity, neuropathologic loss of anterior horn cells as well as sclerosis in the lateral columns of the spinal cord (Charcot et al, 1869) and named the disease accordingly, Amyotrophic Lateral Sclerosis.



Figure i 1 : Jean-Martin Charcot
National Library of Medicine

As a result, ALS is also referred as Charcot's disease. Interestingly, even before Charcot's work, the first clinical case reports seem to be attributed to Charles Bell and date back to 1824 and were followed by several other case descriptions during the 1850's notably by other Frenchmen Guillaume Duchenne de Boulogne (1806-1875) and Jean Cruveilhier (1791-1974) (Cruveilhier et al. 1863; Duchenne de Boulogne et al. 1851). In the United States, as ALS caused the death of baseball

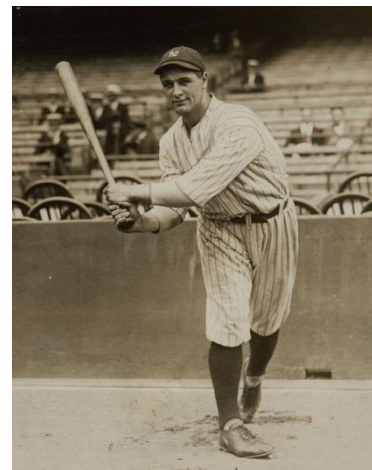


Figure i 2 : Lou Gehrig, 1923
Pacific and Atlantic photos

Hall of Famer Henry Louis Gehrig (1903-1941; Fig i 2) the pathology is often called Lou Gherig's disease.

ALS can be described as a late onset motor neuron disease where degeneration of both upper and lower motor neuron systems is observed (UMN and LMN) and leads to a rapidly progressive and ultimately lethal muscular paralysis. “Lateral Sclerosis” is related to UMN damage as it translates hardness of the lateral columns of the spinal cord. This hardening is due to gliosis, which appears after degeneration of the corticospinal tracts. “Amyotrophic”, on the other hand, indicates LMN damage as it refers to muscle fiber atrophy causing overall muscle weakness and fasciculation, which is a consequence of their denervation.

Reminder:

A minimum of two motor neurons are required in somatic motor pathways: an upper motor neuron and a lower motor neuron. The body cell of the upper one is in the central nervous system. It projects from the cortex to the brainstem and spinal cord (corticospinal tracts). For the lower motor neuron, its cell body resides in the brainstem for cranial nerves or in the spinal cord for peripheral nerves and innervates a single motor unit. LMN axons are the only one extending in the peripheral nervous system in order to innervate skeletal muscles **(Fig i 3)**.

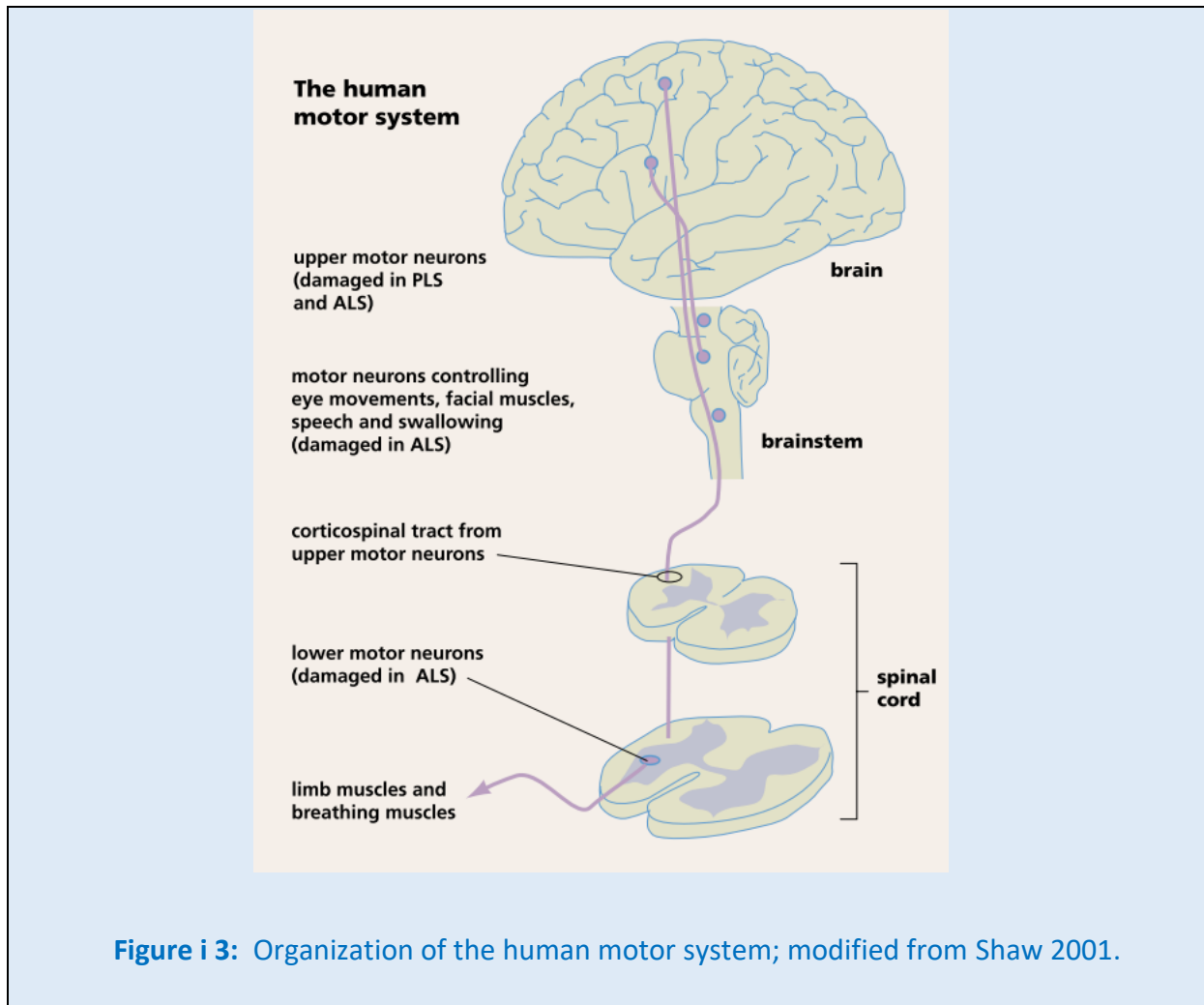


Figure i 3: Organization of the human motor system; modified from Shaw 2001.

B) The clinic

(1) Symptoms, diagnosis and prognosis

On a clinical point of view, the initial presentation of ALS holds great heterogeneity. Some patients first present muscle weakness of the limbs, which is referred to a spinal-onset, while other present dysarthria and dysphagia (respectively difficulty of speech and swallowing), which indicate a bulbar-onset (Hardiman *et al.*, 2017). More globally, the spectrum of symptoms is quite wide considering any voluntary muscle can suffer damage. In the majority of cases, the onset is focal and clinical signs progressively spread locally but also

to other regions that show anatomical connections (van Es *et al.*, 2017). There is no definitive diagnostic test to demonstrate ALS but according to El Escorial and Awaji criteria, patients must present combined evidence of UMN and LMN being affected that cannot be explained by any other pathology. In that purpose, clinical, electrophysiological, muscle biopsies, serological or cerebrospinal fluid (CSF) testing and neuroimaging studies can be performed (Campanari, Bourefis and Kabashi, 2019). Moreover, the progression must be comparable to a neurodegenerative disorder (Wijesekera and Leigh, 2009). Apart from muscle weakness, speech and swallowing difficulties, clinical features supporting ALS diagnosis also include abnormal larynx and/or pulmonary function as patient ultimately develop respiratory insufficiency and pulmonary complications which are the usual cause of death.

Even though ALS first symptoms concern the motor behavior, phenotyping studies reveal that up to 50% of patients also show behavioral and cognitive defects (Phukan *et al.*, 2012). Interestingly, ALS and frontotemporal dementia (FTD, a progressive neurodegenerative disorder affecting both frontal and temporal cortices and causing gradual cognitive impairment) share pathobiological features and genetic causes. It is estimated that up to 15% of ALS patients show symptoms consistent with FTD while around the same portion of FTD patients also meet ALS criteria (Ringholz *et al.*, 2005; Wheaton *et al.*, 2007; Phukan *et al.*, 2012) giving rise to an ALS-FTD clinical spectrum of disease. Behavioral signs are mostly related to apathy and loss of sympathy while cognitive defects often translate into language, social cognition, executive function and more rarely memory impairments (Beeldman *et al.*, 2016).

As diagnosis takes up to one year after the first signs appear (Zoccolella *et al.*, 2006), the fact that ALS is a rapidly progressing disease makes the discovery of a reliable molecular biomarker critical for earlier diagnosis. Although quantification of neurofilament light chain

NEFL was described as relevant in order to support a diagnosis, no biomarker has been validated and integrated into standard procedure for ALS diagnosis. More precisely, NEFL serum levels were efficient to distinguish ALS patients from healthy individuals and also FTD, Alzheimer's and Parkinson's diseases (Verde *et al.*, 2019) but only cerebrospinal fluid (CSF) levels were shown to be accurate to differentiate ALS patients from patients suffering from an "ALS mimic" syndrome (Steinacker *et al.*, 2016; Xu *et al.*, 2016).

Although for the previously described reasons prognosis is highly variable, from onset the median survival is around 2 or 3 years for bulbar onset and 3 to 5 years for spinal onset (Sejvar *et al.*, 2005). Overall, large cohort study show that survival of 3 years for 48% of patients, 5 years for 24% and only 4% survive for more than 10 years (Turner *et al.*, 2003; Testa *et al.*, 2004). Algorithms have been developed in order to try to predict prognosis (Hothorn and Jung, 2014; Elamin *et al.*, 2015). Two different systems are currently used to stage the disease, the King's and Milano-Torino (MITOS) systems (Tramacere *et al.*, 2015). They are respectively based on the number of anatomical regions or body functions that are being affected. King's staging is known to be more accurate in the earlier stages of the disease and MITOS in the later. As combined FTD and associated symptoms have a negative influence on the prognosis, one could argue that these staging methods should integrate cognitive or behavioral changes. Weight loss has also been correlated to shortened life expectancy of patients (Körner *et al.*, 2013).

[Stephen Hawking: exceptional survival for an exceptional man.](#)

On March 14, 2008, Stephen Hawking (**Fig i 4**), one of the world's most famous physicists, cosmologist and ALS patient passed away at the age of 76 years old. After he was diagnosed with ALS in 1963, he was given a life expectancy of only 2 years. That is to say, he outlived this

tragic but yet logical estimation by more than 50 years. Hawking showed the first signs of the disease in his 20s, which could partly explain this exceptional life span as younger patients tend to show slower progression and thus longer survival. Besides the length, it is the stability of his condition that was also peculiar. Long surviving patients usually still show gradual deterioration whereas Hawking was relatively stable even after severe disabling paralysis had spread to his body. It was also speculated Hawking displayed a rare variation of the disease but overall the physicians and researchers of the ALS community fall short of explaining why he went on to live several decades where he brilliantly theorized on black holes and quantum gravity. Although statistics do account for such exceptional ALS patients (Westeneng *et al.*, 2018), they are unfortunately too rare to allow a significant study. They however illustrate the need to adapt and personalize medical care for each patient (Dobson, 2002).



Figure i 4: Stephen Hawking, 1974, NASA

(2) Treatments and patient management

As to date, there is no cure for ALS. However, Riluzole and Edaravone have shown efficiency in positively modifying ALS normal course of action in patients and have come to the market (Beghi *et al.*, 2011; Jaiswal, 2019). Heterogeneous clinic and pathogenetic have made research for compounds quite challenging. Indeed, more than 50 compounds, implicating various mechanisms of action have been tested and discarded after clinical trials (Beghi *et al.*, 2011). Riluzole was the first compound to be approved by the FDA in the treatment for ALS. The molecule may inhibit glutamatergic neuro-transmission but the precise mechanism of action remains unclear. The pioneer clinical trial for riluzole (Lacomblez *et al.*, 1996) outlined that after 18 month of treatment, patients survival was increased by 3 months as compared to a placebo group. For Edaravone, presumably a antioxydating agent, showed positive results in terms of disease progression but it is important to highlight the cohort was exclusively composed of selected early onset ALS patients displaying rapid progression (Hardiman and van den Berg, 2017). FDA approved edaravone but the European Medicine Agency did not. A controversy remains as whether the drug should be given to all ALS patients considering the selectivity of the patients that participated in the trial (Hardiman and van den Berg, 2017).

Apart from these disease-modifying therapies, ALS patients mainly receive symptomatic treatments and interventions (pharmacological or non-pharmacological). As previously described, the spectrum of symptoms that patient display is large hence treatments targeting dysphagia, dysarthria, hypersalivation (sialorrhoea), spasticity, cramps, pain, thrombosis, respiratory insufficiency as well as behavioral and cognitive impairments. Management of ALS thus require multidisciplinary approach for optimal survival and quality of life (Traynor *et al.*, 2003; Rooney *et al.*, 2015). Indeed, considering the inevitable fatality of ALS, much of the patient management is not only focused on treatment and survival but on

ameliorating the quality of life of the patient especially in the “end-of-life management”. Multidisciplinary approach requires a multitude of health professionals and caretakers without discarding psychological therapy as patients are very likely to experience powerlessness facing the rapid decay of their body towards near death.

Among life-prolonging procedures, and in case of menacing respiratory failure, tracheostomy (incision of the neck and trachea to open a direct airway) can be performed for long-term mechanical ventilation. Few patients agree to the procedure as it definitely ensures total immobility and loss of oral communication as well as extreme discomfort. When kept alive in such conditions, patients eventually enter a state of profound paralysis with loss of all voluntary muscle command called “totally locked-in state” (Hayashi and Kato, 1989; Wijesekera and Leigh, 2009). Communication often relies on their remaining oculomotor capacities and adapted technological devices. Weight loss is a serious issue in ALS and a well-established negative prognosis factor (Reich-Slotky *et al.*, 2013). In case of severe dysphagia resulting in important weight loss, the patient can choose to be fed by insertion of a gastrostomy tube (enteral nutrition). Even though no precise criteria has been established, this technique is generally considered after a weight loss superior to 5% (Miller *et al.*, 1999). The procedure relieves the patient and care takers from the stress of difficult meals as it ensures safe route for food and medication but are counterbalanced by practical and psychological complications (Stavroulakis *et al.*, 2016). However, weight loss isn't only caused by difficulties to swallow. Loss of appetite and muscle mass as well as hypermetabolism are also responsible. As a result, high calorie diets, nutritional supplements and overall supervision by a nutritionist are highly recommended and show significant results (Körner *et al.*, 2013).

(3) Epidemiology and risk factors

Remainder:

- Prevalence: proportion of the population presenting the said condition. It refers to all existing cases.
- Incidence: proportion of the population that newly contract the said condition. It refers to the new cases that appear.

Global ALS epidemiology knows limitations. First of all, the vast majority of studies concern populations from Europe and the United States (about 80%) and mainly integrate cohorts of patients from European descent. Populations with greater ancestral and genetic diversity show lower incidence and prevalence of ALS (Cronin, Hardiman and Traynor, 2007; Bucheli *et al.*, 2014). For example, the median age of onset is about 10 years earlier in such heterogeneous populations as compared to European ancestry populations. In order to fully elucidate ALS epidemiology studies will have to integrate data from admixed populations from all continents. Epidemiological studies also suffer from recent evolution of our understanding on the disease (notably ALS's link to FTD), heterogeneity of cases and complexity to establish a diagnosis and thus a precise onset. These factors must be taken into account for more rigorous studies (Rooney, Brayne, *et al.*, 2017).

The prevalence of the disease in populations of Europe or of European ancestry is of 2,6 to 3 cases per 100 000 people (Logroscino *et al.*, 2010; Huisman *et al.*, 2011). Interestingly, studies regularly depict a gender difference in terms of lifetime risk as for women it is about

1:400 and 1:350 for men (Johnston *et al.*, 2006; Al-Chalabi and Hardiman, 2013). Furthermore, when men mostly display spinal-onset ALS, the majority of women seem to develop a bulbar-onset (Logroscino *et al.*, 2010). Geographic localization seems to play an important role. For example, populations from Asia show reduced incidence as compared to Europe (0.79 and 0.89 per 100 000 people respectively in East and South Asia versus 1.89 for northern Europe) and proportionally lower bulbar-onset. Overall survival is twice as short in Europe than in Asia (24 months versus 48 months) (Marin *et al.*, 2017; Logroscino and Piccininni, 2019). Latitude gradient may also correlate with data. Within the European continent, a north to south gradient has been described as percentage of spinal-onset was higher in the south. In Italy, northern areas of the country have higher incidence than in the south (Chiò *et al.*, 1999).

Sporadic and Familial form of the disease respectively show peak onset at 58-63 years and 47-52 years (Logroscino *et al.*, 2010; Al-Chalabi and Hardiman, 2013). Data show increasing age-specific incidence over the years, as population of Western countries have been aging over the past decades. ALS records a peak of incidence around 75 years old (Marin *et al.*, 2017). Some studies have attempted to estimate future distribution of ALS and have predicted an increase of 69% of total number of patients going toward 2040 (Arthur *et al.*, 2016).

A few risk factors were outlined from epidemiologic studies. Athletes seem to show higher frequency of ALS cases than the rest of the population, but the reason remains unclear. Preliminary data suggest that while type 2 diabetes, high levels of circulating lipids and female contraceptive hormones may be protective, smoking and overall exposure to smoking tend to be detrimental (Rooney *et al.*, 2017; Wang *et al.*, 2017).

The Island of Guam:

Located in Micronesia in the Pacific Ocean, the small island of Guam, which is under US sovereignty, presented in the 1950's the highest ALS incidence in the world thus focusing great interest from the ALS community. When about 3 cases per 100 000 people were reported in Europe and North America, almost 100 per 100 000 people were affected by ALS on this island (Plato *et al.*, 2003). More globally, the island was also home to high incidence of Alzheimer's and Parkinson's diseases which, combined with ALS, was sometimes referred to as "Guam disease". For reasons that remain unclear, in the following decades, this number drastically dropped to become equivalent to western country standards (Wiederholt, 1999). However, in such a short period of time, and as the island gradually adopted a westernized life-style, genetics could not explain these statistical changes. A role from a neurotoxic compound, β -methylamino-l-alanine, was suggested. The molecule is found in cycad seeds, which served in the preparation of "tortillas" in local cuisine after being dried and grounded into a flour. Similarly, the same seeds were used in traditional medicine in the Japanese Kii peninsula, which also ranked at the top of the list of areas with the highest ALS incidence. Exposure to water containing high concentration of cyanobacterias producing β -methylamino-l-alanine was also thought to increase risk of ALS (Bradley *et al.*, 2013).

(4) Genetics

In all previously described aspects of the disease, ALS showed a complex profile. The genetic aspect is no exception. Certain ALS patients display a Mendelian pattern of inheritance whereas the majority do not show any identifiable family history. They are traditionally referred to as familial ALS (fALS) and sporadic ALS (sALS) and account for around 5-10% and 90-95% of cases respectively (Rowland and Shneider, 2001; Hanby *et al.*, 2011). Around 70%

of fALS cases can be explained by the presence of a presumably pathogenic mutation. Most commonly, the mutated gene is *C9orf72* (40%), *SOD1* (20%), *TARDBP* or *FUS* (each accounting for 1 to 5%). In sporadic cases, 10% carry a mutation already identified in fALS. (Chiò *et al.*, 2014; Chia, Chiò and Traynor, 2018). As of yet, more than 30 genes have been associated with ALS (Fig i 5). The particulars of key ALS-associated genes will be discussed further in the following sections of this introduction: *SOD1* in section I.C.3) “Implicated cellular mechanisms”; *TARDBP* and *FUS* in section II. “*TARDBP* and RNA metabolism: a central role in ALS”; *C9orf72*, *OPTN*, *p62/SQSTM1* and *TBK1* in section III.B. “Autophagy’s implication in ALS”.

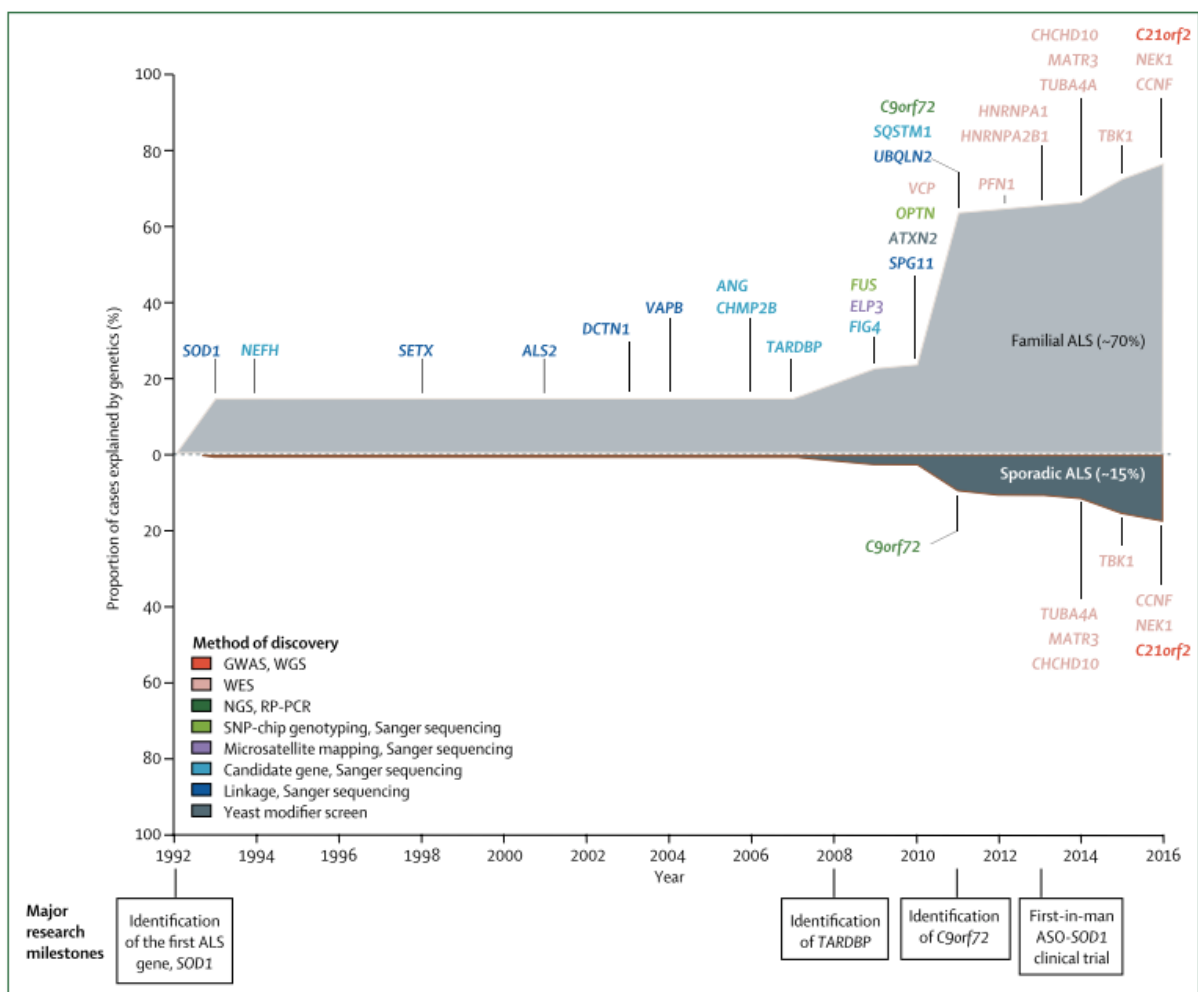


Figure i 5: Genetic landscape of ALS; modified from Chia et al. 2018.

GWAS: genome-wide association study; WGS: whole-genome sequencing; WES: whole-exome sequencing; NGS: next generation sequencing; RP-PCR: repeat-primed PCR; ASO: antisense oligonucleotide

These genes are now identified as major risks of ALS but studies based on mathematical models reveal a tendency for oligogenic inheritance (Heiman-Patterson *et al.*, 2011, 2015). This model of inheritance would explain cases of incomplete penetrance. It is also consistent with the fact that genetically heterogeneous populations show reduced incidence of ALS as previously described (Byrne *et al.*, 2012; van Blitterswijk, van Es, Hennekam, *et al.*, 2012; van Blitterswijk, van Es, Koppers, *et al.*, 2012).

Oligogenic versus polygenic:

An oligogenic trait is caused by more than one but relatively few genes and each of them have a detectable effect with a variable importance on the final phenotype. Wide effect genes (or susceptibility genes) act in a dominant pattern but final phenotype is buffered or aggravated through the interaction with other smaller effect genes and possibly environmental factors. The remaining steps linking these dominant risk factors to the final phenotypic presentation of ALS, that can include epigenetics, remain globally unknown. A polygenic trait is caused by several genes but the effect of each individual one is not detectable. Their additive effect is responsible for the appearance of the particular trait. ALS seem to show an oligogenic profile which separates it from several common disorders such as schizophrenia which displays a polygenic architecture (Loh *et al.*, 2015).

However, this simplified dichotomous “familial versus sporadic” representation is now considered outdated as genome-wide studies depict similar genetic architecture between fALS and sALS (van Rheenen *et al.*, 2016). Furthermore, respective definition show limitations

that do not account for the heterogeneity of cases. First-degree relatives of sALS patients are eight times as likely to develop the disease than any other individual (Hanby *et al.*, 2011). In familial cases, Mendelian inheritance is not perfect as the penetrance is inferior to 50% and evidence suggests genetic pleiotropy (multiple phenotypic presentations for a single incriminated mutation). Pleiotropy of incriminated genes can cover a multitude of phenotypic presentation within the ALS-FTD spectrum with variable severity but also completely distinct pathologies. For example, *C9orf72* is linked to cases of ALS, FTD, Parkinson's and Alzheimer's diseases, Huntington phenocopies, as well as psychiatric disorders (Cooper-Knock, Shaw and Kirby, 2014).

Concurrently to this complex genetic profile and heterogeneity of ALS, studies in mice described an effect of the genetic background on the phenotyping presentation of the disease (Heiman-Patterson *et al.*, 2011, 2015). Overall, the genetic contribution to sporadic ALS is estimated at around 60% in two different studies working on twins (Al-Chalabi *et al.*, 2010; Graham, Macdonald, & Hawkes, 1997).

Genetic investigations can also be limited by practical details and lack of information. For example, some familial cases are believed to be falsely considered sporadic because of missing information or premature deaths in the familial history of patients (Al-Chalabi and Lewis, 2011).

(5) Histopathology

Gross abnormalities are not constantly found in ALS patients however, several characteristic traits can be observed. As previously described skeletal muscle show atrophy and signs of denervation but can also show aberrant reinnervation with clustering of atrophic

fibers (Hardiman *et al.*, 2017). Logically, abnormalities can be observed in UMN and LMN systems. For UMN features, the motor cortex may present atrophy with degeneration of Betz cells (Hammer, Tomiyasu and Scheibel, 1979; Nihei, McKee and Kowall, 1993) potentially combined with astrocytic gliosis. In patients with signs of dementia or established overlapping FTD, frontal and temporal atrophy may be observed (Chang *et al.*, 2005; Murphy *et al.*, 2007). Some cases of atrophy of the central gyrus have been reported (Kiernan and Hudson, 1994; Saberi *et al.*, 2015). Axonal loss and subsequent gliosis of descending corticospinal tracts are particularly showing in the lateral and anterior horns of the spinal cord. Myelin pallor can also be associated. For overall UMN and LMN, spinal cord may reveal more than 50% of depletion as well as microglial infiltration but show disparities within cases. Ventral horn motor neurons seem to be particularly affected concerning LMN pathology (Wijesekera and Leigh, 2009). Other ALS associated pathological features also comprise vacuolization and spongiosis (Saberi *et al.*, 2015). These terms respectively depict large empty spaces adjacent of neurons and sponge-like appearance due to the presence of microscopic holes.

Spared motor neurons:

ALS causes degeneration and loss of all types of motor neurons but show relative sparing of 2 particular nuclei: Onufrowicz's and oculomotor nuclei (Iwata and Hirano, 1978; Okamoto *et al.*, 1991; Murayama, Bouldin and Suzuki, 1992). They respectively command sphincter and eye movement control. The reason for this particularity remains unclear but presence of Calcium-binding proteins may protect the neurons from toxic high calcium concentrations (Elliott and Snider, 1995; Vanselow and Keller, 2000). In any case, relative preservation of eye movement can be used as a mean of communication after the loss of oral expression (Beukelman, Fager and Nordness, 2011; Lenglet *et al.*, 2019).

Regardless of their frequency, these abnormalities do not compare with cytoplasmic inclusions or aggregates that are indisputably the pathological hallmark of ALS. Before key protein constituent were identified, the inclusions were classified into different subsets (according to appearance and required staining method to detect them), Lewy body-like, skein-like or Bunina bodies being the predominant forms. Inclusions are mainly reported in degenerating motor neurons of the spinal cord but also, to a lesser degree, in various brain regions (frontal cortex, temporal cortex, hippocampus, cerebellum, striatum...) and occasionally in other cell types namely oligodendrocytes and glial cells (Arai *et al.*, 2003; Piao *et al.*, 2003; Saberi *et al.*, 2015).

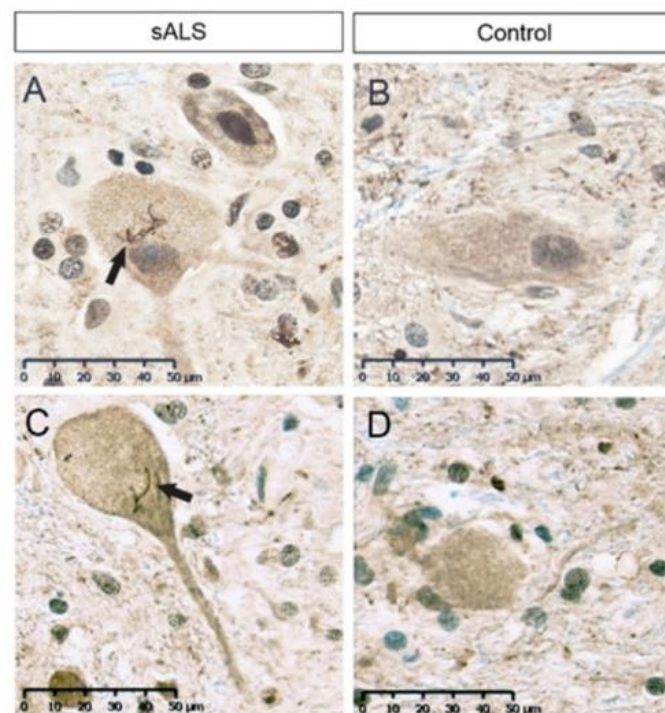


Figure i 6 Ubiquitin-positive inclusions in ALS; modified from Saberi et al. 2015

Ubiquitin skein-like inclusions (arrows) in spinal MN of the lumbar anterior horn (A) and Betz cells of the motor cortex (C) in ALS but not control (B, D).

The first important step towards characterization of ALS related inclusions was when the majority of inclusions (Lewy body-like and skein-like) were described as ubiquitin-positive protein aggregates (Leigh *et al.*, 1988; Lowe *et al.*, 1988) (**Fig i 6**).

As ubiquitination is known to mark specific substrates for degradation, ALS started to be considered as a potential proteinopathy. Indeed, ubiquitin suggested the presence in the inclusions of other proteins displaying misfolding and aggregating behavior. Since these landmark studies, the ALS research community has been driven by the identification of protein constituents that have fed discoveries in the genetic aspects of the disease and the deciphering of pathological mechanisms. Ubiquitin-positive inclusions, regardless of their subtype, are close to be universal in ALS patients as they are thought to be present in 95% of cases (Leigh *et al.*, 1991).

The first aggregating protein to be identified in inclusions was SOD1 (D R Rosen *et al.*, 1993). SOD1 is prone to aggregation and found in inclusions in both fALS and sALS patients. SOD1 importance in the disease is also to be linked with another characteristic cellular abnormality of ALS being markers of oxidative stress and mitochondrial defects. More details about SOD1's implication in ALS will be further discussed throughout this introduction and notably in section **I.C.3) "Implicated cellular mechanisms"**. Within ubiquitin-positive inclusions, SOD1 inclusions were found to be TDP-43-negative which distinguish them from the vast majority of ALS cases (Tan *et al.*, 2007). Indeed, TDP-43 is by far the major protein component of pathological inclusions estimated to represent 97% of cases (Arai *et al.*, 2006; Neumann *et al.*, 2006; Ling, Polymenidou and Cleveland, 2013). Full-length TDP-43 but also its cleaved forms, TDP-35 and TDP-25 are prone to aggregate (Neumann *et al.*, 2006; Y.-J. Zhang *et al.*, 2007). Other TDP-43-negative inclusions mainly are FUS-positive (Kwiatkowski *et al.*,

2009; Vance *et al.*, 2009). On an interesting note, TDP-43 and FUS immunoreactivity is also observed in 45 and 9% of pathological inclusions in FTD patients hence confirming both diseases belong in a common histopathologic spectrum (Mackenzie, Rademakers and Neumann, 2010). Apart from these 3 proteins, the cytoplasmic inclusions occasionally show simultaneous immunoreactivity for proteins involved in protein clearance and homeostasis pathways (UBQLN2, VCP, p62, OPTN, CHMP2B...) (Ling, Polymenidou and Cleveland, 2013).

C9orf72 repeat expansion patients show particular histopathological traits (*C9orf72* and its implication in ALS will be described in section **III.B.2) "Autophagy in ALS"**). They present typical TDP-43-positive inclusions but also TDP-43-negative p62-positive inclusions (Al-Sarraj *et al.*, 2011). Furthermore, colocalizing with p62 but not TDP-43, dipeptide repeat proteins (DPRs) resulting from aberrant non-ATG translation of the *C9orf72* gene repeat expansions (Ash *et al.* 2013). The expanded repeats also cause signature neuropathological foci of RNA (Wojciechowska and Krzyzosiak, 2011; T. Zu *et al.*, 2013) found in various cell types (motor neurons, microglia and astrocytes) (DeJesus-Hernandez *et al.*, 2011) of the spinal cord, motor, frontal and temporal cortices, hippocampus and cerebellum (Mackenzie, Frick and Neumann, 2014). These RNA foci seem to accumulate in cells presenting TDP-43 pathology (Cooper-Knock *et al.*, 2015).

	Predominant pathology	Associated genes
Classic ALS	TDP-43	ALS2, SETX, TARDBP, VAPB, CHMP2b, ANG, UBQLN2, OPTN, PFN1, TUBA4a, UNC13a, FIG4, ELP3, NEK1, C21orf2, SIGMAR1, DCTN1, MATR3, CHCHD10, VCP, hnRNPA1, hnRNPA2b1, NIPA1, SMN1, TBK1, ATXN2, MOBP, SARM1, UBQLN2, SQSTM1
Classic ALS	SOD1	SOD1
Classic ALS	FUS	FUS
ALS with cognitive or behavioural impairment or comorbid FTD	TDP-43	TARDBP, CHMP2b, TBK1, UBQLN2, SQSTM1, DCTN1, UNC13a
Classic ALS, ALS-FTD, FTD	TDP-43, p62, dipeptide repeats, RNA foci	C9orf72
Multi-system proteinopathy*	TDP-43	VCP, hnRNPA1, hnRNPA2b1, SQSTM1
Behavioural variant FTD	TDP-43	CHMP2, GRN
Behavioural variant FTD	FUS	-
Behavioural variant FTD	Tau	MAPT
Semantic variant primary progressive aphasia	TDP-43	GRN, C9orf72
Semantic variant primary progressive aphasia	Tau	MAPT
Logopenic and non-fluent variant primary progressive aphasia	Tau	MAPT

ALS=amyotrophic lateral sclerosis. FTD=frontotemporal dementia. *A familial disorder in which patients present with ALS, FTD, inclusion body myositis, Paget's disease of the bone, or combinations thereof.

Table 1: The various pathologies reported in the spectrum of ALS/FTD; modified from Van Es et al. 2017

C) State-of-art in ALS research

As described previously, ALS is an incurable disease displaying great clinical complexity. Studies on patients offer limited understanding of disease mechanisms that lead to motor neuron degeneration which is crucial for the development of a potential treatment. This

section will review the different models used in ALS research including the ones used in this study and then expose our current knowledge on ALS pathological mechanisms.

(1) Modeling ALS

Animal models have been unable to fully reproduce the complex aspects of ALS but decades of development throughout various model systems have allowed to emulate certain traits of the disease, highlight pathological mechanisms and elaborate potential therapeutic strategies. Modeling can focus on the reproduction of ALS-related phenotypes, axonopathy, aggregation-related mechanisms, expression of mutant protein or full or partial TDP-43 pathology (which will be thoroughly discussed in section **II.B TARDBP in ALS**) and include a wide range of models including pure *in vitro* biochemical systems, small non-mammalian animal models whether invertebrates or vertebrates, rodent models and cell cultures with notably the promising use of patient-derived stem cell models (Van Damme, Robberecht and Van Den Bosch, 2017; Lutz, 2018). The selection of a suitable model has to be question-oriented and should be made in accordance with the intrinsic advantages and disadvantages of each system (**Fig i 7**). We will be briefly elaborate the specifics of some of these systems and key advances that have been made with their use.

The yeast model *Saccharomyces cerevisiae* has been successfully used for its wide range of available genetic tools and its resulting capacity to allow efficient genetic screening (whether overexpression or deletion of gene) notably through simple growth assay in the context of ALS (Elden *et al.*, 2010; H.-J. Kim *et al.*, 2013; Jovičić *et al.*, 2015). About a third of yeast genes have homologous genes in human which has allowed successful translation of data. This model has notably allowed identification of ATXN2 as a genetic risk factor in ALS by

unveiling ATXN2's capacity to interact with TDP-43 and modify TDP-43-related toxicity (Elden *et al.*, 2010).

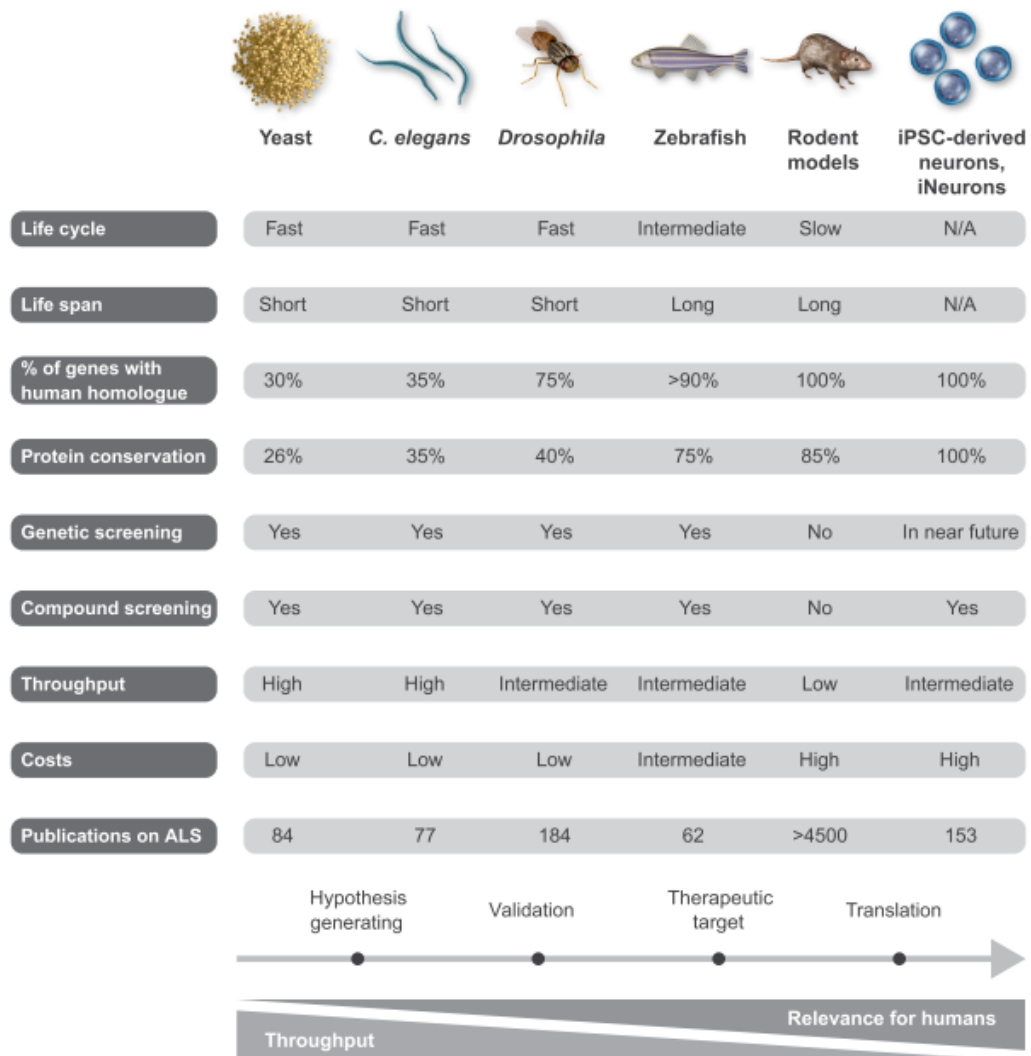


Figure i 7: Model systems in ALS research; modified from Van Damme *et al.* 2017.

A variety of cell lines have been used in ALS research notably in the study of disease-associated gene function through their depletion or overexpression (mutant or wild-type). For example, our understanding of C9orf72's implication in autophagy has benefited from cellular systems (Webster *et al.*, 2016). Cell cultures have been particularly useful in the mechanistic deciphering of aggregation and notably the importance of low-complexity

domains of key ALS proteins such as TDP-43 (Molliex *et al.*, 2015). Showing reduced practicability but enhanced context-relevance, primary cultures of rodent neurons and more particularly motor neurons have helped unveil their vulnerability to several factors and ALS mutant-related mechanisms (Nagai *et al.*, 2007; Van Damme *et al.*, 2007; Song *et al.*, 2016) and possible neuroprotective factor (Bordet *et al.*, 2007). The generation of induced pluripotent stem cells (iPSCs) from patient tissues and their differentiation in neurons (Son *et al.*, 2011) or even spinal motor neurons (Maury *et al.*, 2014) appears very promising for ALS research. iPSCs have the unique ability to model sporadic ALS which cannot be achieved in other model systems (Matus, Medinas and Hetz, 2014). iPSC ALS-models were successful in recapitulating multiple aspects of the pathology as well as in highlighting disease-related functional phenotypes (Bilican *et al.*, 2012; H. Chen *et al.*, 2014; Kiskinis *et al.*, 2014; Matus, Medinas and Hetz, 2014; Zhang *et al.*, 2015) that will hopefully allow genetic screening in the future (Li-Juan *et al.*, 2016). By combining this stem cell system to the use of genetic tools such as gene-editing techniques, iPSCs offers the possibility to study the effects of disease-related mutation correction (Kiskinis *et al.*, 2014; Sances *et al.*, 2016). Nonetheless, iPSC ALS modeling still need improvement (Sances *et al.*, 2016; Van Damme, Robberecht and Van Den Bosch, 2017) and would benefit from the integration of disease-relevant components such as improved cellular context with realistic interactions with non-neuronal cells or an aging component. For these reasons, the translational potential of iPSCs are still questioned (Van Damme, Robberecht and Van Den Bosch, 2017). However, they show great promises for the future of ALS research notably for potential therapeutic strategies (Egawa *et al.*, 2012; Lagier-Tourenne *et al.*, 2013; Sareen *et al.*, 2013).

Observations made in cellular models are limited by the lack of a realistic biological context and integration of the complex mechanistic interplays found in a living animal model.

Among the models used in ALS research, the vast majority of published studies are conducted in rodent models (**Fig i 7**). There are rodent models for all of the most prevalent genetic mutations in ALS including SOD1, TARDBP, FUS and C9orf72 (Turner and Talbot, 2008; Gendron and Petrucelli, 2011; Philips and Rothstein, 2015; Nolan, Talbot and Ansorge, 2016) displaying certain aspects of the disease such as hallmark TDP-43 pathology or ALS-like motor phenotype (Lutz, 2018). Our current understanding of pathological mechanisms leading to motor neuron degeneration mostly results from the use of rodent models (Ferraiuolo *et al.*, 2011). For example, the “dying-back” hypothesis was revealed through the use of mutant SOD1 mice (Fischer *et al.*, 2004). These mechanisms will be discussed in section **I.C.3** “**Implicated cellular mechanisms**”. Being a mammalian model, they show great genetic homology to humans. Overall, it is the dominant ALS-modeling system before translation to the clinic (Van Damme, Robberecht and Van Den Bosch, 2017). The main drawbacks of these rodent models are the inbred nature of mouse colonies, the high cost and extensive time required in order to generate a rodent model (Lutz, 2018).

As a result, there has been an increasing interest for several alternative vertebrate and invertebrate models in ALS research. These “small animal” models may lose in genetic homology and protein conservation to humans as compared to rodents but gain in throughput and convenience to obtain statistical relevance (Van Damme, Robberecht and Van Den Bosch, 2017). *Drosophila Melanogaster*, or fruit fly, is the most widely used among these alternative animal models as studies take advantage of the relative simplicity of its 4 chromosome-genome (McGurk, Berson and Bonini, 2015), the great range of genetic tools available (Ugur, Chen and Bellen, 2016), its rapid life cycle and rather sophisticated nervous system (Pandey and Nichols, 2011). Among other ALS-related genes, models of deletion and/or overexpression of human wild-type or mutant SOD1 (Watson *et al.*, 2008), TARDBP (Feiguin *et al.*, 2009; Estes

et al., 2011; Vanden Broeck *et al.*, 2013), FUS (Chen *et al.*, 2011; Lanson Jr *et al.*, 2011) and C9orf72 (Xu *et al.*, 2013; Mizielinska *et al.*, 2014) efficiently display toxicity in drosophila and have helped strengthen our knowledge of pathological mechanisms in ALS (Van Damme, Robberecht and Van Den Bosch, 2017).

Caenorhabditis elegans models of these four main ALS-related mutations have also been generated (Therrien and Parker, 2014). This nematode model is extremely convenient for genetic pathways studies and has revealed interplays between ALS-related genes (Vérièpe, Fossouo and Parker, 2015), allowed the identification of disease modifying factors for motor neuron degeneration (Aggad *et al.*, 2014; Jablonski *et al.*, 2015) and TDP-43 proteinopathy (Liachko, Guthrie and Kraemer, 2010; Liachko *et al.*, 2016). *C. elegans* displays an even shorter life cycle than the drosophila (4 days) and its 302-neuron nervous system is precisely mapped and referenced (Therrien and Parker, 2014). Along the spectrum of available animal models, in between these two invertebrate models, that show reduced genetic homology to humans, and rodent models, which development is rather costly, time-consuming and incompatible with high throughput screenings, is the zebrafish model that will be used in this study (see section I.C.2)a) “**The zebrafish**”).

Even though these models have greatly contributed to ALS research, no successful translation to the clinic has been achieved as of yet. In this context, a cross-model strategy may be adequate to ameliorate translation to patients (Van Damme, Robberecht and Van Den Bosch, 2017).

(2) *The models of this study*

(a) *The zebrafish*

Overview:

Danio rerio, commonly called zebrafish, is a small freshwater tropical fish originating from South Asia (**Fig i 8**). It belongs to the Danio genus, a class a teleost fish of the greater Cyprinidae family (Meyers, 2018). Apart from being a common aquarium pet fish it has been used for research purposes for more than 40 years for its ease of care and manipulation, small size, high fecundity (Hisaoka and Firlit, 1962) and rapid development (Kimmel *et al.*, 1995).



Figure i 8: *Danio rerio*; Aquaportail.com

The development of the zebrafish has been extensively described (Kimmel *et al.*, 1995) notably due to the convenient transparency of the cells and surrounding protective chorion. As it shows variability according to temperature, the timing of developmental stages is standardized to a temperature of 28,5°C (Kimmel *et al.*, 1995). Male and female show courtship behavior to synchronize release of oocytes and sperm in water and allow fertilization (Darrow and Harris, 2004). Several hundreds of eggs can be laid by a single gravid female (Hisaoka and Firlit, 1962). After fertilization, the development is rapid. 10 hours post fertilization (hpf), epiboly is complete and distinct head and tailbud are forming. Every 20 minutes, the trunk undergoes segmentation into a new somite thus expanding rapidly

(Kimmel *et al.*, 1995). Neurulation begins and leads to formation of the nervous system. At 36 hpf, most major organs are formed. The motor system is already functional at 2 days post fertilization (dpf) (Kimmel *et al.*, 1995; Drapeau *et al.*, 2002; Schmidt, Strähle and Scholpp, 2013), the larvae hatches from its chorion between 48 to 72 hpf, acquires the capacity for upright swimming (Kimmel *et al.*, 1995) and around 4 dpf, it develops a feeding behavior (Muto and Kawakami, 2013). The larvae will be called a juvenile after 4 weeks and achieve sexual maturity at about 12 weeks (Parichy *et al.*, 2009).

If research on the zebrafish historically concerned genetic, toxicology and development studies (Meyers, 2018) with notably a focus on its regenerating capacities (Bernardos *et al.*, 2007; Gemberling *et al.*, 2013), the model is becoming a model system for studying human diseases of increasing importance (Seth, Stemple and Barroso, 2013). The genome of the zebrafish has been fully sequenced and shows great homology (between 80 and 90%) and protein conservation (around 75%) to humans (Howe *et al.*, 2013). The transparency of the zebrafish is of great use as it enables whole-mount live imaging notably allowing tracking of fluorescent labeled compounds (Kimmel *et al.*, 1995; Kabashi *et al.*, 2010; Kabashi, Brusteijn, *et al.*, 2011). A collection of transgenic lines expressing fluorescent proteins under promoters specific for a population of cell (Higashijima, Hotta and Okamoto, 2000; Flanagan-Steet *et al.*, 2005; Bradford *et al.*, 2010; Naumann *et al.*, 2010; Zelenchuk and Brusés, 2011; Satou *et al.*, 2013). A great range of genetic modifications can be performed in zebrafish such as gene editing strategies based on ZFN (zinc-finger nuclease) and TALEN (transcription activator-like effector nuclease) systems (Doyon *et al.*, 2008; Foley *et al.*, 2009; Bedell *et al.*, 2012; Dahlem *et al.*, 2012; Y. Zu *et al.*, 2013) or the most recent CRISPR/Cas9 system (Hruscha *et al.*, 2013; Hwang *et al.*, 2013; Jao, Wente and Chen, 2013), conditional gene activation or

inactivation techniques (Ni *et al.*, 2012) and the widely used morpholino-mediated transient knockdown technique (Stainier, Kontarakis and Rossi, 2015; Stainier *et al.*, 2017).

Morpholino-mediated knockdown:

Morpholinos (or AMOs) are chemically modified antisense oligonucleotides that are able to bind nucleic acids. They block translation of mRNAs in vitro and in vivo in a RNase-H-independent manner. By binding to their target mRNA, AMOs block the recruitment of the translational machinery through steric inhibition (Summerton and Weller, 1997). They thus allow specific and transient knockdown of a targeted gene and have been used extensively in both *Xenopus* and zebrafish (Heasman, Kofron and Wylie, 2000; Nasevicius and Ekker, 2000). The method offers a rapid way to observe physiological consequences of gene knock down as F0 morphants (embryos injected with AMO) can be analyzed. This rapid observation allows direct visualization of knock down consequences without installation of genetic compensatory mechanisms that can occur during generation and selection of classic loss-of-function mutant (Rossi *et al.*, 2015) thus explaining potential phenotypic differences observed when comparing the two methods (Kok *et al.*, 2015). Potential side effects of AMO use was evoked (Gentsch *et al.*, 2018) but recent data tend to refute these observations (Paraiso *et al.*, 2019).

They are key controls to ensure the specificity of a phenotype obtained upon AMO-based knockdown. A dose-dependent assay is performed to select the lowest dose of AMO that is effective and avoid the toxic effects of excessive dosage. The use of a control mismatch AMO that does not bind to any region of the model's genome is also required. It is necessary to use AMOs that target different regions of the same gene (such as one that binds the initial ATG (ATG-AMO) and one splice-blocking AMO) to confirm that the observed phenotype is specific of the knockdown. Finally, rescue of the induced phenotype through co-injection of

RNA or cDNA of the targeted gene (either of the human orthologue or a modified zebrafish version untargeted by the AMO) constitutes a strong proof of the specificity of the phenotype.

Behavioral tests can be conducted on both adult and larval zebrafish (Meyers, 2018). For example, early motor activity can be assessed at 48 hpf classically due to the larvae touch-evoked escape response or TEER and as soon as 17hpf when the embryo displays stereotyped motor activity within its chorion (Drapeau *et al.*, 2002). *Danio rerio* is the only vertebrate model that enables high-throughput screening of chemical compounds for altered behavioral patterns (Zon and Peterson, 2005; Rihel *et al.*, 2010; Orger and de Polavieja, 2017).

The zebrafish in ALS research:

Danio rerio is increasingly being used in ALS research for its many advantages and vertebrate model qualities (Babin, Goizet and Raldua, 2014; Patten *et al.*, 2014). Studies have been conducted on adults, notably for in mutant SOD1 (Ramesh *et al.*, 2010) , but most of the use of the zebrafish concern embryonic models (Van Damme, Robberecht and Van Den Bosch, 2017). Transient knockdown of an endogenous zebrafish orthologue can eventually be combined with transient expression of the corresponding or a different wild type or mutant human protein through injection of corresponding human mRNA or cDNA. This has allowed the generation of zebrafish models for the main ALS-related genes SOD1, TARDBP, FUS and C9orf72 among others. These strategies have notably helped in the demonstration of gain-of-function mechanisms for mutant SOD1 (Sakowski *et al.*, 2012; Van Hoecke *et al.*, 2012) and combined loss-of-function and gain-of-function mechanisms for pathogenesis concerning

TARDBP, FUS and C9orf72 (Armstrong and Drapeau, 2013b; Ciura *et al.*, 2013; Hewamadduma *et al.*, 2013; Lee *et al.*, 2013; Schmid *et al.*, 2013) through the observation of neuronal toxicity and motor deficits. Moreover, a common pathogenic pathway was suggested for TARDBP and FUS (Kabashi, Bercier, *et al.*, 2011). Screening tests conducted on ALS zebrafish models permitted identification of genetic modifiers of the disease such as EphA4 (Van Hoecke *et al.*, 2012) and confirmation of potentially therapeutic compounds (McGown *et al.*, 2013).

These mechanisms will be further described in the next sections of this introduction **(I.C.3) “Implicated cellular mechanisms”, II. “TARDBP and RNA metabolism: a central role in ALS” and III.B.2) “Autophagy in ALS”**).

(b) SH-SY5Y cells

This thesis will present data conducted both zebrafish and human cells to benefit from the advantages of a cross-model approach. The SH-SY5Y cell line is a cloned subline of SK-N-SH cells that was developed from a bone marrow biopsy of neuroblastoma on a 4-year old female in the 1970's (Biedler, Helson and Spengler, 1973; Biedler *et al.*, 1978). Contrarily to SK-N-SH cell line that contains different cellular phenotypes, SH-SY5Y cell line is a homogenous population of neuroblast-like cells. They can proliferate in culture for long periods with little risks of contamination. As they derive from immature neural crest cells, SH-SY5Y exhibit stem cell properties thus allowing potential differentiation protocols (Påhlman *et al.*, 1981; Singh and Kaur, 2007; Guarnieri *et al.*, 2009). Because they display biochemical and functional properties of neurons, this cell line has been widely used in neurological studies and notably in neuronal differentiation and neurodegenerative mechanisms, neurotoxicity and neuroprotection processes (Xie, Hu and Li, 2010; Xicoy, Wieringa and Martens, 2017).

While investigating novel interactions between TDP-43 and ALS-related autophagy genes, the zebrafish model will provide an insight into integrative whole-organism functional interplay while the SH-SY5Y human cell line will help us in the description of the underlying molecular and cellular mechanisms at play while staying in a neuronal context.

siRNA-mediated knockdown:

In this study, siRNA will be used in SH-SY5Y cells to achieve transient knockdown through direct transfection. Small interfering RNA are double stranded ribonucleic acid of around 20 base pairs that will form a complex with the RISC (RNA-induced Silencing Complex) and unwound into single strand RNA that will be able to bind its complementary endogenous RNA target with high specificity and induce its cleavage. The cleavage will cause degradation of the target RNA thus preventing translation into protein (Carthew and Sontheimer, 2009).

(3) Implicated cellular mechanisms

Several defective cellular mechanisms have been incriminated for ALS pathogenicity. Their discovery directly benefited from advances in the genetic etiology (**Fig i 9**) and ALS modeling. Although witnessed in animal models or patients, these mechanisms are not necessarily relevant in all presentation of ALS and their relative impact is still under debate. Among these mechanisms, impaired autophagy and RNA metabolism, two major incriminated processes in ALS, will each benefit from in-depth descriptions in their dedicated sections later in this introduction (respectively in sections **III.B “Autophagy’s implication in ALS”** and **II.C.2 “RNA metabolism defects in ALS”**).

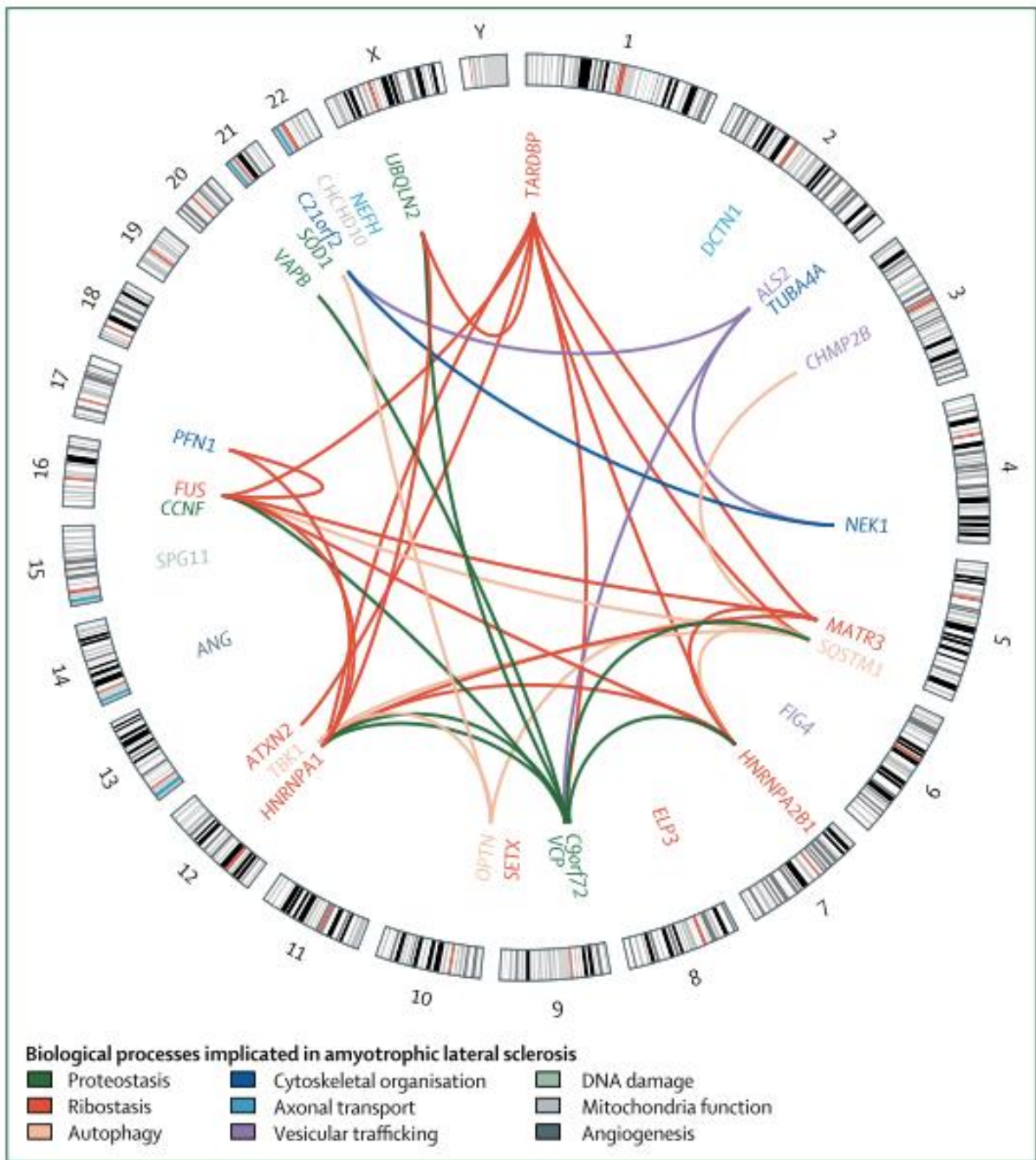


Figure i 9: Biological processes implicated in ALS; modified from Chia et al. 2018.

Ideogram of the human karyotype showing location of each implicated genes.

Impaired protein homeostasis:

A healthy and functional cell requires maintenance of its protein homeostasis or proteostasis. This equilibrium is achieved by multilevel balancing between protein synthesis, folding, trafficking but also degradation (Kurtishi *et al.*, 2019).

Numerous genes found mutated in ALS patients, encode for proteins involved in several mechanisms of protein degradation namely autophagy, UPS (ubiquitin proteasome system), ER stress and stress granule (SG). These mechanisms have been clearly related to neurodegeneration when impaired (Medinas, Valenzuela and Hetz, 2017). As described previously, some of these proteins such as p62/SQSTM1, OPTN, VCP or UBQLN2 can also be found in the cytoplasmic proteinaceous aggregates and inclusions (Ling, Polymenidou and Cleveland, 2013), a hallmark of ALS pathology, which concurrently, is a clear sign of impaired proteostasis (for more information on these proteins, see section **III.B “Autophagy’s implication in ALS”**).

Protein clearance is mainly endorsed by two pathways that are both relevant to ALS: autophagy (which will be thoroughly described later in this introduction) and Ubiquitin Proteasome System (UPS).

Ubiquitin Proteasome System:

UPS is a eukaryote intracellular system in which, proteins targeted for degradation are tagged by a small protein called ubiquitin. The polyubiquitinated substrate will then undergo degradation by an enzymatic complex, the proteasome, through proteolysis (Tanaka, 2013).

ALS related mutations have been associated with impaired UPS (Rueggsegger and Saxena, 2016). Mutant UBQLN2 and VCP alter substrate delivery to the proteasome (Barthelme *et al.*, 2015; Deng *et al.*, 2011; Le *et al.*, 2016; Nalbandian *et al.*, 2012; Seok Ko *et*

al., 2004). In a mouse model (Kabashi *et al.*, 2004) or in neuronal cell cultures (Urushitani *et al.*, 2002), SOD1 mutation cause global decrease of UPS activity.

Intracellular accumulation of misfolded proteins locally causes endoplasmic reticulum stress (ER stress). This triggers the activation of the UPR, the unfolded protein response that will, depending on the importance of the stress, help with degradation and recovery of normal ER function or will engage in apoptosis (Lee and Chae, 2015). Once again, this protective proteostasis mechanism has been implicated in ALS (Matus *et al.*, 2013). Mutant SOD1 has been shown to inhibit ER stress related degradation through direct binding to the ER membrane (Nishitoh *et al.*, 2008). Interestingly, in a mouse model of ALS, drug-mediated reduction of ER-stress attenuated the symptoms and delayed progression of the disease while chronic enhancement promoted ALS manifestations (Saxena, Cabuy and Caroni, 2009).

Cellular stress can also trigger another protective mechanism, the formation of stress granules (SG). They are membrane-less granules composed of both protein and RNAs that are able to assemble and disassemble quickly to suppress translation of non-essential proteins and sequester unnecessary mRNAs (Lin *et al.*, 2015). However, aberrant accumulation of SG has been reported in ALS and reflects impairments at a crossroad between proteostasis and RNA metabolism (Taylor, Brown and Cleveland, 2016; Weishaupt, Hyman and Dikic, 2016). ALS-related mutations in *VCP* have been reported to alter SG dynamics and clearance (Buchan *et al.*, 2013; Seguin *et al.*, 2014). RNA-binding proteins (RBPs) such as ALS-related TDP-43 or FUS are components of SG (Lin *et al.*, 2015; Monahan, Shewmaker and Pandey, 2016).

Mitochondrial damage and oxidative stress:

Several mutations in genes involved in mitochondrial function have been linked to ALS among which CHCHD10, SIGMAR1, C21orf2, NEK1 and ALS very first associated gene SOD1 (Hardiman *et al.*, 2017).

Superoxide dismutase 1:

SOD1 is a dimeric antioxidant enzyme containing a Cu and a Zn ion in its core. Its main function is the conversion of superoxide radicals into hydrogen peroxide thus reducing the quantity and toxicity of reactive oxygen species (ROS) (McCord and Fridovich, 1969; Fridovich, 1995; Wang *et al.*, 2018). The protein is abundant and located in various cellular compartment including cytosol, nucleus but also in the inner membrane space of the mitochondria (Chang *et al.*, 1988; Crapo *et al.*, 1992; Liou *et al.*, 1993; Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001). SOD1 is logically believed to be involved in protection against oxidative stress and mitochondrial metabolism. SOD1 was the first protein identified in ubiquitin-positive inclusion in ALS and the first identified genetic cause of the disease (Daniel R Rosen *et al.*, 1993). As of yet, it is still one of the main genetic cause identified in ALS accounting for around 12% of fALS and up to 2% of sALS (Chiò *et al.*, 2008). Among the more than a hundred SOD1 mutations identified, only a few show evidences of pathogenicity which indicate two potential gain-of-function mechanisms: specific protein cytotoxicity and protein aggregation (Andersen, 2006). Overall, SOD1-related ALS is now recognize as a distinct form of the disease as their peculiar histopathologic profile without TDP-43-positive inclusions indicate (Mackenzie *et al.*, 2007) (see section **I.B.5) “Histopathology”**).

ALS has been related to multiple mitochondrial impairments: production of ROS (reactive oxygen species), altered oxidative phosphorylation and calcium buffering as well as abnormal apoptotic signaling (Pasinelli *et al.*, 1998, 2000; Wiedemann *et al.*, 1998, 2002; Li *et al.*, 2000; Parone *et al.*, 2013). Such defects are critical in motor neurons as their length and activity particularly requires efficient mitochondrial function. In patients and mouse models (SOD1 and TDP-43 ALS models), changes in morphology and distribution of mitochondria have been reported (Vande Velde *et al.*, 2011; Magrané *et al.*, 2013). Mice with mutant SOD1 present aggregates of the mutated protein that colocalize with VCP in the inner-membrane space of mitochondria thus impairing protein import (Higgins, Jung and Xu, 2003) but also show oxidative damage to mitochondria resulting in defective respiratory chain function (Parone *et al.*, 2013). Defective axonal transport of mitochondria, concurrent with axonopathy in ALS, was observed in several animal models (Laird *et al.*, 2008; Bilslund *et al.*, 2010).

Other ALS-related proteins are related to mitochondrial defects. Both FUS and TDP-43 were reported to cause perturbations in ER/mitochondria signaling by inhibiting binding of VAPB, an ER protein, to PTPIP51, a regulatory mitochondrial protein (De Vos *et al.*, 2011; Stoica *et al.*, 2014, 2016). As described previously, TDP-43 is prone to aggregation in cytosol but also in mitochondria where it also leads to defects. In yeast, its mitochondrial localization is linked with disturbed electron transport chain and overall respiratory function impairment (Ralf J. Braun *et al.*, 2011) while it activates mitophagy and alter general mitochondrial function in murine motor neurons (Lu *et al.*, 2012). TDP-43 and particularly ALS mutant is able to enter mitochondria where it can bind to mitochondrial mRNAs. Mutant TDP-43 (and to a lesser degree wild type TDP-43) inhibits translation of two respiratory chain complex I subunits

mRNAs (ND3 and ND6) through direct binding thus impairing assembly of the complex and its function. Inhibiting TDP-43 localization in mitochondria interestingly reduced its related toxicity (W. Wang, Li, W. L. Lin, *et al.*, 2013; W. Wang *et al.*, 2016).

Furthermore, C9orf72 derived DPRs seem to disturb mitochondrial function and cause oxidative stress as well as DNA damage (Lopez-Gonzalez *et al.*, 2016). ALS associated CHCHD10 mutation had a deleterious impact on mitochondrial genome, altered apoptosis signaling and promoted loss of mitochondrial cristae junctions (Genin *et al.*, 2016).

Accumulation of ROS leads to oxidative stress which causes damages to cell structure, protein, DNA and RNA and is linked to motor neuron loss (McCord and Fridovich, 1969; Daniel R Rosen *et al.*, 1993; Rao *et al.*, 2008; Tsang *et al.*, 2014). Sub-products of these oxidative stress damages are increased in CSF and serum from both fALS and sALS patients (Lyras *et al.*, 1996; Smith *et al.*, 1998; Simpson *et al.*, 2004; Mitsumoto *et al.*, 2008), as well as in cellular or animal model of the disease (Barber and Shaw, 2010; Parakh *et al.*, 2013).

Impaired cellular trafficking:

TDP-43 is involved in regulation of endosomal trafficking (Schwenk *et al.*, 2016). Its loss-of-function was reported to alter traffic of dendritic endosomes which affected global neuronal health. As described previously, mitochondrial transport throughout the axon was reduced by ALS-related proteins TDP-43, FUS, VAPB and SOD1 (Y.-F. Xu *et al.*, 2011; Mórtz *et al.*, 2012; Magrané *et al.*, 2013; W. Wang, Li, W. L. Lin, *et al.*, 2013; Baldwin *et al.*, 2016) in several ALS models. Motor neuron degeneration, due to defective anterograde/retrograde transport, was also reported in *SOD1* transgenic mice (Ligon *et al.*, 2005; Bilsland *et al.*, 2010).

Axonal transport, a mechanism of crucial importance for lengthy motor neuron function, relies on proteins that were found to be mutated in ALS patients and associated to

defective axonal transport: DCTN, PFN1, CHMP2B and TUBA4A (Puls *et al.*, 2003; Urwin *et al.*, 2010; Wu *et al.*, 2012; Smith *et al.*, 2014). Two other ALS-related mutated gene, ALS2 and UNC13A were related to trafficking defects as they are respectively involved in endosome trafficking and fusion (Topp *et al.*, 2004; Devon *et al.*, 2006) and synaptic release of vesicles for neurotransmission (Böhme *et al.*, 2016). Mutations in NEFH (neurofilament heavy polypeptide) is present in few ALS patients but direct link to axonal dysfunction isn't clear (Garcia *et al.*, 2006). Similarly, rare mutations in PRPH are involved in neurofilament function such as protein cargo trafficking (Gros-Louis *et al.*, 2004; Corrado *et al.*, 2011). TDP-43 mutations alters the axonal transport of its RNA partners both *in vitro* and *in vivo* (Alami *et al.*, 2015).

Apart from genetic evidence, defective axonal transport in ALS also benefited from histopathological observations made in patients motor neurons such as abnormal accumulation of phosphorylated neurofilament, mitochondria and lysosomes (Hirano, Donnemfeld, *et al.*, 1984; Hirano, Nakano, *et al.*, 1984; Okada *et al.*, 1995; Rooke *et al.*, 1996).

Altered function of glial cells and neuroinflammation:

Neuronal loss has been associated with several non-cell-autonomous mechanisms in ALS, whether it is in animal models or in ALS patients. Interestingly, these mechanisms have been observed early on in the disease, before the appearance of symptoms (Endo, Komine and Yamanaka, 2016). For example, autopsies performed on ALS patients demonstrated activation of astrocyte, microglia and infiltration of T cells at degenerating motor neurons (Engelhardt and Appel, 1990; Engelhardt, Tajti and Appel, 1993; Henkel *et al.*, 2004; Brettschneider *et al.*, 2012), suggesting the implication of neuroinflammation. Evidence of neuroinflammation was

indeed found in both patients and mice (Corcia *et al.*, 2013; Brites and Vaz, 2014). In mutant *SOD1* mouse model, activation of astrocytes and microglia exacerbates pathology (Yamanaka *et al.*, 2008; Haidet-Phillips *et al.*, 2011) while specific loss of mutant *SOD1* in astrocytes delayed disease onset and progression (Wang, Gutmann and Roos, 2010). Interestingly, specific expression of mutant *SOD1* in motor neurons didn't show such impact on the progression of the disease (Wang, Gutmann and Roos, 2010).

Mouse *C9orf72* loss of function model displays hyperactivated immune responses of macrophage and increased circulating proinflammatory cytokines (O'Rourke *et al.*, 2016). Loss of function of *TBK1*, a known cause of ALS (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015), is another concurrent sign of immune response impairments as it plays a role in innate immunity and inflammation by interacting with mTOR complex (Hasan *et al.*, 2017; Bodur *et al.*, 2018) and ALS-related *SQSTM1/p62* and *OPTN* (Richter, Danielle A. Sliter, *et al.*, 2016; He, Chen and Li, 2017; Li *et al.*, 2018) (interactions also involved in autophagy). *TBK1* is indeed linked to several autoimmune diseases and stands at a crossroad between autophagy and neuroinflammation (Oakes, Davies and Collins, 2017).

Oligodendrocytes provide metabolic support and allow healthy neuronal function (Rinholm *et al.*, 2011; Lee *et al.*, 2012), their dysfunction, degeneration and failure to regenerate have been observed in ALS (Kang *et al.*, 2013; Philips *et al.*, 2013) thus contributing to axonopathy. In mutated *SOD1* mice, oligodendrocytes seem to contribute to motor neuron death in a *SOD1*-dependant manner (Ferraiuolo *et al.*, 2016). If correlation is evident, oligodendroglia impairments clear contribution to the pathogenesis in ALS patients is still under debate.

Excitotoxicity (Kretschmer, Kratzer and Schmidt, 1998; Wang, Wang and Wang, 2004) and failure of DNA repair (W.-Y. Wang *et al.*, 2013; Sama, Ward and Bosco, 2014) are other notable pathological mechanisms investigated in ALS.

The identified key molecular actors of ALS and their related pathologic mechanisms reveal a paradoxical trait of the disease: the specific vulnerability of motor neurons to defects presumably caused by ubiquitous proteins. The nature and morphology of motor neurons are thought to play a role but partially explains the phenomenon which widely remains a mystery for the ALS community (Hardiman *et al.*, 2017). However, on a grosser scale, animal models allowed insights into the early stages of the disease which was inaccessible in patients leading to the hypothesis of pathogenesis through distal axonopathy in ALS. In the “dying-back” hypothesis, axonal retraction from neuromuscular junctions and degeneration would happen prior to the degeneration of the cell body of neurons in the spinal cord (Fischer *et al.*, 2004). This process may involve several molecular mechanisms that we evoked in this section as well as defects in RNA metabolism and autophagy that will be extensively described later in this introduction (see sections **II.C “RNA metabolism and ALS”** and **III.B “Autophagy’s implication in ALS”**).

II) TARDBP and RNA metabolism: a central role in ALS

TDP-43 holds a central role in ALS pathology and is essential to this study as we investigate novel aspects of its interaction with autophagy. In this section, I will review its

normal function and implication in ALS before tying it to more global RNA metabolism defects in ALS.

A) TARDBP

(1) Discovery and initial characterization

TDP-43 is now mainly known for its RNA-binding activity but it was not its first attributed function when it was characterized in 1995. It was described as a repressive transcription factor of HIV1 (Human Immunodeficiency Virus type 1) gene expression (Ou *et al.*, 1995). TDP-43, which was reported to be 43kDa, binds to a polypyrimidine-rich motif in HIV's transactive response DNA element, TAR. The authors thus decided to designate it TAR DNA binding protein (*TARDBP* encoding for TDP-43).

The *TARDBP* gene, located on chromosome 1p36.22, is composed of six exons (five coding and one non-coding). It encodes for TDP-43, a highly conserved and ubiquitously expressed protein (Ayala *et al.*, 2005) (**Fig i 10**). Although there are some exceptions (pathologic or not), TDP-43 is predominantly nuclear (Buratti *et al.*, 2001). In its N-terminal region, the peptide contains two RNA recognition motifs, RRM1 and RRM2, that allow binding to RNA but also single stranded DNA (Buratti and Baralle, 2001; Buratti *et al.*, 2001; Wang *et al.*, 2004; Ayala *et al.*, 2005). These motifs are flanked by a nuclear localization and a nuclear export signal that allow the protein to shuttle between nucleus and cytosol (Ayala *et al.*, 2008; Matthew J. Winton *et al.*, 2008). Interestingly, the N-terminal domain was found to be crucial for the RNA-binding activity as it enabled TDP-43 dimer and oligomer formation that are distinct from pathological aggregates and necessary for physiological TDP-43 function (Afroz

et al., 2017; Jiang *et al.*, 2017). The C-terminal region, which contains a Gly-rich domain, allows recognition of proteins including other RNA-binding proteins (Buratti *et al.*, 2005). The region is also called Low Complexity domain (LCD) or prion like domain as the protein is prone to pathological aggregation (King *et al.* 2012). A role of the prion-like domain in RNA granule assembly has been proposed (Kato *et al.*, 2012; T. W. Han *et al.*, 2012).

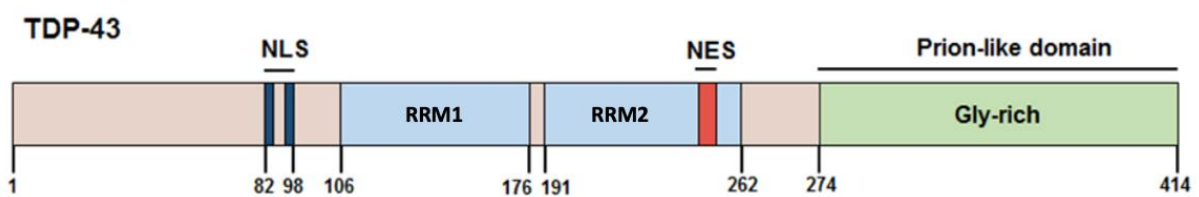


Figure i 10: TDP-43's domains; modified from Shynrye Lee *et al.* 2015.

NLS: Nuclear Localization Signal; NES: Nuclear Export Signal; RRM: RNA Recognition motif

(2) TDP-43's normal function

TDP-43, being able to bind to RNA, DNA and proteins, is involved in numerous interactions and serves a wide range of cellular processes. Early developmental expression of the protein was reported in both brain and spinal cord of zebrafish (Shankaran *et al.*, 2008). In mice, TDP-43 was described to be widely expressed at embryonic stages with particularly high levels in the CNS. The study indicated that TDP-43 was developmentally regulated and vital in early development as full knock-out of the protein was lethal at embryonic stage (Sephton *et al.*, 2010).

TDP-43 is able to act on gene expression in several ways. Although the complete spectrum of target genes is poorly known and requires genome wide studies, TDP-43 can act

as a transcription repressor (Ling *et al.*, 2010; Sephton *et al.*, 2011). It also interacts with other transcription protein including, among others, MeCP2 (Ling *et al.*, 2010; Sephton *et al.*, 2011). TDP-43 displays a splicing activity on pre-mRNAs that is linked to its C-terminal domain and binding to other nuclear splicing factors (Buratti *et al.*, 2005; Buratti and Baralle, 2010). Another way TDP-43 can act on transcriptional regulation is by promoting microRNA biogenesis (Kawahara and Mieda-Sato, 2012). TDP-43 promoting effect comes from direct binding to particular primary microRNAs or later on through interaction with nuclear complex Drosha which acts on precursor microRNAs. The interaction follows through even later as cytoplasmic TDP-43 interacts with the Dicer complex thus promoting the processing of precursor microRNAs (Kawahara and Mieda-Sato, 2012). Long noncoding RNA are also bound by TDP-43 thus implicating the protein in their various regulatory functions (Tollervey *et al.*, 2011; Ling, Polymenidou and Cleveland, 2013).

More globally, the ribonucleoprotein TDP-43 is involved in nearly all steps of RNA metabolism which includes splicing, maturation, transport, stability and translation but also SG formation whether acting alone or in complexes with other proteins (Lagier-Tourenne, Polymenidou and Cleveland, 2010; Buratti and Baralle, 2012; Lagier-Tourenne *et al.*, 2012). Unlike DNA targets, the range of target RNAs of TDP-43 has benefited from numerous studies in various models (Sephton *et al.*, 2011; Xiao *et al.*, 2011; Colombrita *et al.*, 2012) including genome-wide approaches notably in human and mouse brain (Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). We now know that TDP-43 binds to about a third of total transcriptome, which in the brain comprises about 6000 RNAs targets. The studies confirmed the importance of TDP-43 for the CNS as regulated and spliced RNA targets include transcripts encoding for neuronal development regulating proteins or proteins implicated in various neurological disorders. These studies have shown that TDP-43 binds preferentially to long

clusters of UG-rich sequences (Tollervey *et al.*, 2011; Colombrita *et al.*, 2012) mostly found in the 3'UTR of bound transcripts. Importantly, TDP-43 has been reported to bind to its own mRNA hence outlining self-regulation through a negative feedback loop (Ayala *et al.*, 2011; Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). By interacting with similar RNPs and notably the transcript of ALS-related FUS, TDP-43 can also act on RNA metabolism without any direct binding. It was estimated that roughly 300 RNAs that do not bind TDP-43 were indirectly affected by reduced levels of TDP-43. TDP-43 has been associated with cytoplasmic RNA granules of several sorts including processing bodies (P bodies, involved in RNA decay), stress granules (involved in translation regulation) and transporting RNP granules containing transcripts for local translation (Kiebler and Bassell, 2006; Anderson and Kedersha, 2009; Buchan and Parker, 2009).

Overall, antisense oligonucleotide mediated knock-down of TDP-43 resulted in more than 600 transcripts having affected levels and 950 having affected splicing patterns in mouse nervous system (Polymenidou *et al.*, 2011). Numerous RNA metabolism impairments have been observed in the context of ALS notably some directly related to TDP-43. They will be described in section **II.C.2) "RNA metabolism defects in ALS"**.

TDP-43's influence surpasses sole gene expression and RNA metabolism as its interactors are known to be involved in various mechanisms including protein degradation, cytoskeleton, cell cycle, trafficking, signaling and morphology, synaptic function, neuronal development, and metabolic function (Sephton *et al.*, 2011; Tollervey *et al.*, 2011; Colombrita *et al.*, 2012). Furthermore, its interaction with the key cellular process of autophagy will be discussed in section **IV.B "TARDBP and autophagy"**. This large spectrum of influence and key role in cell health justifies the scrutinous investigation of TDP-43 pathology in ALS.

B) TDP-43 in ALS

(1) TDP-43 pathology

As mentioned in section **I.B.5) “Histopathology”**, ALS histological hallmark is the presence of pathological inclusions of TDP-43 in neurons and glia in the majority of ALS cases and a good portion of FTD cases (Ling, Polymenidou and Cleveland, 2013). These inclusions are predominantly observed in the cytoplasm and correlate with nuclear depletion of TDP-43 (Arai *et al.*, 2006; Neumann *et al.*, 2006) (**Fig i 11**).

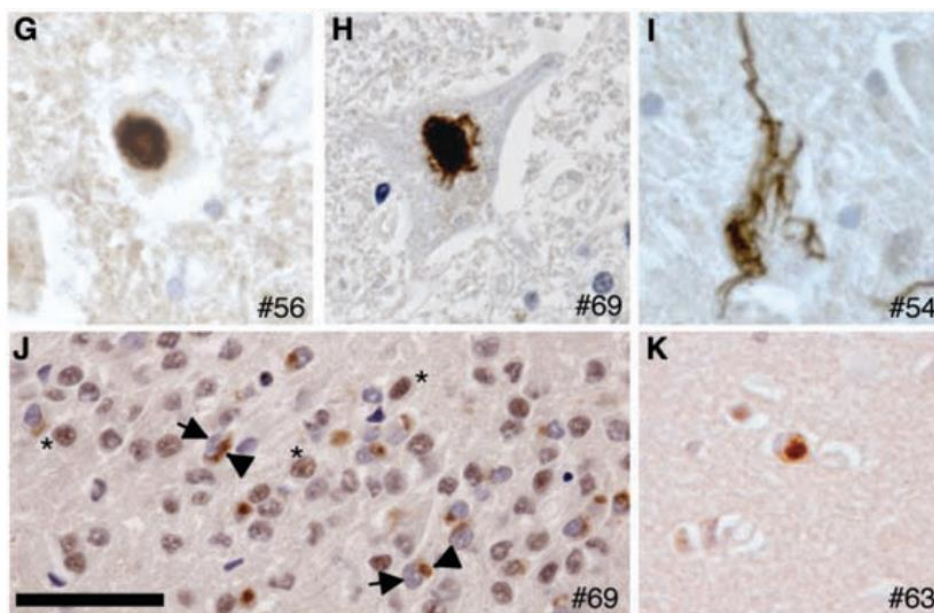


Figure i 11: TDP-43-positive inclusions in ALS patients (Neumann *et al.*, 2006)

Immunostaining with anti TDP-43 labeled Lewy body-like (G), round (H), and skeinlike inclusions (I) in motor neurons of the spinal cord. Cytoplasmic ubiquitin-positive inclusions in hippocampal dentate granule neurons (J) and frontal cortex (K) were stained by TDP-43. Asterik: normal nuclear staining; Arrows: absence of nuclear staining

TDP-43 nuclear aggregates:

However rare, the pioneer studies that unveiled the presence of TDP-43 in aggregates of ALS-FTD patients (Arai *et al.*, 2006; Neumann *et al.*, 2006) described the presence of nuclear ubiquitin-positive inclusions that were also positive for TDP-43. Both studies reported that these nuclear inclusions were related to particular subset of FTD cases but one of the studies described (without showing data) a single case of ALS presenting nuclear TDP-43-positive inclusions (Arai *et al.*, 2006). In HeLa cells, nuclear but reversible aggregation of TDP-43 after Heat-shock treatment was reported (Udan-Johns *et al.*, 2014). Although interesting, these cases should not overshadow the strong tendency of TDP-43 to stay soluble in the nucleus and prone to aggregation in the cytoplasm. A tendency also shared with other prion-like RBPs like FUS. The fact that these proteins show physiological high concentrations in the nucleus but yet weak tendency to aggregate in this compartment as compared to cytosol may seem paradoxical. High nuclear RNA concentration was shown to buffer aggregation tendencies of prion-like RBPs like TDP-43. Reduction of nuclear RNA concentration or genetic inhibition of their RNA-binding abilities cause phase separation and trigger solid-like aggregation of these RBPs (Maharana *et al.*, 2018).

In these aggregates, both full-length TDP-43 (FL TDP-43) and truncated C-terminal fragments are present and display characteristic modifications: abnormal phosphorylation and ubiquitylation (Arai *et al.*, 2006; Neumann *et al.*, 2006). ALS-related truncation of TDP-43 is caspase-mediated and theoretically leads to various forms depending on the site of cleavage (Y.-J. Zhang *et al.*, 2007; Li *et al.*, 2015). However, the ALS community widely focuses on the forms that are found in patients, TDP-35 and TDP-25 (named according to their reported

molecular weights). These two forms have in common a total or partial loss of the N-terminal domain of the protein. TDP-35 still contains the RRM and thus ability to bind and affect RNAs (Kitamura *et al.*, 2016) but misses its nuclear localization signal. The fragment is thus localized in cytosol where it is highly prone to aggregate (Bozzo *et al.*, 2016). The TDP-25 fragment no longer contains any functional RNA binding motif. Caspases activation has been linked to ALS-related mutations in patient tissues, cellular and animal models and is related to pro-apoptotic signaling observed in the disease (Pasinelli *et al.*, 1998, 2000; Li *et al.*, 2000; Guégan *et al.*, 2001; Wengenack *et al.*, 2004). The relevance of TDP-43 truncation to ALS pathogenesis is still poorly understood. Truncation of TDP-43 is not required to observe nuclear depletion, cytoplasmic aggregation and toxic behavior (Wobst *et al.*, 2017) however, sole over-expression of C-terminal fragments is sufficient to exert toxicity (Wang *et al.*, 2015) with each fragment presenting specificities (Kitamura *et al.*, 2016; Salvatori *et al.*, 2018). They show enhanced aggregating behavior and overall cellular toxicity (Zhang *et al.*, 2009) and are believed to drive co-aggregation of FL TDP-43 and dominantly impair its normal function (Yang *et al.*, 2011).

Aggregation of TDP-43, the defining trait of ALS histopathology, is toxic on its own (Yang *et al.*, 2011) but doesn't seem to be a requirement for TDP-43-mediated toxicity (Igaz *et al.*, 2011). Nevertheless, patients with unusual long duration disease present relatively few TDP-43 aggregates as compared to the average rapidly progressing case (Nishihira *et al.*, 2008). In addition, pathologic TDP-43 aggregates have been reported to display prion-like spreading properties both *in vitro* (Nonaka *et al.*, 2013) and *in vivo* (Porta *et al.*, 2018). TDP-43 localization and shuttling in the cell is a key element of its normal function. Its aggregation is in direct link with its mislocalization that seem to be influenced by several factors. Increased cytoplasmic levels of TDP-43 were observed in response to genetic factors (Matthew J Winton

et al., 2008; Barmada *et al.*, 2010; Ritson *et al.*, 2010), chemical stressors that will notably lead to formation of granules (I.-F. Wang *et al.*, 2008; Colombrita *et al.*, 2009; Volkening *et al.*, 2009; Freibaum *et al.*, 2010; Nishimoto *et al.*, 2010; Dewey *et al.*, 2011) or physical neuronal injury (Moisse, Mephram, *et al.*, 2009; Moisse, Volkening, *et al.*, 2009; Sato *et al.*, 2009). Even the rare cases of nuclear inclusions are the reflect of a mislocalization within the nucleus as the RBP is normally located in euchromatic regions of nucleoplasm (Thorpe *et al.*, 2008; Casafont *et al.*, 2009).

In an intriguing manner, cells displaying aggregates of mislocalized TDP-43 also present nuclear clearance of normal TDP-43 (Neumann *et al.*, 2006; Giordana *et al.*, 2010). This trait is not necessarily observed in other cases of pathological mislocalization and aggregation of protein (DiFiglia *et al.*, 1997) and may be related to TDP-43's ability to self-regulate (Ayala *et al.*, 2011; Igaz *et al.*, 2011; Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). Nuclear depletion of TDP-43 leads to partial or total loss of function causing a variety of defects (Igaz *et al.*, 2011; Polymenidou *et al.*, 2011) and is likely to play a role in degeneration. However, TDP-43's range of direct or indirect interactors is so important that it buffers the identification of precise disease mechanisms linking its loss of normal nuclear function to pathogenesis.

While ubiquitylation is related to protein degradation mechanisms (and will be discussed later in section **III.A "Autophagy: an overview"**), the role of aberrant phosphorylation of FL TDP-43 and its C-terminal fragments in disease mechanisms is still unclear. However, it is consistently found in patients with TDP-43 proteinopathy and observed in various TDP-43 over-expression models (Matthew J. Winton *et al.*, 2008; Igaz *et al.*, 2009, 2011; Nonaka, Arai, *et al.*, 2009; Nonaka, Kametani, *et al.*, 2009; Xu *et al.*, 2010; Zhang *et al.*, 2010). Phosphorylation may enhance TDP-43's already extensive half-life, tendency to

aggregate and toxicity (Liachko, Guthrie and Kraemer, 2010; Zhang *et al.*, 2010) but isn't necessarily required for toxicity (Dormann *et al.*, 2009; Zhang *et al.*, 2009).

Nuclear clearance appears quite early in the disease pathogenesis (**Fig i 12**). Mislocalized in the cytoplasm, progressive phosphorylation intervenes seemingly before ubiquitylation and TDP-43, and its newly truncated forms, are described in a state of pre-inclusions. Mature round and dense inclusions or skein-like inclusions then form and correlates with high phosphorylation and ubiquitylation of TDP-43 and C-terminal fragments (Davidson *et al.*, 2007; Mori *et al.*, 2008; Pamphlett *et al.*, 2009; Giordana *et al.*, 2010; Lee, Lee and Trojanowski, 2012).

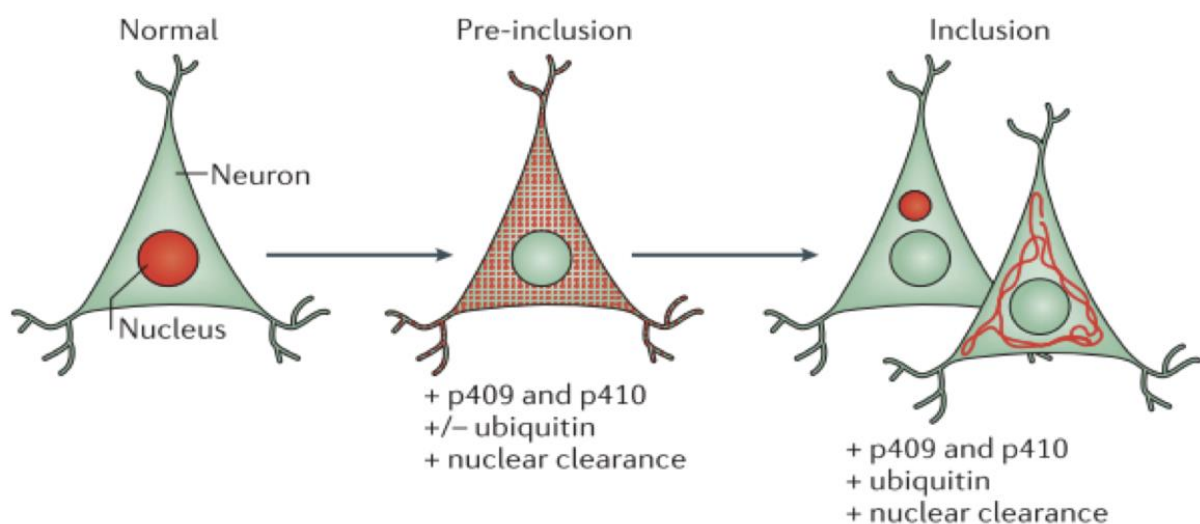


Figure i 12: Chain of events of TDP-43 pathology; modified from E. B. Lee et al. 2012.

Schematic TDP-43 immunostaining shown in red. p409 and p410: phospho-TDP-43 epitopes.

Even though they account for a rather small portion of ALS cases with about 4% of fALS and 1% of sALS (Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008; Van Deerlin *et al.*, 2008; Chiò *et al.*, 2014; Renton, Chiò and Traynor, 2014) and few rare cases of FTD (Borrioni *et al.*, 2009; Kovacs *et al.*, 2009), more than 40 different mutations have been related to ALS and FTD and

are massively clustered in the C-terminal region of the protein with the exception of a few being adjacent to it and only one known mutation in the RRM2s (**Fig i 13**) (Lagier-Tourenne et al. 2010; Lattante et al. 2013). Logically, mutations are not necessary to observe TDP-43 neuropathology (Pamphlett *et al.*, 2009) but seem to increase TDP-43's tendency to be cleaved (Chiang *et al.*, 2016), to aggregate (Nonaka, Kametani, *et al.*, 2009) and form inclusions that may display even more toxic properties (Gopal *et al.*, 2017).

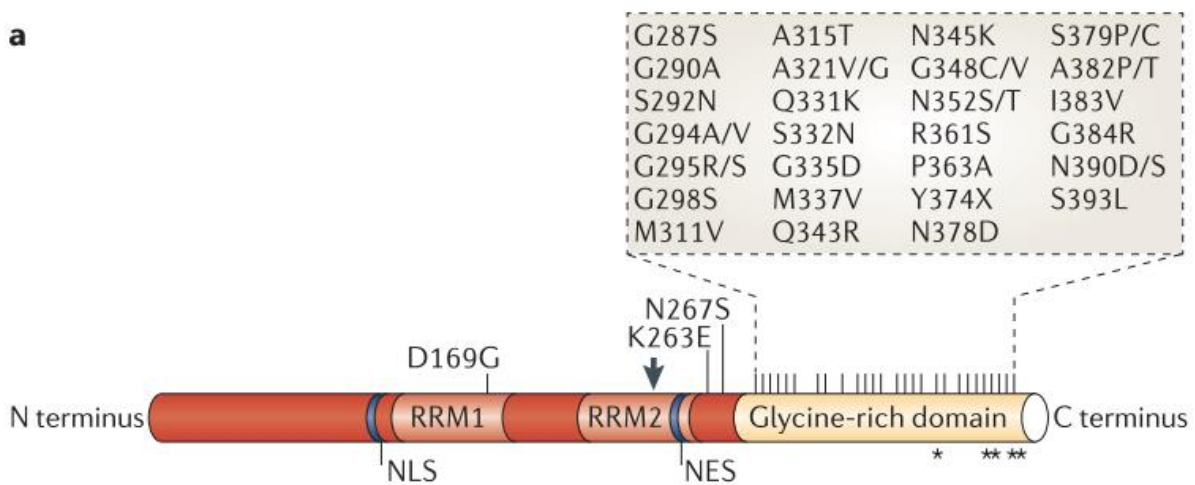


Figure i 13: TDP-43 ALS-related mutations; modified from E. B. Lee et al. 2012.

NLS: Nuclear Localization Signal; NES: Nuclear Extraction Signal; RRM: RNA Recognition motif

(2) TDP-43's ALS-related partners of interaction

TDP-43 has been shown to interact with several ALS-related genes either at the protein level or at the RNA level. In pathological inclusions, TDP-43 can be found colocalized in aggregates with several proteins found mutated in ALS cases such as protein clearance-related p62 (Brady *et al.*, 2011), OPTN (Maruyama *et al.*, 2010; Deng, Bigio, *et al.*, 2011) and UBQLN2 (Deng, Chen, *et al.*, 2011; Groen *et al.*, 2013) as well as other RNA-binding protein FUS (Ling *et al.*, 2010; Keller *et al.*, 2012) and stress-granule related TIA-1 (Volkening *et al.*, 2009; Liu-

Yesucevitz *et al.*, 2010). Ataxin2, involved in RNA metabolism, has been reported to colocalize with TDP-43 in inclusions in FTD cases but not ALS (Farg *et al.*, 2012).

Pathology aside, physiological *TARDBP* and *FUS* appear to be involved in an epistatic interaction. *TARDBP* seem to act upstream of *FUS* as overexpression of human RNA of the later can rescue the altered motor phenotype caused by knock-down of *tardbp* in zebrafish (Kabashi, Bercier, *et al.*, 2011). TDP-43 was shown to bind to *FUS* mRNA as well as several other ALS-related transcripts encoding proteins involved in RNA metabolism namely Ataxin2, Matrin 3, hnRNPA1, hnRNPA2B1, TIA-1 among others (Sephton *et al.*, 2011). Interestingly, Ataxin2 has been described as a modifier of TDP-43-mediated toxicity and a genetic risk factor for ALS (Elden *et al.*, 2010). Consistent with its RNA-binding function, TDP-43 also associates and form granules with several proteins of the hnRNP family (Buratti *et al.*, 2005; Freibaum *et al.*, 2010; Ling *et al.*, 2010).

TDP-43 shares an intriguing relation with VCP (valosin containing protein) which is involved in various functions notably in protein degradation and stress granule formation (J. S. Ju *et al.*, 2009; Tresse, Florian A. Salomons, *et al.*, 2010; Seguin *et al.*, 2014; Wang *et al.*, 2019) and found mutated in some ALS cases (Janel O. Johnson *et al.*, 2010) that will be discussed later in this introduction. TDP-43 interacts with PGRN or progranulin mRNA (Sephton *et al.*, 2011) which is a major cause of FTD when mutated (Mackenzie *et al.*, 2006; Beck *et al.*, 2008). Interestingly, PGRN seem to mediate caspase-dependent cleavage of TDP-43 (Y.-J. Zhang *et al.*, 2007) and protect against mutant TDP-43-mediated axonopathy in zebrafish (Laird *et al.*, 2010).

Although evidence for direct binding isn't consistent (Sephton *et al.*, 2011), TDP-43 was found to colocalize in ribonucleoprotein granules with NEFL mRNA and affect its transport

when mutated (Alami *et al.*, 2014). *NEFL* isn't found mutated in patients but as previously explained, it is one the most relevant candidate in the search of a molecular biomarker of ALS (Steinacker *et al.*, 2016; Xu *et al.*, 2016; Verde *et al.*, 2019).

(3) Modeling TDP-43 pathology

Since the description of TDP-43 as a key pathological feature of ALS and FTD and the discovery of its pathology-related mutations (Arai *et al.*, 2006; Neumann *et al.*, 2006), numerous studies have tried to model TDP-43 pathology in various host systems including yeast, cell cultures and animal models (Wegorzewska and Baloh, 2011; Van Damme, Robberecht and Van Den Bosch, 2017) as described in section **I.C.1) “Modeling ALS”**. Considering the multitude of traits that characterize TDP-43 pathology and, as evidence for both loss of function and gain of function mechanisms are reported, modeling has been attempted through different strategies that include knock-down and over-expression of WT or mutated FL or C-terminal fragments of TDP-43 under various promoters (Wegorzewska and Baloh, 2011; Van Damme, Robberecht and Van Den Bosch, 2017). These attempts have highlighted the multiple ways in which TDP-43 modulation can be toxic. Maintaining TDP-43 levels within normal parameters seems crucial as full knock-out has been reported to be lethal at early stages (Fiesel *et al.*, 2010; Sephton *et al.*, 2010), knockdown displays clear toxicity (Feiguin *et al.*, 2009; Kabashi *et al.*, 2009; Lu, Ferris and Gao, 2009; Li *et al.*, 2010) and increased levels of TDP-43 by overexpression of the wild-type or ALS-causing mutant protein is highly deleterious (Ash *et al.*, 2010; Hanson *et al.*, 2010; Li *et al.*, 2010; Ritson *et al.*, 2010; Wils *et al.*, 2010; Igaz *et al.*, 2011). In fact, simple overexpression of TDP-43 and its related toxic effects observed in both animal and cellular models are under debate concerning their

relevance to ALS pathology (Wegorzewska and Baloh, 2011). However, overexpression rodent models have highlighted the specific vulnerability of spinal motor neurons as compared to the rest of nervous system (Wegorzewska *et al.*, 2009; Wils *et al.*, 2010; Zhou *et al.*, 2010).

Animal models have globally failed to emulate key histopathological traits such as formation of TDP-43 aggregates and TDP-43 nuclear depletion thus impacting our understanding of their respective implication in disease mechanisms (Wegorzewska *et al.*, 2009; Hanson *et al.*, 2010; Laird *et al.*, 2010; Wils *et al.*, 2010; Zhou *et al.*, 2010; Joyce *et al.*, 2011; Devoy *et al.*, 2017; White *et al.*, 2018). In rodents, ubiquitin-positive inclusions were found in spinal motor neurons of the animals but rarely contain TDP-43 (Wegorzewska *et al.*, 2009; Wils *et al.*, 2010; Zhou *et al.*, 2010). *Drosophila* models show contradicting results as overexpression models display either aggregates of FL TDP-43 (Li *et al.*, 2010), soluble C-terminal fragments (Fiesel *et al.*, 2010) or do not show any of these traits despite overall toxicity of overexpression (Hanson *et al.*, 2010). Several cellular models have however been successful in reproducing histopathological hallmarks notably unveiling the toxicity of C-terminal fragments and their role in promoting aggregation of the protein (Igaz *et al.*, 2009; Zhang *et al.*, 2009; Yang *et al.*, 2011) but full phenocopy of TDP-43 pathology remains challenging (Van Damme, Robberecht and Van Den Bosch, 2017).

There are paradoxical challenges in generating TDP-43 pathology models: reproducing the numerous aspects of TDP-43 pathology to gain in realism and individualizing each aspect to understand its relevance for pathogenesis.

(4) TDP-43 pathogenesis

Nearly all cases of ALS or FTD display TDP-43 proteinopathy, with the exception of SOD1 or FUS mutation ALS patients and FTD patients presenting tauopathies (Ling, Polymenidou and Cleveland, 2013). This puts the protein at the center of attention of disease pathogenesis. Nevertheless, the precise mechanisms through which neuronal malfunction, degeneration and death occur is still not known. Much progress has however been made since the focus was made on TDP-43 in 2006 (Arai *et al.*, 2006; Neumann *et al.*, 2006). It is now believed that several pathways may be involved in neurodegeneration. The different aspects of TDP-43 pathology that were described above play in favor of a probable combination of both gain- and loss-of-function (Lee, Lee and Trojanowski, 2012; Ling, Polymenidou and Cleveland, 2013).

Indeed, gain of toxic function appears to be linked to aggregation, prion-like properties and abnormal cytoplasmic function of mislocalized and truncated TDP-43. Loss of function, on the other hand, is highlighted by nuclear clearance, partial or total loss of RNA-binding function of C-terminal fragments and loss of high impact interactions. The challenge in understanding TDP-43 proteinopathy is in direct correlation with the complexity of its normal function, adaptability to cellular and environmental context and vast interactome. Indeed, the number of RNAs and proteins that interact with TDP-43, and are potentially affected by TDP-43 pathology, is so extensive that it makes the pinpointing of disease-relevant candidates complex (Lee, Lee and Trojanowski, 2012). Although one could argue that reproduction of TDP-43 pathology was overly simplified, an interesting study attempted to quantify the respective contribution of loss-of-function (represented by nuclear depletion of TDP-43) and gain-of-function (represented by cytoplasmic TDP-43 aggregates) mechanisms in TDP-43 pathology. They were respectively estimated to contribute to 55% and 45% of induced cytotoxicity (Cascella *et al.*, 2016).

Besides wild-type TDP-43 pathology, ALS-related mutations may not only display increased tendencies to generate the aspects of TDP-43 pathology but also show specific toxic gain-of function properties affecting RNA metabolism (Fratta *et al.*, 2018; Rouaux, Gonzalez De Aguilar and Dupuis, 2018). In section **II.C.2) “RNA metabolism defects in ALS”**, we will describe the overall defects of this key homeostatic function in ALS in which TDP-43 is heavily involved. However, TDP-43-related pathogenesis may also involve other potential mechanisms of action.

Defective endocytosis:

Impairments in this crucial process in which various substances are internalized in the cellular compartment may contribute to TDP-43 pathogenesis. Colocalization of TDP-43 to endocytosis-associated proteins such as Rab5 was observed in both patients and cellular models (G. Liu *et al.*, 2017). Inhibition of endocytosis correlates with TDP-43 aggregation while increased endocytosis ameliorates TDP-43 toxicity (G. Liu *et al.*, 2017). In human iPSC-derived neurons, TDP-43 knockdown reduced the number and functionality of endosomes and TDP-43 overexpression causes the opposite effect (Schwenk *et al.*, 2016).

Role of TDP-43 in mitochondrial function and oxidative stress:

Over-expression of both mutant or wild-type TDP-43 lead to abnormal colocalization at the mitochondria and was linked with mitochondrial dysfunction (Ralf J Braun *et al.*, 2011; W. Wang, Li, W.-L. Lin, *et al.*, 2013; Stribl *et al.*, 2014). TDP-43 pathology also correlates with the inducement of oxidative damage (Duan *et al.*, 2010; Bharathi *et al.*, 2016; Bharathi,

Girdhar and Patel, 2017). Depletion in endogenous anti-oxidant factors has been reported to aggravate TDP-43 insolubility and aggregating behavior (Iguchi *et al.*, 2012). Subjecting TDP-43 to oxidative agents promotes its aggregation (Cohen *et al.*, 2012) and loss of its nucleic acid binding activity (Chang *et al.*, 2013). Mutant SOD1 aggregates have been reported to cause TDP-43 mislocalization and promote its aggregation (Zeineddine *et al.*, 2017). Mitochondria-related and ALS protein CHCHD10 was described to interact with TDP-43 (Lehmer *et al.*, 2018). Finally, TDP-43 normally penetrates mitochondria and interact with mitochondrial mRNAs involved in the respiratory function and its truncated forms show differential sub-mitochondrial localization and toxicity (Salvatori *et al.*, 2018).

Defective chromatin remodeling:

Impairments in this process have been studied in several neurodegenerative diseases including ALS (Berson *et al.*, 2018; Bastle and Maze, 2019). TDP-43 was linked with impaired nucleosomal traffic (Berson *et al.*, 2017). It was also reported to bind CHD1 (chromodomain helicase DNA binding protein 1), a protein which modifies TDP-43-mediated toxicity (Berson *et al.*, 2017). Nuclear depletion of TDP-43 and FUS correlates with retention of mRNAs, including transcripts corresponding to proteins involved in the formation of nBAF complex (an important chromatin remodeling protein complex). Furthermore, mutant TDP-43 are associated with altered expression of key epigenetic markers (Masala *et al.*, 2018).

Overall, several mechanisms may be involved in TDP-43-mediated pathogenesis and alternatively reflect implication of both LoF and GoF components (**Fig i 14**). The protein, its

homeostasis and correct localization play a crucial role in cellular health and notably for its key role in RNA metabolism, a process that displays important impairments in ALS.

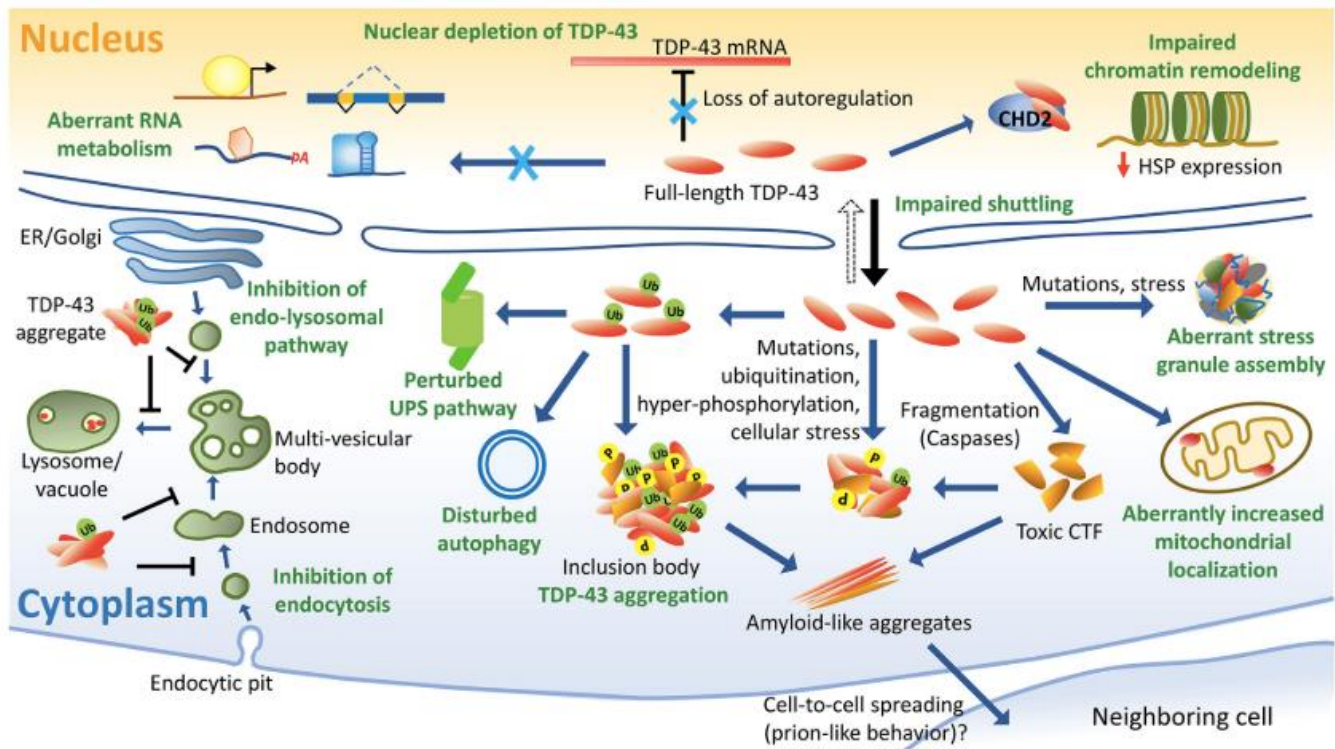


Figure i 14: Schematics of TDP-43 induced pathology; modified from Prasad et al. 2019.

C) RNA metabolism and ALS

(1) ALS-related genes involved in RNA metabolism

Alterations in RNA metabolism is a highly recurrent theme in ALS pathogenesis that was driven by the identification of several mutated genes in patients that are involved in this crucial cellular process. At the basis of this hypothesis of pathogenesis was, naturally, the discovery of mutations in *TARDBP* and *FUS*, both encoding for RNA-binding proteins containing low-complexity domains that render them prone to aggregation (Arai *et al.*, 2006;

Neumann *et al.*, 2006; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). For TDP-43, its involvement in RNA metabolism has been thoroughly highlighted by the previous description of its normal function in the process (see section **II.A.2** “**TDP-43’s normal function**”).

Much like TDP-43, FUS (or fused in sarcoma), is a protein composed of an RNA-recognition motif, a low-complexity/prion-like domain and a nuclear localization signal (NLS) that is able to shuttle between nucleus and cytoplasm and can bind RNA, DNA and proteins (Iko *et al.*, 2004). It was found mutated in approximately 4-5% of fALS and in a lower percentage of sALS (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Corrado *et al.*, 2010; DeJesus-Hernandez *et al.*, 2010). ALS-related mutations of FUS can be categorized in two groups as they seem to cluster in the low-complexity domain or in the NLS at the C-terminal part of the protein. This later group of mutations was reported to lead to abnormal cytoplasmic accumulation of the protein in patients (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009) as discussed in section **I.B.5** “**Histopathology**”. FUS was reported to bind to thousands of RNAs in studies conducted in brain tissues or in cell lines (Hoell *et al.*, 2011; Colombrita *et al.*, 2012; Ishigaki *et al.*, 2012; Lagier-Tourenne *et al.*, 2012; Rogelj *et al.*, 2012) and is involved in multiple steps of RNA metabolism notably by interacting with other proteins in RNP complexes. In particular, the role of FUS in splicing regulation is directly in relation with its ability to interact with key splicing factors (hnRNP A1, YB-1, U1 snRNP) (Rapp *et al.*, 2002; Meissner *et al.*, 2003; Yamazaki *et al.*, 2012; Yu *et al.*, 2015; Kamelgarn *et al.*, 2016) and integrate spliceosomal complexes (Rappsilber *et al.*, 2002; Zhou *et al.*, 2002). FUS seems to be involved in transcription regulation via its interactions with RNA polymerase II and III (RNAP2 and RNAP3) or cyclin D1 (X. Wang *et al.*, 2008; Tan and Manley, 2010; Brooke *et al.*, 2011; Schwartz *et al.*, 2012; Tan *et al.*, 2012). Interestingly, FUS and TDP-43 seem to mostly bind different RNA targets (Lagier-Tourenne *et al.*, 2012; Rogelj *et al.*, 2012).

Besides from *TARDBP* and *FUS*, mutations in several other genes involved in RNA metabolism have been reported in ALS patients. Indeed, mutations in angiogenin (*ANG*) (Greenway *et al.*, 2006; Yamasaki *et al.*, 2009), ataxin-2 (*ATXN2*) (Ostrowski, Hall and Mekhail, 2017), matrin-3 (*MATR3*) (Coelho *et al.*, 2015), heterogeneous nuclear ribonucleoprotein A1 and A2 B1 (*hnRNPA1* and *hnRNPA2B1*) (Dreyfuss *et al.*, 1993; Alarcón *et al.*, 2015), stress-granule related T-cell-restricted intracellular antigen-1 (*TIA-1*) (Förch *et al.*, 2000) have been associated with ALS and all play a role in RNA processing (Butti and Patten, 2019). Furthermore, mutations in TATA-box binding protein associated factor 15 (*TAF15*) (Ibrahim *et al.*, 2013) and Ewing's sarcoma breakpoint region 1 (*EWSR1*) (Duggimpudi *et al.*, 2015), coding for two proteins that show structural and functional similarities with *FUS* and have been implicated in regulation of transcription and splicing (Q. Han *et al.* 2008; Huang *et al.* 2005), were also identified.

(2) RNA metabolism defects in ALS

Evidence for alterations in various steps of RNA metabolism have been observed in the context of ALS (Butti and Patten, 2019). Both *FUS* and *TDP-43* models show alterations of transcriptome. Indeed, iPSC-derived motoneurons from patients carrying mutant *FUS* display transcriptomic changes (De Santis *et al.*, 2017). Similar differences were reported in both mutant *FUS* transgenic mice (Scekic-Zahirovic *et al.*, 2016) and *FUS* knockdown in cell culture (Lagier-Tourenne *et al.*, 2012; Nakaya *et al.*, 2013). In several of these *FUS* models, expression of other RNA metabolism-related *TAF15* was altered as well as genes involved in synaptic development and neurodegenerative mechanisms (Fujioka *et al.*, 2013; Scekic-Zahirovic *et al.*, 2016; De Santis *et al.*, 2017). Concerning *TDP-43*, a simple oligonucleotide-mediated knock-

down resulted in more than 600 transcripts having affected levels (Polymenidou *et al.*, 2011). Among the range of TDP-43 RNA targets, some are key to synaptic development, function and neurotransmission (Godena *et al.*, 2011; Sephton *et al.*, 2011; Colombrita *et al.*, 2012; Narayanan *et al.*, 2013; Chang *et al.*, 2014; Coyne *et al.*, 2014). Dysfunctional regulation of their expression might be relevant to ALS pathology as it may explain synaptic dysfunction due to expression of ALS-related TDP-43^{G348C} in zebrafish (Armstrong and Drapeau, 2013a), reduced synaptic formation and dendritic branching in drosophila TDP-43 depletion models (Feiguin *et al.*, 2009; Lu, Ferris and Gao, 2009), altered dendritic development, spine morphology and synaptic transmission in TDP-43^{A315T} mice (Handley *et al.*, 2016) and reduced dendritic branching in mammalian primary neuronal cultures with depletion or overexpression of TDP-43 (Herzog *et al.*, 2017). TDP-43 may also cause neurodegeneration via dysregulation of NEFL mRNA. TDP-43 was reported to bind and stabilize this transcript (Strong *et al.*, 2007) for which we have found that stoichiometry imbalance caused motor neuron degeneration and development of an ALS-related phenotype in animal models (Xu *et al.*, 1993; Julien, Côté and Collard, 1995). In ALS patients, NEFL mRNA is interestingly abnormally regulated (Wong, He and Strong, 2000). More globally, TDP-43 mutations are associated with impaired axonal transport of RNAs (Alami *et al.*, 2014) and the protein is known to be involved in RNA stability (Strong *et al.*, 2007; Fiesel and Kahle, 2011).

microRNAs play a key role in expression regulation as they impact RNA and subsequent protein levels (Catalanotto, Cogoni and Zardo, 2016) and are involved in ALS-relevant cellular mechanisms such as the development of the nervous system (Johnston and Hobert, 2003) and notably of motor neurons for which they also play a role in normal function and survival (Asli and Kessel, 2010; Haramati *et al.*, 2010; Chen and Wichterle, 2012; Luxenhofer *et al.*, 2014; Thiebes *et al.*, 2015; Bhinge *et al.*, 2016). Altered expression of disease-relevant microRNAs

was reported in CNS of fALS and sALS patients (Helferich *et al.*, 2018) as well as in iPSC-derived motor neuron progenitors from ALS patients (Rizzuti *et al.*, 2018). Both FUS and TDP-43 (as discussed in section **II.A.2) “TDP-43’s normal function”**) are known to be implicated in microRNA biogenesis and regulating activity (Freibaum *et al.*, 2010; Da Cruz and Cleveland, 2011). Depletion of TDP-43 leads to changes in the profile of expression of microRNAs in cell culture (Buratti *et al.*, 2010) while mutations in TARDBP lead to altered expression of several microRNAs key to motor neurons (Kawahara and Mieda-Sato, 2012; Z. Zhang *et al.*, 2013). FUS depletion also impacts expression of key microRNAs (Morlando *et al.*, 2012).

Splicing regulation seems altered in both TDP-43 and FUS models. TDP-43 depletion or over-expression models cause alternative splicing of numerous targets (Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). In CNS tissues from TDP-43 ALS patients, alternative splicing of several genes has been reported (Shiga *et al.*, 2012; Yang *et al.*, 2014). Alterations of RNA metabolism is often attributed to a loss of TDP-43 normal function, notably hinted by nuclear depletion and truncation-mediated loss of RNA-recognition motifs (Chiang *et al.*, 2010; Wu, Cheng and Shen, 2012; Iguchi *et al.*, 2013; Yang *et al.*, 2014). For instance, in a mouse embryonic stem cell model, depletion of TDP-43 caused the abnormal splicing of cryptic exons (exons which splicing into mRNA is normally repressed by TDP-43) which lead to toxicity (Ling *et al.*, 2015). However, gain-of-function of mutant TDP-43 involving abnormal splicing has also been described (Fratta *et al.*, 2018; Rouaux, Gonzalez De Aguilar and Dupuis, 2018). Indeed, mice expressing TDP-43 mutated in its low-complexity domain suffered from neurodegeneration that correlated with the consistent skipping of constitutive exons normally spliced by wild-type TDP-43 into mRNAs (named skiptic exons) (Fratta *et al.*, 2018; Rouaux, Gonzalez De Aguilar and Dupuis, 2018). In both phenomena, the abnormal splicing leads to altered stability and translation of the mRNA. A role of loss of FUS splicing function in

pathogenesis has been suggested as ALS-related mutation in NLS of FUS, causing cytoplasmic retention of the protein (Dormann *et al.*, 2010), results in splicing defects (Reber *et al.*, 2016). Moreover, FUS LoF in mice brains leads to altered splicing in more than 300 genes (Lagier-Tourenne *et al.*, 2012). These splicing defects may have repercussions on neuronal development, signal transmission to skeletal muscles (Reber *et al.*, 2016), cytoskeleton and subsequent effects on axonal growth and function (Ishigaki *et al.*, 2012; Orozco *et al.*, 2012; Rogelj *et al.*, 2012; Nakaya *et al.*, 2013).

Markers of stress granules (SG) have been identified in ubiquitin-positive inclusions in ALS and FTD patients as well as in cell culture (Liu-Yesucevitz *et al.*, 2010; Wolozin, 2012). In response to various stressors, eukaryotic cells form SG, cytoplasmic foci of RNAs and RNPs (notably RNA-binding proteins, translation initiation factors and small ribosomal subunits) to temper with RNA metabolism, stock RNAs and prevent unnecessary translation (Kedersha and Anderson, 2007; Protter and Parker, 2016). Numerous ALS-related proteins are known components of SG such as TDP-43 (Colombrita *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010; Bentmann *et al.*, 2012), FUS (Sama *et al.*, 2013), ANG (Emara *et al.*, 2010; Thiyagarajan *et al.*, 2012), ATXN2 (Nihei, Ito and Suzuki, 2012), EWS, TAF15 (Andersson *et al.*, 2008; Blechingberg *et al.*, 2012) or TIA-1 (Kedersha *et al.*, 1999). Interestingly, prolonged stress and subsequent sequestration of RNA-binding proteins, other protein partners and their RNA targets is thought to be relevant to ALS pathology (Bentmann, Haass and Dormann, 2013; Li *et al.*, 2013; Ramaswami, Taylor and Parker, 2013; Yasuda *et al.*, 2013). For example, expression of Pur-alpha interacts with FUS and is a disease modifier of FUS-mediated pathology. The protein was found sequestered with mutant FUS in SG in ALS patient-derived cell culture (Di Salvio *et al.*, 2015; Daigle *et al.*, 2016). Mutant TDP-43 has been shown to be recruited more efficiently to SG than wild-type TDP-43 (Liu-Yesucevitz *et al.*, 2010).

Interestingly, RNA metabolism defects are also associated with two other key ALS proteins, C9orf72 and SOD1. An hexanucleotide repeat expansion in C9orf72 has been identified as the major known cause of ALS (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). The genetic abnormality leads to the formation of C9orf72 dipeptide repeats (DPRs) (Ash *et al.* 2013) but also C9orf72 repeat RNA foci (T. Zu *et al.*, 2013) in ALS patients (see section **III.B.2) “Autophagy defects in ALS”** for more description concerning C9orf72 pathology). The formation of such RNA foci promotes recruitment of RNA-binding proteins such as TDP-43, FUS or hnRNP A1 among others (Donnelly *et al.*, 2013; Lee *et al.*, 2013; Sareen *et al.*, 2013; Xu *et al.*, 2013; Cooper-Knock, Shaw and Kirby, 2014; Haeusler *et al.*, 2014) and alters RNA metabolism by disturbing their normal function (Simón-Sánchez *et al.*, 2012; Donnelly *et al.*, 2013; Lee *et al.*, 2013; Mori, Lammich, *et al.*, 2013; Gitler and Tsuiji, 2016). In C9orf72-ALS motor neurons derived from iPSCs, suppressing RNA foci formation reduces sequestration of RNA-binding proteins and subsequent impairments in gene expression (Donnelly *et al.*, 2013; Lagier-Tourenne *et al.*, 2013). DPRs may also influence RNA metabolism. Mice expressing a C9orf72-related DPR revealed altered expression of more than 6000 genes through RNA sequencing. Certain types of DPRs have been associated with altered splicing of several RNAs (Kwon *et al.*, 2014) and seem to colocalize and potentially sequester proteins involved in RNA translation such as ribosomal subunits and transcription factor eIF3n (Zhang *et al.*, 2018) and lead to reduced levels of ribosomal RNAs resulting in neuronal death (Suzuki *et al.*, 2018). The DROSHA protein, a key protein of microRNA biogenesis, was found colocalized in DPRs in tissues of C9orf72 ALS/FTD patients (Porta *et al.*, 2015). Mutant C9orf72 has also been reported to interfere with the transcription and splicing of its own RNA (Mori, Weng, *et al.*, 2013; Haeusler *et al.*, 2014; Highley *et al.*, 2014) which supports a C9orf72-

haploinsufficiency model for pathogenesis (Ciura *et al.*, 2013). C9orf72 has been associated with SG regulation (Maharjan *et al.*, 2017) and a particular DPR specie has been directly implicated in their formation (Zhang *et al.*, 2018). Overall, several studies highlighted major transcriptome alteration in C9orf72-ALS context (Donnelly *et al.*, 2013; Prudencio *et al.*, 2015; Selvaraj *et al.*, 2018).

Concerning SOD1, the mutant protein has been found to gain the faculty to bind particular RNAs (Kenan, Query and Keene, 1991; Tiwari, Xu and Hayward, 2005) such as VEGF and NEFL causing altered expression, stability and ultimately altered function of the proteins (Menzies *et al.*, 2002; Lu *et al.*, 2009; H. Chen *et al.*, 2014). In addition, reduced levels of NEFL mRNA was observed in motor-neurons of SOD1-ALS patients and in transgenic mice (Menzies *et al.*, 2002). The interaction between mutant SOD1 and NEFL mRNA may involve an interaction with TDP-43 (Volkening *et al.*, 2009). Mutant SOD1 mice also display upregulated expression of disease-relevant microRNA-9 (Zhou *et al.*, 2013). More broadly, transcriptomic studies revealed altered gene expression including some involved in neuroinflammation, oxidative stress, mitochondrial, synaptic and neurodevelopmental pathways in mutant SOD1 models and patient sample (Hedlund *et al.*, 2010; Lincecum *et al.*, 2010; Bandyopadhyay *et al.*, 2013; Kumimoto, Fore and Zhang, 2013; Sun *et al.*, 2015; D'Erchia *et al.*, 2017). Interestingly, these changes seem to be mostly different from the one observed in a mutant TDP-43 model (Rotem *et al.*, 2017).

In other words, several ALS-related genes, whether normally implicated in RNA metabolism or not, affect various steps of RNA metabolism (**Fig i 15**). Together with defective autophagy (see section III. **“Autophagy and its implication in ALS”**), with which converging mechanisms exist (see section IV. **“ALS: at a crossroad between autophagy and RNA**

metabolism”), RNA homeostasis is one of the most incriminated dysregulated machinery in ALS pathogenesis.

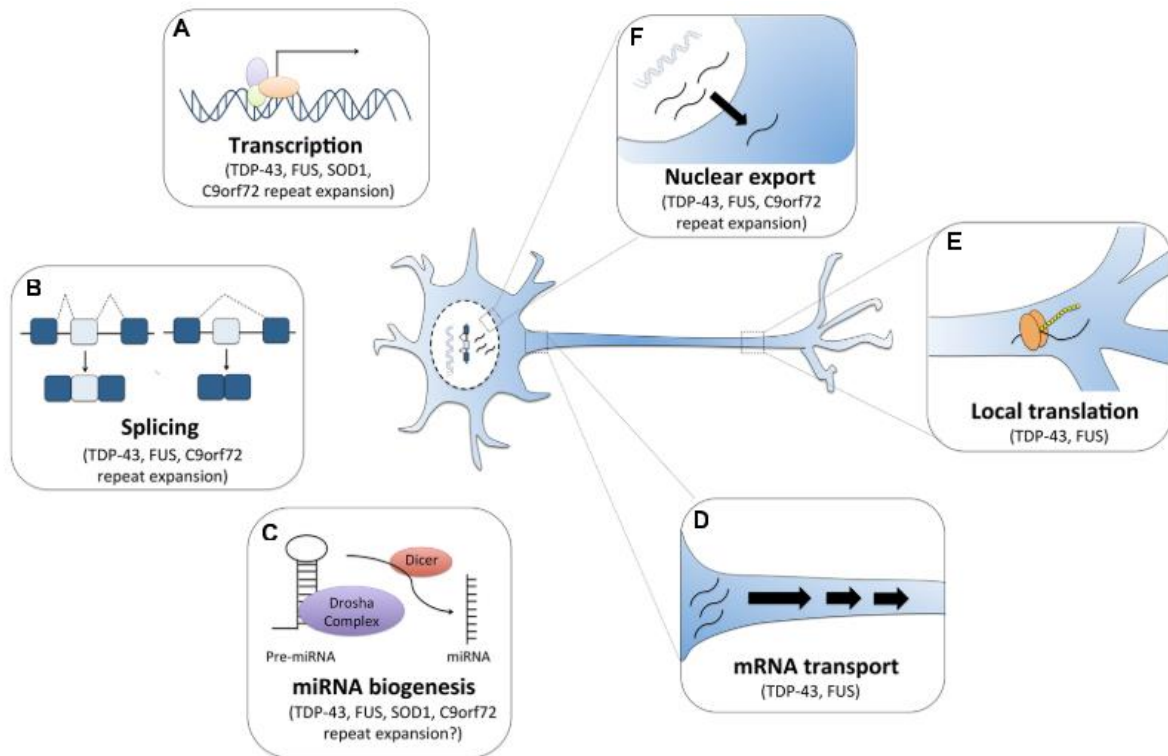


Figure i 15: Affected RNA metabolism functions in ALS; modified from Butti and Patten 2019.

III) Autophagy and its implication in ALS

A) Autophagy: an overview

(1) Definition

In 1963, future Nobel Prize winner and Belgium biochemist Christian de Duve, made the first description of lysosomes and their mediated intracellular degradation process. By pure

accident, while aiming to study insulin and carbon hydrates metabolism, he isolated by centrifugation a membraned structure containing various acid hydrolases to which he logically attributed a “digestive function” and dedicated his work to, despite his previous focus on biochemistry (de Duve, 1963, 2005). This catabolic process was widely studied and later found to be highly conserved and of crucial importance for homeostasis (Takeshige *et al.*, 1992; Tsukada and Ohsumi, 1993). The autophagic process was found to be depending on a class of ATG genes (for “autophagy related”) encoding for proteins that regulate the process throughout its multiple steps (Mizushima, Yoshimori and Ohsumi, 2011). These ATG genes were first described in yeast and the names of their orthologues may vary among species. Autophagy can be divided in several subtypes: macroautophagy, microautophagy and CMA or chaperone-mediated autophagy (Klionsky, 2007). In CMA, proteins are targeted to the lysosome by cytosolic chaperones (Kaushik and Cuervo, 2012). Microautophagy consists in direct engulfment of cytosolic proteins and degradation by the lysosome (Li, Li and Bao, 2012). Macroautophagy is the most characterized subtype and is often referred simplistically, and hereafter in this thesis, as autophagy. It refers to the forming of the autophagosome, a double-membrane vesicle that entraps proteins, organelles and various other cytosolic materials, and its trafficking and fusing with the lysosome for degradation of its content through hydrolyzation (Mizushima, 2007; Ravikumar *et al.*, 2009; Rubinsztein, Shpilka and Elazar, 2012) **(Fig i 16).**

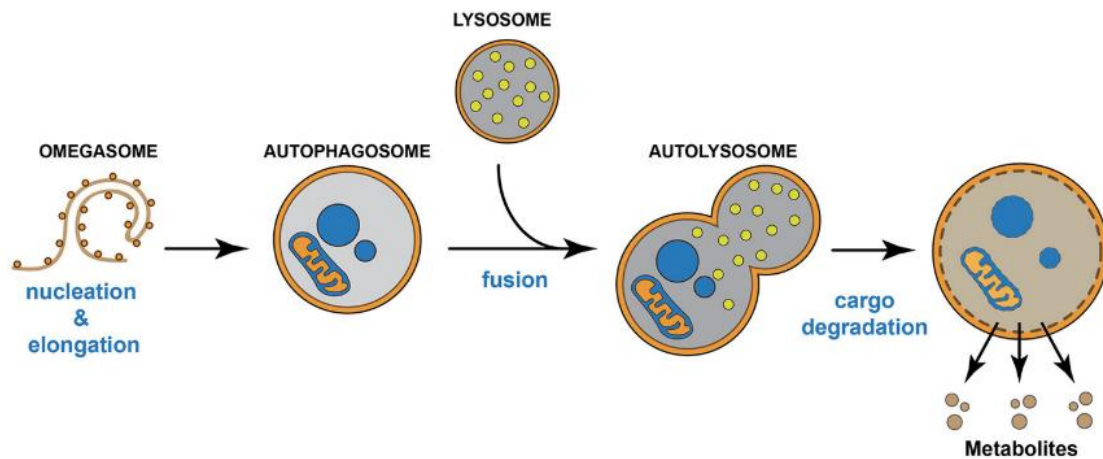


Figure i 16: Overview of the autophagic pathway; modified from Budini et al. 2017.

For a long time, autophagy was thought to be a non-selective pathway of cellular degradation activated by nutrient deprivation (de Duve and Wattiaux, 1966). The cell “digest” its own content to recycle nutrients when energy supplies are limited. The catabolic process has then been linked to several pathological mechanisms including cancer, neurodegeneration, immune response and ageing that ameliorated our understanding of autophagy (Liang *et al.*, 1999; Mizushima *et al.*, 2008; Levine, Mizushima and Virgin, 2011; Choi, Ryter and Levine, 2013). Autophagy is now known to also serve as an intracellular quality control system and allow selective degradation of various cargoes such as misfolded and aggregated proteins or damages organelles (Shaid *et al.*, 2013). Autophagy is highly active during differentiation and development (Mizushima and Levine, 2010). Conventional knockout of numerous ATG genes cause neonatal lethality in animal models (Kuma *et al.*, 2004; Komatsu *et al.*, 2005; Saitoh *et al.*, 2008, 2009; Sou *et al.*, 2008).

The autophagic process comprises several steps: initiation and nucleation (which results in the formation of phagophore, precursor of the autophagosome), elongation,

autophagosome maturation and degradation of its cytosolic content through fusion with the lysosome (Choi, Rytter and Levine, 2013).

(2) *The stages of autophagy*

Initiation and nucleation:

The mechanisms required for initiation of autophagy are complex and not fully understood as a multitude of multifunctional proteins, involved in various pathways, are at play. However, initiation of formation of the phagophore starts with the activation of the ULK complex and its translocation to a precise membrane source site (Yu, Chen, and Tooze 2018; Egan et al. 2011; Chan et al. 2007). This “budding” site will more likely be on the ER and seem to be marked by ATG9 (Karanasios *et al.*, 2016). This autophagy initiation complex ULK is composed of ULK1 (UNC51-like Ser/Thr kinase and ATG1 orthologue), ULK2, ATG13, FIP200 (Kinase-family-interaction protein and ATG17 orthologue) and ATG101 (Wirth, Joachim and Tooze, 2013) (**Fig i 17**).

Although independent pathways have been described (Sarkar *et al.*, 2008; He and Klionsky, 2009; Stephan *et al.*, 2009), the recruitment of ULK1 for autophagy appears to be mainly regulated by other kinases mTORC1, AMPK and ubiquitination (Egan *et al.*, 2011; Kim and Guan, 2013; Yuan, Russell and Guan, 2013; Reidick *et al.*, 2015). Indeed, ULK1 was reported to work directly downstream of mTORC1 for autophagy induction (Kamada *et al.*, 2000; Yin, Pascual and Klionsky, 2016) (**Fig i 17**).

mTORC1:

mTOR (mechanistic Target of Rapamycin) is a protein coordinating eukaryotic cell growth, proliferation, survival (thus having great implication in cancerous mechanisms) as well as metabolism (including protein synthesis and degradation) and in response to environmental inputs like growth factors, stress signals, insulin signaling and through its nutrient sensor ability (Mortimore and Pösö, 1987; Saxton and Sabatini, 2017; Paquette, El-Houjeiri and Pause, 2018). It is the main actor of kinase mTOR Complex 1 which is autophagy's most characterized regulator. mTORC1 is composed of mTOR, RAPTOR (regulatory-associated protein of mTOR, which will be mentioned in this thesis), mLST8, DEPTOR and PRAS40 (Saxton and Sabatini, 2017). The kinase activity of mTORC1 directly influences autophagy as it will be described hereafter but also indirectly. Inactivation of mTORC1 allows translocation of TFEB (transcription factor EB) to the nucleus. TFEB is a master transcriptional regulator of genes involved in the biogenesis of both autophagosome and lysosome (Settembre *et al.*, 2011, 2012).

Kinase activity of mTORC1 causes binding and inactivation of ULK1 and consequently inhibits autophagy in basal conditions (nutrient rich conditions) (Kim *et al.*, 2011; Meijer *et al.*, 2015). In a situation of nutrient deprivation, ULK1 is freed from its inhibitory binding with mTORC1 and rendered able to initiate autophagy. Energy starvation also leads to ULK1 activation. Low ATP/AMP ratio causes activation of AMPK (AMP-activated protein kinase), which will in turn activate ULK1. ULK1 is then able to inhibit the mTORC by phosphorylating its subunit RAPTOR and thus initiate autophagy (J. W. Lee *et al.*, 2010; Bach *et al.*, 2011; Egan *et al.*, 2011). Concerning ubiquitination, mTORC1 is known as an inhibitor of AMBRA1 (PI3KIII complex subunit) in basal conditions. Upon starvation, inactive mTORC1 enables AMBRA1 to

promote poly-ubiquitylation of ULK1 which fosters its mediated initiation of autophagy (Grumati and Dikic, 2018). Consistently with the fact that mTOR has a close link to mitochondrial function, ROS have been described to induce autophagy and mitophagy (Desai, Myers and Schreiber, 2002; Kim *et al.*, 2002).

Upon induction, several molecular actors gather on location to promote formation of the phagophore. This is referred as nucleation and it primarily involves the recruitment of the PI3K-III kinase complex or class III phosphatidylinositol 3-kinase complex 3, which is composed of tumor suppressor Beclin-1 (ATG6 orthologue), ATG14, AMBRA1, p150/VPS15, VPS34 (Schu *et al.*, 1993; Kihara *et al.*, 2001). Activation of this complex relies on activation of VPS34 by Beclin-1, itself being activated upon release from Beclin-2, an anti-apoptotic protein (Di Bartolomeo *et al.*, 2010; He and Levine, 2010; Budini *et al.*, 2017) (**Fig i 17**).

The site of nucleation of the phagophore, called omegasome (Roberts and Ktistakis, 2013), is most likely sprouting from the ER but can also be from Golgi, mitochondria, endosomes or plasma membrane (Dunn, 1990; Ueno, Munro and Kominami, 1991; Axe *et al.*, 2008; Vaccaro *et al.*, 2008). The birthing phagophore is made of a lipidic bilayer membrane enriched in phosphatidylinositol 3-phosphate, PI(3)P (Budini *et al.*, 2017) (**Fig i 17**).

Elongation:

The newly sprouting phagophore expands to form an isolation membrane. In order to engulf the cellular contents that will be degraded, two ubiquitin-like conjugation systems are required to be integrated to the membrane (Polson *et al.*, 2010): the ATG12-ATG5-ATG16L1 system (which formation is ATG7 and ATG10-dependent (Itakura and Mizushima, 2010; Hamasaki *et al.*, 2013; Walczak and Martens, 2013)) and the ATG8 family system. The ATG8

family is notably comprised of LC3 (microtubule-associated protein 1A/1B light chain 3), GABARAP and GATE16. These proteins from the ATG8 family need to undergo lipidation in order to serve their ubiquitin-like conjugating function, which requires ATG4b-dependent cleavage of their C-terminal domain and integration within the autophagic vesicle by ATG7 and ATG3-mediated conjugation with a lipid phosphatidylethanolamine (PE) (Tanida *et al.*, 2004; Katahira *et al.*, 2007). During lipidation, the most characterized ATG 8 family member, LC3, is transformed in LC3 I after cleavage and in LC3 II after its integration in the membrane (Otomo *et al.*, 2012) (**Fig i 17**). It allows cargo sequestration by associating with autophagy receptors (Mizushima, Yoshimori and Ohsumi, 2011). In its lipidated form, LC3 seems to mediate membrane tethering and hemifusion, drive expansion and control the size of the autophagosome (Xie *et al.* 2008; Nakatogawa *et al.* 2007).

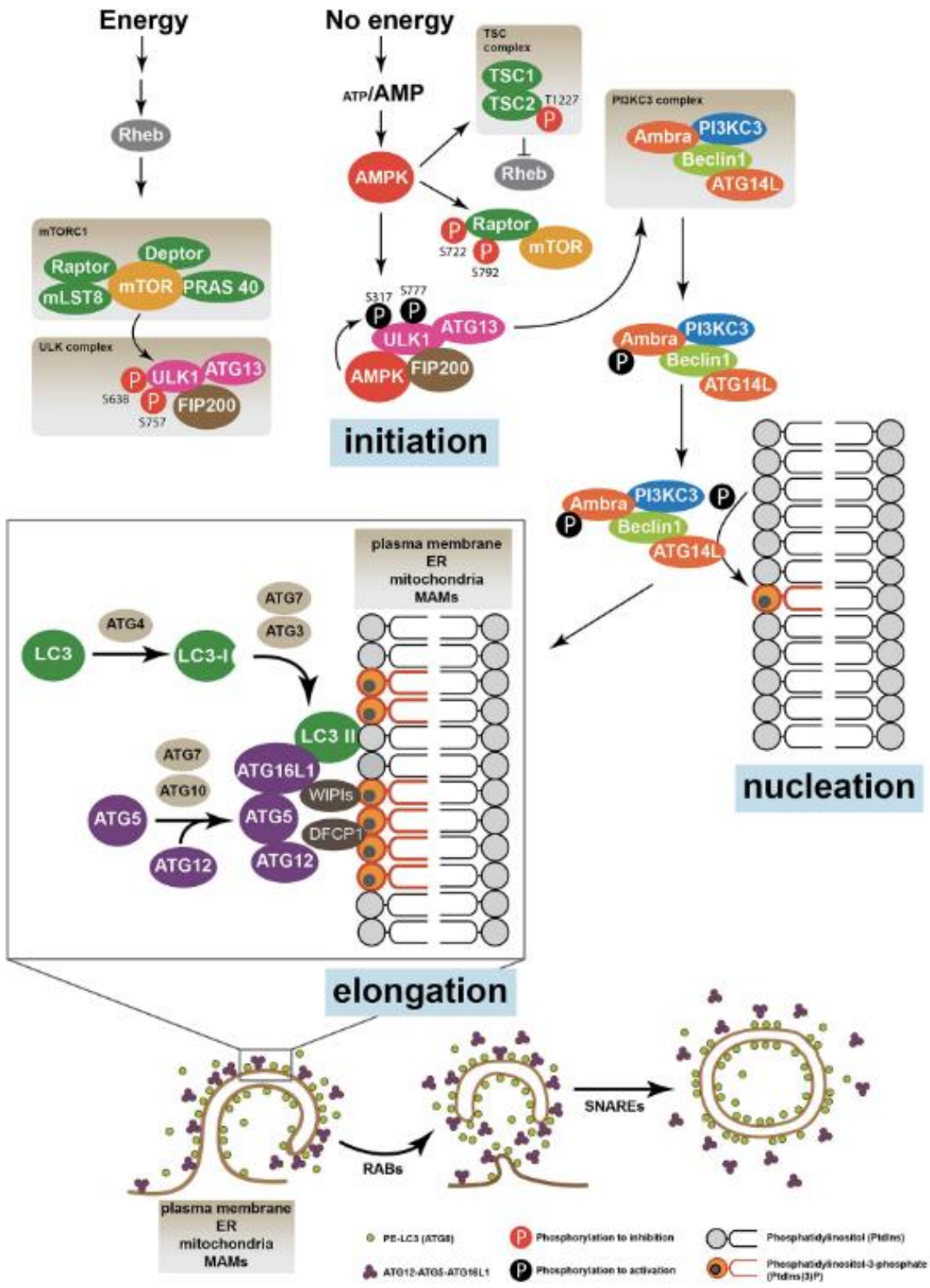


Figure i 17: Schematics of the autophagic machinery; modified from Budini et al, 2017.

Representation of the key molecular actors of the different stages of autophagy

Cargo selection and targeting to the autophagosome:

The selective ability of autophagy was highlighted by the characterization of autophagy receptors. Unlike bulk autophagy, selective autophagy needs to differentiate the cell content that need to be targeted to the autophagosome and degraded. This targeting process mainly involves ubiquitylation of the cargo.

Ubiquitin, the “kiss of death”:

Ubiquitin is a small globular protein (8 kDa) that is found in all tissues of eukaryotes (thus the term “ubiquitous”) and shows great conservation (Hershko and Ciechanover, 1998). Ubiquitin covalently conjugates to a lysine substrate and act as a modifier. This tagging process is called ubiquitylation (or ubiquitination) and serves multiple cellular mechanisms such as gene transcription, DNA repair, cell cycle, signaling and death but is also the most prevalent form of substrate targeting in degradation pathways. Ubiquitylated proteins or organelles can be targeted to the proteasome (UPS), lysosome or the autophagosome (Grumati and Dikic, 2018). The type of ubiquitylation can act on the targeting as a substrate can undergo single or multiple mono-ubiquitylation but also binding of chains of ubiquitin. Furthermore, the nature of the poly-Ubiquitin chain seems to play a role on the substrate fate and activation of a preferred degradation pathway (Kirisako *et al.*, 2006; Husnjak and Dikic, 2012; Grumati and Dikic, 2018). Importantly, ubiquitin is the key substrate of the overwhelming majority of inclusion bodies in neurological diseases; and has been used for the past decades as the keystone marker for pathological studies (Grumati and Dikic, 2018).

Autophagy receptors are responsible for the recognition of ubiquitylated substrates (**Fig i 18**). Two dozen of autophagy receptors have been identified but only a few have been

well characterized, among which, SQSTM1/p62, OPTN, UBQLN2 have been associated with ALS when mutated (Maruyama *et al.*, 2010; Deng, Chen, *et al.*, 2011; Fecto, Yan, P. Vemula, *et al.*, 2011). These proteins share in common two particular structures: ubiquitin-binding domains (UBA or UBAN) that allow recognition of the cargo; and LC3-interacting region (LIR) that permits binding to lipidated proteins from the ATG8 family and thus engulfment into the autophagosome (Lippai and Low, 2014; Rogov *et al.*, 2014; Stolz, Ernst and Dikic, 2014; Wild, McEwan and Dikic, 2014). TBK1 (Ser/Thr Tank-binding kinase 1) was shown to phosphorylate and thus activate SQSTM1/p62 and OPTN to promote autophagy (Wild *et al.*, 2011; Komatsu, Kageyama and Ichimura, 2012b; Pilli *et al.*, 2012; Matsumoto *et al.*, 2015; Richter, Danielle A Sliter, *et al.*, 2016). The role in ALS of TBK1, p62/SQSTM1 and OPTN will be further in section III.B.3 “Focus on p62/SQSTM1, OPTN and TBK1: key ALS-related genes”.

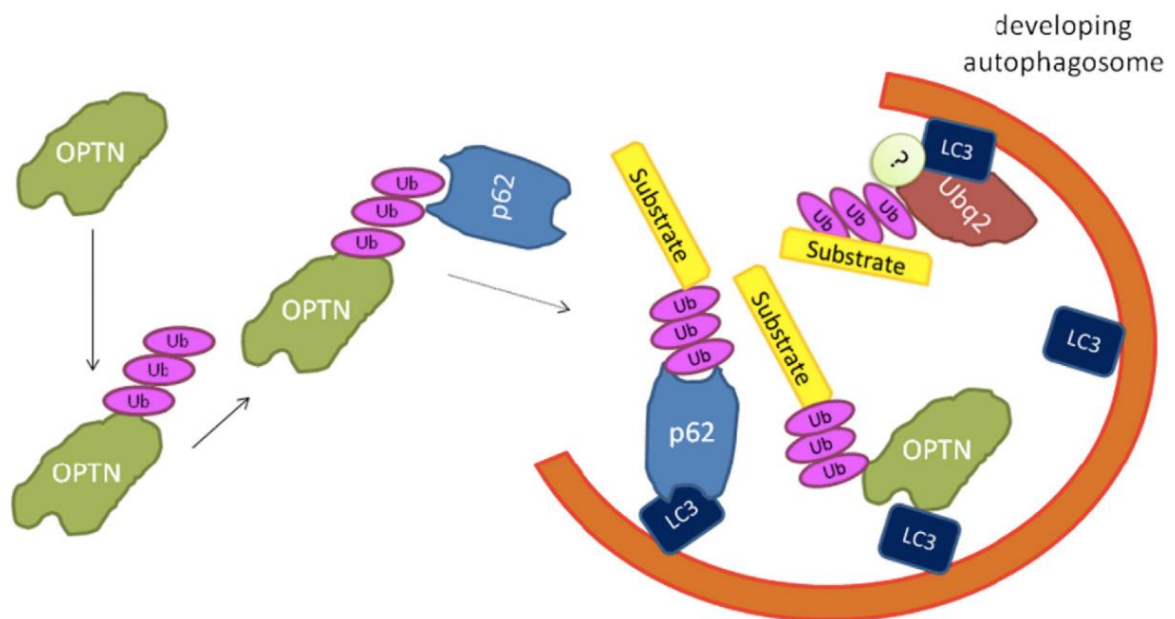


Figure i 18: Schematic model of the role of p62/SQSTM1, OPTN and UBQLN2 as autophagy receptors; modified from Majcher *et al.* 2015.

Maturation and Degradation:

As cargo selection occurs, the phagophore expands until it closes on itself and forms an independent and double-membraned vesicle, the autophagosome. The autophagosome matures and is able to traffic using the microtubule network. It will eventually encounter the lysosome and fuse with it to form the autolysosome. The enzymatic lysosomal content enables hydrolytic degradation of the sequestered content and inner membrane of the autophagosome (Mizushima, 2007). Fusion is mediated by a series of tethering factors among which is the HOPS complex (homotypic fusion and protein sorting) (Rieder and Emr, 1997; Seals *et al.*, 2000; Wurmser, Sato and Emr, 2000) and adapting proteins called SNAREs (soluble N-ethyl-maleimide-sensitive fusion attachment protein receptor) (Moreau, Renna and Rubinsztein, 2013). The specificity of fusion between autophagosome and lysosome is still unclear but LC3 may play a role (McEwan *et al.*, 2015; Z. Wang *et al.*, 2016). The macromolecules resulting from degradation are released in the cytosol through the action of permeases and recycled by the cell (Suriapranata *et al.*, 2000; Yang *et al.*, 2006).

(3) Mitophagy

Mitophagy is the process of degradation of mitochondria. Their removal can be part of normal turnover, to accommodate metabolic conditions and meet the cellular requirements of particular steps of development (Schweers *et al.*, 2007; Palikaras, Lionaki and Tavernarakis, 2018). It can also serve as a quality control and selectively degrade damaged or exhausted mitochondria (Kim, Rodriguez-Enriquez and Lemasters, 2007; Novak *et al.*, 2010). Mitochondria enter in contact with the autophagosome through lipidated LC3-docking via receptors that have LIR domains but not necessarily the faculty to bind ubiquitin (Palikaras,

Lionaki and Tavernarakis, 2018). Overall, mitophagy can be classified into ubiquitin-dependent or -independent (Khaminets, Behl and Dikic, 2016). Ubiquitin-dependent mitophagy is regulated by the PINK1/Parkin pathway as it catalyzes the attachment of ubiquitin chains on mitochondrial outer membrane (MOM or OMM) and promotes their autophagy (Clark *et al.*, 2006; Park *et al.*, 2006; Narendra *et al.*, 2008). Several autophagy receptors have been described to occasionally participate in mitophagy such as p62/SQSTM1, NDP52 or TAXIBP1 (Moore and Erika L.F. Holzbaur, 2016) but only OPTN was shown to be sufficient for mitophagy and promoted engulfment of mitochondria in the autophagosome via LC3 binding (Wong and Holzbaur, 2014; Heo *et al.*, 2015; Moore and Erika L. F. Holzbaur, 2016; Richter, Danielle A Sliter, *et al.*, 2016). In these studies, OPTN and TBK1 (Ser/Thr Tank-binding kinase 1) were described to form an axis essential for mitochondrial quality control and cellular homeostasis (He, Chen and Li, 2017).

(4) Measuring autophagy

Decades of scientific advances have outlined the essential role of autophagy in normal physiology as well as in pathological mechanisms. Hence, the need to monitor and measure autophagy is crucial for our understanding of this process. Unfortunately, available methods often give partial information, are complex and can be subjects of misinterpretation. These methods can be separated in two different type of approaches: simple observation of autophagy-related structures; and quantification of the degradation of proteins and organelles (also known as assessing the autophagic flux) (Yoshii and Mizushima, 2017).

Observation of autophagic structures.

Being the most upstream autophagy specific actor, ULK1 complex subunits as well as ATG9 can be used as markers to visualize the site of initiation of autophagosome formation (Itakura and Mizushima, 2010). Later on, markers of the ULK1 complex, PI3KIII complex or autophagy conjugation systems such as LC3 allow visualization of the protruding isolation membrane (Kishi-Itakura *et al.*, 2014). The omegasome site is also characterized by the presence of the DFCP1 protein (Axe *et al.*, 2008). Completion of the autophagosome will mark the detachment of these early ATG proteins but not LC3 and other ATG8 family members. Although it also localizes to ER and mitochondria, STX17, an autophagosomal SNARE can be a marker of closed autophagosomes (Itakura, Kishi-Itakura and Mizushima, 2012). The LC3-positive, STX17-positive mature autophagosomes fuse, and thus colocalize, with lysosomes that can be marked by LAMP1/2 or LysoTracker. The LysoTracker will give more temporal information on the autolysosome as it will mark differently early fusion vesicles from later ones that already suffered from inner membrane degradation (Tsuboyama *et al.*, 2016). Accumulation of these structures can indicate degradation defects.

These molecular markers are good evidences of the presence of autophagosomes but not definitive proof. Although incorrect identification is possible, electron microscopy can allow direct visualization of double-membrane vesicles that are autophagosomes and single-membrane ones that are autolysosome (Eskelinen, 2008b, 2008a). However successfully used on cell cultures, electron microscopy is challenging on tissues (Kishi-Itakura *et al.*, 2014).

Measurement of autophagic flux:

In order to quantify the degradation of autophagosomes, in other words the end result of autophagy, LC3 is by far the most widely used marker because of its useful characteristics. The preform of LC3 is cleaved to form LC3 I and LC3 II after integration in the autophagosome

membrane (Otomo *et al.*, 2012). After this lipidation process, LC3 II is present in both outer and inner membranes of the autophagosome and as the inner membrane is degraded, a portion of LC3 II is also degraded (Kabeya *et al.*, 2000). The amount of LC3 II can hence reflect the number of autophagosomes. A ratio between LC3 I and LC3 II is often used to quantify LC3's integration in autophagosomes as compared to its cytosolic presence but variable efficiency to detect LC3 I by immunoblotting in comparison with LC3 II renders any interpretation difficult (Mizushima and Yoshimori, 2007). The autophagy receptor p62/SQSTM1 is also used to quantify autophagosomes as it binds to LC3 and is degraded by autophagy (Bjørkøy *et al.*, 2005; Pankiv *et al.*, 2007). In tissues especially, a deficiency in autophagy is indicated by accumulation of soluble p62/SQSTM1 and p62-positive puncta. Nevertheless, as p62 expression is transcriptionally regulated, a combined quantification of protein and RNA levels is advised (Jamart *et al.*, 2013; Sanchez *et al.*, 2014).

However, at a given time point, an accumulation of LC3 II or p62/SQSTM1 and by extent autophagosomes can mean an activation of autophagy as well as an inhibition of their degradation thus leading to misinterpretation. To actually quantify the autophagic flux, one is required to determine the lysosome-dependent degradation of these autophagosomal markers. This can be achieved by comparing accumulation of the markers in a control condition to a condition where lysosomal degradation is inhibited, by using Bafilomycin A1 or B1 for example. The difference in amount of autophagosomes represents the autophagic flux (Yoshii and Mizushima, 2017).

Various techniques using exogenous constructs and reporters are also used to assess autophagic flux (extensively reviewed in (Yoshii and Mizushima, 2017)) and some of them use LC3 properties. One example is the GFP-LC3-RFP-LC3ΔG probe. This single fusion protein can

be cleaved by ATG4b on the C-terminal of the first LC3 but not on the second modified LC3 resulting in the equivalent production of GFP-LC3 that can be integrated into the membranes of autophagosomes and RFP-LC3ΔG that will stay in the cytosol and unintegrated to autophagosomes. As autophagosomes and a portion of GFP-LC3ΔG undergo degradation, its levels can be compared to the internal control that is cytosolic RFP-LC3ΔG and give an estimate of the autophagic flux (Kaizuka *et al.*, 2016).

(5) Modulating autophagy

As previously discussed, autophagy is an highly adaptative system that integrates inputs from various signaling pathways and stress signals that are mainly integrated by autophagy's upstream master regulators mTORC1 and AMPK as well as ubiquitylation but also through cross-signaling events that involve autophagy-related proteins that also have roles in other pathways. The importance of autophagy in normal and pathological mechanisms has also powered the characterization of pharmacological and nutritional modulators notably for their therapeutic potential. As the process is complex and spreads out in several stages that involves at least 100 different proteins (Noda and Inagaki, 2015) it provides numerous targets of modulation for both inhibition or activation at each step of autophagy (Galluzzi *et al.*, 2017) **(Fig i 19).**

In this section, we will focus on drugs that will be used in this study. Bafilomycin is a family of macrolide antibiotics produced by gram-positive bacteria, Streptomyces (Dröse and Altendorf, 1997). The different compounds of the family exhibit various biological functions such as anti-tumoral, -parasitic, -fungal and immunosuppressant activities (Hayashi

et al., 2000; Marchesini *et al.*, 2005; Padrón, 2010; Keller, Schmidt and Lünemann, 2017; Whitton *et al.*, 2018). As mentioned in section III.A.4) “Measuring autophagy”,

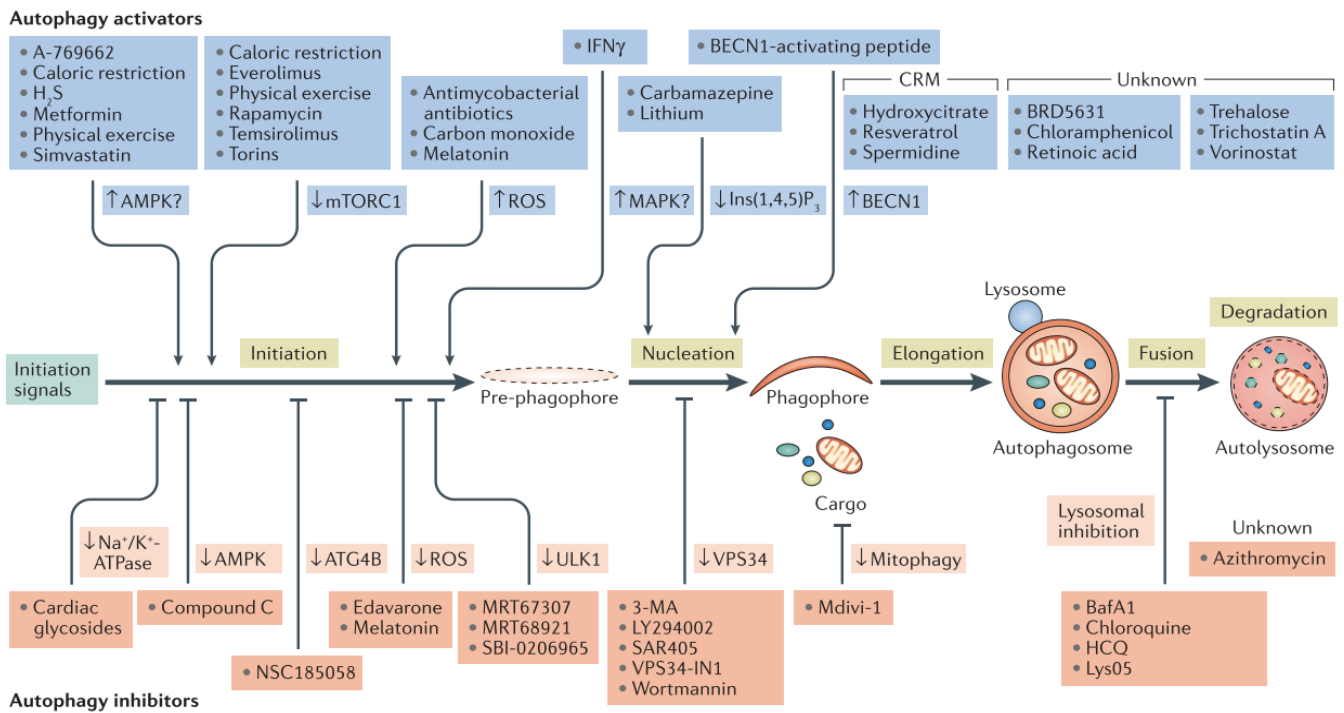


Figure i 19: Schematics of pharmacological or nutritional modulators of autophagy; modified from Galluzzi *et al.* 2017.

Bafilomycin B2 is a known inhibitor of lysosomal degradation (**Fig i 19**). More precisely, and similarly to the widely used Bafilomycin A1, the molecule inhibits vacuolar or V-ATPase (Hayashi *et al.*, 2000) which normally promotes acidification and normal function of the lysosome (Yamamoto *et al.*, 1998; Vinod *et al.*, 2014). In addition, several studies have shown that the molecule also blocks fusion of autophagosomes with lysosome (Shacka, Klocke and Roth, 2006) with variability depending on treatment (Klionsky *et al.*, 2008). Use of Bafilomycin A1 or B2 thus induce accumulation of autophagosomes (Boya *et al.*, 2005; Shacka, Klocke and Roth, 2006) and related markers, such as p62 and LC3 notably in neurons (Lumkwana *et al.*,

2017). Strong inhibition of autophagy via Bafilomycin is however concomitant with toxicity, autophagic stress and apoptosis (Shacka, Klocke and Roth, 2006).

Also used in this study, Torin1 is a powerful autophagy activator (Kaizuka *et al.*, 2016; Galluzzi *et al.*, 2017). It is an ATP-competitive inhibitor of kinase activity of mTOR-related complexes mTORC1 and mTORC2 (Thoreen *et al.*, 2009; Liu *et al.*, 2010) (**Fig i 19**). Torin1 presents high selectivity towards mTOR (a 1000-fold selectivity over PI3KIII complex) and, as compared to Rapamycin, a commonly used inhibitor of mTOR and activator of autophagy, disrupts mTORC1-dependant phenotypes with more completion. Concerning the transcriptional machinery notably, Torin1 can affect Rapamycin-resistant (or partially resistant) transcription factors and mTORC1 substrates TFEB, 4EBP, S6K that feed autophagy, lysosome biogenesis but also protein synthesis, cell proliferation and inflammation (Thoreen *et al.*, 2009; Kim and Guan, 2015; Napolitano *et al.*, 2018). Torin1 is indeed vastly used in the field of cancer (Sun, 2013; Xie, Wang and Proud, 2016).

Altogether, these 2 autophagy modulators are used in this study to evaluate autophagic flux but also appreciate TDP-43's effect on autophagy and adaptability to autophagy modulations.

B) Autophagy's implication in ALS

(1) Autophagy in neurons

Neurons are particularly vulnerable to defective proteostasis and organelle damage. Several neurodegenerative diseases such as ALS, Alzheimer's, Parkinson's and Huntington's diseases, are characterized by accumulation of misfolded proteins (Cushman *et al.*, 2010;

Nixon, 2013). Aging being a major risk factor for such diseases, it reflects the struggle of neurons to maintain their viability and functionality for a lifetime (Carroll, Hewitt and Korolchuk, 2013). Apart from their long life, neurons face other challenges that justify this vulnerability. As post-mitotic cells, they cannot undergo division to discard some of their damaged and dysfunctional content (Tooze and Schiavo, 2008; Kulkarni, Chen and Maday, 2018). Their morphology and complex polarized structure present a logistic challenge that require important trafficking of vesicles. Autophagosomes are able to form and engulf cargoes in the entirety of the neuron and notably distally (Maday and Holzbaur, 2014). However, lysosomal activity seems to be concentrated in the soma which requires retrograde transport of distal autophagosomes (Lee, Sato and Nixon, 2011; Maday, Wallace and Holzbaur, 2012; Cheng *et al.*, 2015; Maday and Holzbaur, 2016) (**Fig i 20**).

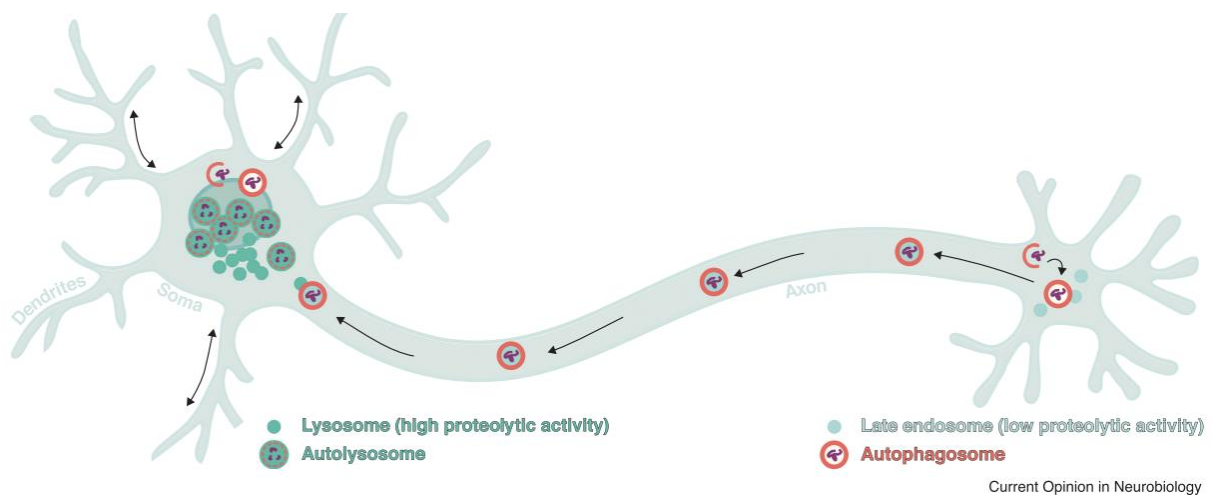


Figure i 20: Mechanism of neuronal autophagy; modified from Kulkarni et al, 2018.

Anterograde transport of autophagosomes towards lysosomes concentrated at the cell body.

Efficient autophagy is essential for homeostasis and survival of neurons (Yamamoto and Yue, 2014; Ariosa and Klionsky, 2016; Kulkarni and Maday, 2018). In mice, specific genetic depletion of ATG 7 and 5 in neurons leads to axonal degeneration and cell death (Komatsu *et*

al., 2007; Nishiyama *et al.*, 2007). This neuronal death is associated with increased ubiquitin-positive protein aggregates indicating defective proteostasis and more specifically autophagy as no UPS dysregulation was observed (Hara *et al.*, 2006; Komatsu *et al.*, 2006). Autophagy was described to play a key role in synaptic development, maintenance and function (Shen and Ganetzky, 2009; Hernandez *et al.*, 2012; Tang *et al.*, 2014; Stavoe *et al.*, 2016; Rudnick *et al.*, 2017). Surrounding glia might play a role in regulating autophagy and notably in neuropathologic mechanisms but clear evidence is still missing (Kulkarni, Chen and Maday, 2018).

As autophagy appears crucial for normal neuronal function and implicated in neuropathologic mechanisms, the degradative process may be a potential therapeutic target in neurodegenerative disorders (Sarkar *et al.*, 2007; Vidal *et al.*, 2014; Maiuri and Kroemer, 2019).

(2) *Autophagy defects in ALS*

Defective autophagy appears as one of the most recurring theme in ALS pathology as the presence of ubiquitin-positive proteinaceous inclusions is close to universal in patients (Nguyen, Thombre and Wang, 2019). Protein misfolding, aggregation and subsequent proteotoxicity has been linked to mutations in key actors TDP-43, SOD1, FUS and C9orf72 (Peters, Ghasemi and Brown Jr., 2015). Proteinopathy is interestingly not only related to mutant but also to wild-type proteins such as TDP-43 in a large portion of ALS cases (Arai *et al.*, 2006; Neumann *et al.*, 2006; Kwong *et al.*, 2007) and to wild-type SOD1 to a lesser degree (Bosco *et al.*, 2010; Forsberg *et al.*, 2010). All these inclusions can also be positive for numerous autophagy-related proteins such as p62/SQSTM1, OPTN, VCP or UBQLN2 (Ling,

Polymenidou and Cleveland, 2013). C9orf72 patients, in addition to the presence of DPRs and TDP-43-positive inclusions, also present unique p62/SQSTM1 inclusions that are negative for TDP-43 (Al-Sarraj *et al.*, 2011; Mackenzie, Frick and Neumann, 2014). Autophagy may be a starting point of pathogenesis as these observations suggest that formation of these inclusions appears to be secondary to autophagy and overall protein quality control failure (Levine and Kroemer, 2008; Tooze and Schiavo, 2008; Menzies, Fleming and Rubinsztein, 2015).

The histopathological hallmark that is protein aggregation has already been described in section **I.B.5) Histopathology** and is not the only striking evidence for autophagy defects in ALS. Indeed, an important portion of ALS genes are directly involved in the autophagic process and associated pathways and have helped in the development of animal models that highlight autophagy related pathological mechanisms such as C9orf72, p62/SQSTM1, OPTN, UBQLN2, TBK1, CHMP2B, VCP or DCTN among others and will be discussed hereafter. However, concerning the two autophagy receptors p62/SQSTM1 and OPTN and their upstream activator TBK1, section **III.B.3) “Focus on p62/SQSTM1, OPTN and TBK1: key ALS-related genes”** will be entirely focused on them as they have an important role to play in this study.

C9orf72:

The hexanucleotide GGGGCC repeat expansion in the first intron of the *C9orf72* gene is the most common genetic cause for the development of ALS/FTD with 23-47% of fALS and 4-21% of sALS (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). C9orf72 patients present unique histopathologic traits apart from TDP-43-positive inclusions which is the presence of TDP-43-negative but p62-positive inclusions (Al-Sarraj *et al.*, 2011), C9orf72 DPRs (Ash *et al.* 2013) and RNA foci (T. Zu *et al.*, 2013) that were previously described in section **I.B.5) “Histopathology”**.

Since its unveiling in 2011, three C9orf72-related mechanistic explanation have been proposed in the contribution of pathogenesis: 1) Haploinsufficiency and thus reduction or loss of C9orf72 protein (Belzil *et al.*, 2013; Ciura *et al.*, 2013; Therrien *et al.*, 2013; Waite *et al.*, 2014); 2) C9orf72 RNA foci-mediated toxicity (Donnelly *et al.*, 2013; Haeusler *et al.*, 2014); 3) C9orf72 DPR-mediated toxicity (Ash *et al.* 2013; Mizielinska *et al.* 2014; Tao *et al.* 2015) (**Fig i 21**).

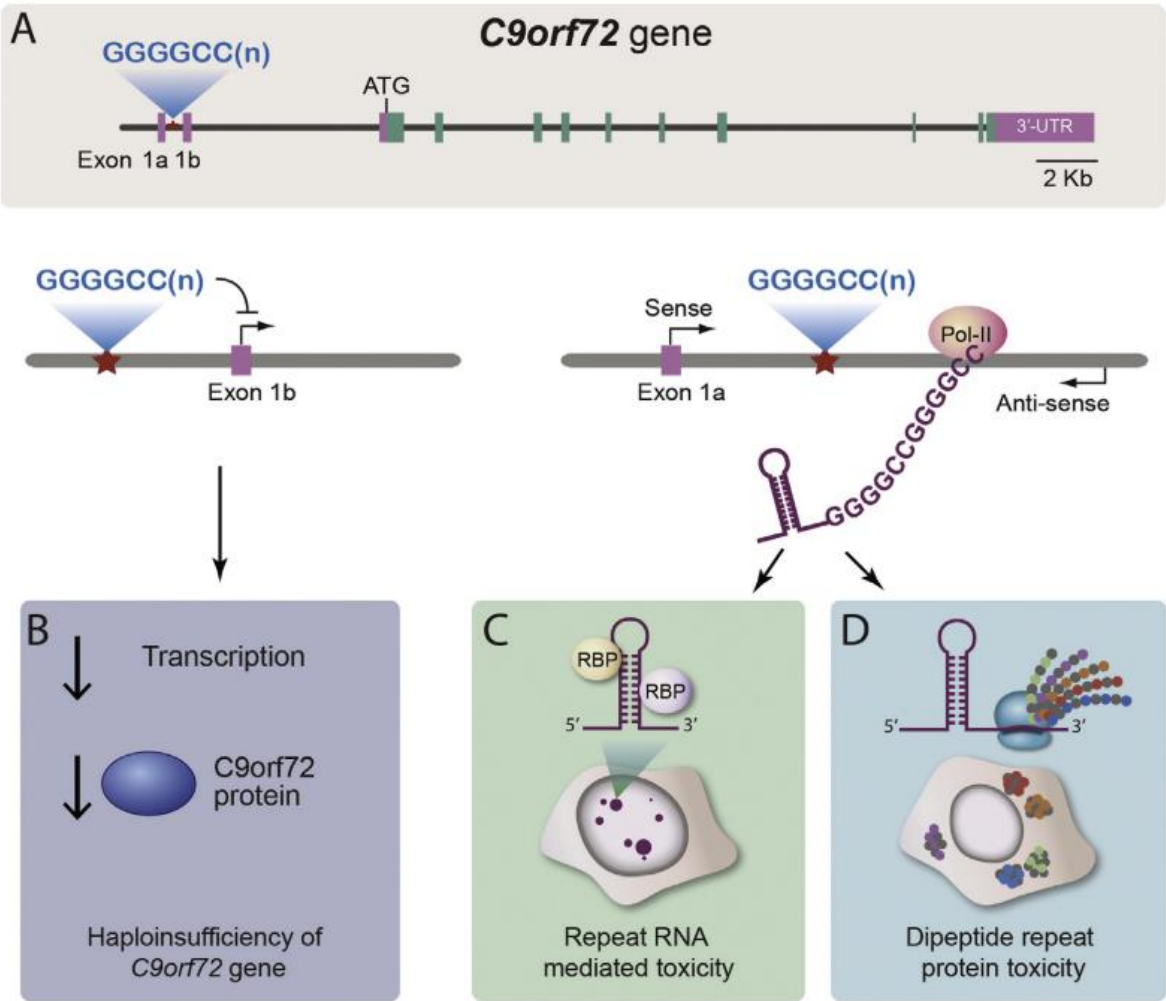


Figure i 21: The proposed mechanisms for C9orf72-mediated pathology; modified from Gitler *et al.*, 2016.

C9orf72 hexanucleotide repeat expansion could be toxic through three mechanisms: loss-of-function (B); gain of toxic function of RNA foci (C) and Dipeptide repeats (D).

As of today, C9orf72's exact function remains unclear, but evidence point towards a multifunctional role and a particular involvement in autophagy. C9orf72 was found to affect autophagy initiation through ULK1 in several potential ways. First, by interacting with the ULK1 complex directly, by binding to SMCR8 (a regulator of ULK1 activity and gene expression) and by interacting with several autophagy related small GTPases, Rabs. C9orf72 may affect trafficking and activation of the ULK1 complex and compromise its downstream effects in a Rab-dependent manner (Szatmári and Sass 2014; M. Yang et al. 2016; Sellier et al. 2016; Amick et al, 2016; Ugolino et al. 2016; Jung et al. 2017; Sullivan et al. 2016; Webster et al. 2016). By interacting with multiple Rabs (Aoki *et al.*, 2017; Farg *et al.*, 2017) and F-actin (Sivadasan *et al.*, 2016) notably, C9orf72 might affect the autophagy-lysosome pathway through an effect on dynamic and structure of the cytoskeleton (J.-Y. Lee *et al.*, 2010; Aguilera, Berón and Colombo, 2012; Zhuo *et al.*, 2013). Upstream of ULK1, loss of C9orf72 function seem to inhibit mTOR (Amick, Rocznik-Ferguson and Ferguson, 2016; Ugolino *et al.*, 2016) and promote autophagy by translocation of TFEB, the transcription factor inducer of autophagy and lysosome biogenesis (Ugolino *et al.*, 2016; Yang *et al.*, 2016). C9orf72 presents a starvation-induced localization at the lysosome and seem to modulate lysosomal function (Amick et al. 2016; Farg et al. 2017; O'Rourke et al. 2016). Overall, C9orf72's implication in autophagy seems complex and will benefit from future studies.

Ubiquilin 2:

UBQLN2 is another example of autophagy receptor found mutated and present in neuronal cytoplasmic inclusions in ALS patients (Deng, Chen, *et al.*, 2011). The mutation is relatively rare as it is estimated to represent about 0,4% of sALS cases (Daoud *et al.*, 2012; Millecamps *et al.*, 2012). The protein is able to bind both ubiquitin and autophagosome-

integrated LC3 (Rothenberg *et al.*, 2010). In animal models, expression of ALS-related mutant UBQLN2 leads to dysfunctional proteostasis, aberrant accumulation of p62 and LC3-II, promotion of protein aggregation and neurodegeneration (Ceballos-Diaz *et al.*, 2015; Hjerpe *et al.*, 2016; Huang *et al.*, 2016; Picher-Martel *et al.*, 2019). These studies however underlined that some of these aspects are due to impairments of the proteasomes. UBQLN2 precise role in autophagy remains poorly characterized as compared to other autophagy receptors p62/SQSTM1 and OPTN (Nguyen, Thombre and Wang, 2019).

Valosin containing protein:

VCP is a AAA+-ATPase involved in protein clearance with clear implication in proteasomal degradation (DeLaBarre *et al.*, 2006) and emerging evidence of an involvement in autophagy (J.-S. Ju *et al.*, 2009; Tresse, Florian A Salomons, *et al.*, 2010). It also plays a role in multiple cellular processes such as cell cycle regulation, DNA repair and replication (Kakizuka, 2008). VCP mutations account for approximately 1 to 2% of fALS (Janel O Johnson *et al.*, 2010; Renton, Chiò and Traynor, 2014). Additionally, VCP mutations are causal in other diseases like FTD, Paget's disease of bone and inclusion body myopathy (Watts *et al.*, 2007). In these diseases, VCP patients show colocalized accumulation of autophagosomes, p62/SQSTM1 and LC3 (J.-S. Ju *et al.*, 2009; Tresse, Florian A Salomons, *et al.*, 2010). VCP was described to inhibit mTOR activity and thus promoting autophagy initiation and autophagosome biogenesis. VCP could also play a later role in the autophagic process as expression of ALS or other VCP-related disease mutations where shown to alter autophagosome maturation and promoted ubiquitin-positive aggregate accumulation in mice (J.-S. Ju *et al.*, 2009; Tresse, Florian A Salomons, *et al.*, 2010) and interfere in autophagosome-lysosome fusion and degradation in cells (Tresse, Florian A Salomons, *et al.*, 2010). An

implication in SG clearance was also suggested as Cdc48, VCP's orthologue in yeast, promotes SG targeting for autophagy (Buchan *et al.*, 2013). In cell culture, VCP seem to regulate the amount of TDP-43-positive SG where mutant VCP promoted their accumulation (Buchan *et al.*, 2013).

Charged multivesicular protein 2B:

Mutations in CHMP2B accounted for 1% of ALS cases in a large cohort study (Cox *et al.*, 2010). The protein is a subunit of ESCRT-III, endosomal sorting complex required for transport III, which enables autophagic degradation of membrane proteins through the formation of multivesicular bodies (Rusten and Simonsen, 2008; Bodon *et al.*, 2011). Examination of CHMP2B ALS patients' brains revealed accumulation of immature autophagosomes and high levels of p62/SQSTM1 and LC3-II (Parkinson *et al.*, 2006; Cox *et al.*, 2010). Mutations lead to truncation of the C-terminal part of the protein (J.-H. Han *et al.*, 2012) and were associated with inhibition of autophagy and subsequent formation of p62- and ubiquitin-positive inclusions (Filimonenko *et al.*, 2007; Lee *et al.*, 2007; Ghazi-Noori *et al.*, 2012) as well as altered autophagosome-lysosome fusion (Lee *et al.*, 2007; J.-Y. Lee *et al.*, 2010), inhibited autophagosome maturation via alteration of SNARE activity (Lu *et al.*, 2013) and impaired endocytic trafficking (J.-H. Han *et al.*, 2012).

Dynactin:

DCTN is estimated to account for 0,5 to 3% of sALS (X. Liu *et al.*, 2017) and, by binding to molecular motor dynein, is required for retrograde transport of autophagosomes (Laird *et al.*, 2008). Indeed, DCTN mutations have been associated with reduced affinity for microtubules hence repressing retrograde transport and resulting in aggregation (Levy *et al.*,

2006). In mice, mutant DCTN alters vesicular trafficking and causes accumulation of autophagosomes enriched in LC3-II and ubiquitin (Laird *et al.*, 2008). Retrograde transport of autophagosomes was also decreased upon knockdown of DCTN in *C. elegans* and lead to their accumulation and incomplete maturation resulting in degeneration of motor neurons (Ikenaka *et al.*, 2013).

Other notable ALS-related genes that appear to be involved in autophagy are VAPB, PFN1, ALS2 and FIG4 (Nguyen, Thombre and Wang, 2019). Interestingly, key ALS genes like SOD1, TDP-43 or FUS, which primary function does not consist in regulating autophagic machinery, also exhibit an effect on autophagy in the context of ALS.

Indeed, induction of autophagy has been observed in transgenic mutant SOD1 mouse model (Morimoto *et al.*, 2007) as well as in the spinal cord of SOD1 ALS patients. Further post mortem examination of patients revealed signs of perturbed autophagy (Sasaki, 2011). ALS-related SOD1^{G85R} mice display reduced levels of Ser757-phosphorylated ULK1 (an inhibited form of the protein) suggesting mTOR repression and AMPK activation (Kim *et al.*, 2011; Bandyopadhyay *et al.*, 2014) while SOD1^{G93A} was linked to increased production and turnover of LC3-II in mice (Morimoto *et al.*, 2007; Li, Zhang and Le, 2008; An *et al.*, 2014; Chen *et al.*, 2015) and motor neuron cultures (Wei, 2014), both of which are consistent with autophagy induction. In addition, this later mouse model showed up persisting upregulation of autophagy-promoting transcription factor TFEB (Settembre and Ballabio, 2011) and autophagy initiation-related protein Beclin 1 (Kang *et al.*, 2011). Furthermore, aberrant interaction and clustering of mutant SOD1 with dynein lead to impaired retrograde axonal transport of autophagosomes thus contributing to failure of autolysosome formation (F.

Zhang *et al.*, 2007; Bilsland *et al.*, 2010). Modulation of autophagy to counteract on SOD1-related pathology showed contrasting result as promoting autophagy seems protective in early SOD1 ALS pathology but aggravating in later stages (K. Zhang *et al.*, 2013; Rudnick *et al.*, 2017).

As these two major mechanistic hypotheses for pathogenesis in ALS are at the center of the thesis, the interaction between autophagy and the RNA-binding proteins FUS and more particularly TDP-43 will be thoroughly discussed in section IV **“ALS: at a crossroad between autophagy and RNA metabolism defects”**.

(3) Focus on p62/SQSTM1, OPTN and TBK1: key ALS related genes

Mutations in autophagy receptors p62/SQSTM1 and OPTN and their activating kinase TBK1 were all identified in forms of ALS (Maruyama *et al.*, 2010; Fecto, Yan, S. P. Vemula, *et al.*, 2011; Cirulli *et al.*, 2015). These three proteins share in common a multi-functional nature and are key actors of the autophagic pathway reinforcing the importance of the process in ALS pathology. They will take on a significant role in this thesis as we will investigate new interactions between autophagy and TDP-43.

p62/SQSTM1:

sequestosome 1, or *SQSTM1*, encodes for p62 which mutations are estimated to cause approximately 1% of ALS cases (Fecto, Yan, S. P. Vemula, *et al.*, 2011; Renton, Chiò and Traynor, 2014) and about 2% of FTD cases (Le Ber *et al.*, 2013). As previously mentioned in section III.A.2) **“The stages of autophagy”**, it is one of the most characterized autophagy

receptor and is highly expressed in spinal motor neurons (Arai *et al.*, 2003). Indeed, its structure allows it to act as an important molecular adaptor during cargo selection and engulfment into the autophagosome. Its C-terminal UBA domain enables binding to ubiquitylated substrates while its LIR domain binds ATG8 family proteins such as LC3 (Johansen and Lamark, 2011) (**Fig i 22**). As compared to similar autophagy receptor, p62/SQSTM1 can oligomerize as it also contains a PB1 domain (Protein Binding 1) at the N-terminal part that is essential for its autophagic function (Gal *et al.*, 2007) (**Fig i 22**). In addition, this domain can allow binding to other autophagy receptors OPTN and NBR1 (Johansen and Lamark, 2011; Liu *et al.*, 2014). p62/SQSTM1 was reported to have a regulating effect on mTORC1's nutrient-sensing pathway that feeds autophagy. The autophagy receptor can bind RAPTOR, key subunit of mTORC1, and mediates activation of downstream S6K1 and 4EBP1. In addition, by binding to specific lysosomal Rags, p62/SQSTM1 permits translocation of mTORC1 to the lysosome and interaction with the Rags, a key process for mTOR signaling in autophagy (Duran *et al.*, 2011). Apart from its determinant role in autophagy, the multifunctional p62/SQSTM1 is implicated in UPS-degradation notably through activation of Nrf2; and inflammation-related NF- κ B pathway through its TRAF1-binding sequence (TBS) and KIR domain (Keap1 interacting region) (Komatsu, Kageyama and Ichimura, 2012a; Rea *et al.*, 2013; Bitto *et al.*, 2014) (**Fig i 22**), all of which are also investigated in ALS pathology and logically ties p62/SQSTM1 to other pathological mechanisms. The protein is linked to Paget's disease of bone (mutations in p62/SQSTM1 being the most commonly identified genetic cause), several age-related diseases and notably cancer (Moscato and Diaz-Meco, 2012; Rea *et al.*, 2013, 2014; Bitto *et al.*, 2014; Moscato, Karin and Diaz-Meco, 2016).



Figure i 22: The structure of p62/SQSTM1; modified from Rea et al, 2014.

ALS-related mutations can be found relatively anywhere on the protein but seem to concentrate on key domains, UBA, LIR, TBS and p62/SQSTM1 promoting region and if several are truncating mutations, most of them are missense mutations (Fecto, Yan, S. P. Vemula, *et al.*, 2011; Rubino *et al.*, 2012; Hirano *et al.*, 2013; Kwok, Morris and de Bellerocche, 2013; Le Ber *et al.*, 2013; Shimizu *et al.*, 2013; Teyssou *et al.*, 2013; van der Zee *et al.*, 2014; Y. Chen *et al.*, 2014). Mutations in these respective domains logically impairs ubiquitin binding, substrate delivery, autophagosome formation (through inhibition of TRAF1) and p62/SQSTM1 expression (Pankiv *et al.*, 2007; Ichimura *et al.*, 2008; Rea *et al.*, 2013). The promoting region of p62/SQSTM1 was reported to be prone to oxidative damage that lead to reduced expression in several neuropathological disorders (Du, Wooten and Wooten, 2009).

As mentioned in **I.2.e) Histopathology** and consistently with p62/SQSTM1's role in targeting substrates to degradation and specifically protein inclusions (Wooten *et al.*, 2006) , p62/SQSTM1 is found in neuronal and glial inclusions in ALS patients, carrying p62/SQSTM1 mutations or not, notably in typical TDP-43-positive inclusions but also in unique TDP-negative inclusions in C9orf72-related ALS cases (Arai *et al.*, 2006; Mizuno *et al.*, 2006; Hiji *et al.*, 2008; Al-Sarraj *et al.*, 2011; Troakes *et al.*, 2012; Mackenzie, Frick and Neumann, 2014). Inclusions enriched in p62/SQSTM1 are found in other inclusion-related pathologies such as in some cases of Parkinson's and Alzheimer's diseases thus strengthening p62/SQSTM1 implication in neurodegenerative mechanisms (Kuusisto, Salminen and Alafuzoff, 2002; Zatloukal *et al.*, 2002).

p62/SQSTM1 also holds a noteworthy relationship with SOD1 in the context of ALS and may contribute to mutant SOD1-related pathology. The autophagy receptor interacts with mutant SOD1 in inclusions through its UBA but also in a direct manner. A precise domain of p62/SQSTM1 was reported to bind mutant SOD1 and target it to LC3 and for degradation in a ubiquitin-independent manner: the SMIR or SOD1 mutant interacting region (Gal *et al.*, 2009). SOD1^{G93A} is linked to higher expression and accumulation of p62/SQSTM1 in model systems, contrarily to wildtype SOD1 (Gal *et al.*, 2007). Loss of p62/SQSTM1 lead to shorter lifespan of SOD1^{G93A} and SOD1^{H46R} mice and was accompanied with aggravated ALS-related symptoms. A mechanism that seem to involve other autophagy and ALS-related protein Alsin 2 (Hadano *et al.*, 2016). The intricacies of p62/SQSTM1 with TDP-43 will be discussed in section **IV.2 “TARDBP and autophagy”** and further investigated in this thesis.

Optineurin:

The autophagic receptor OPTN participates in several cellular processes such as NF-κB activation, antibacterial host defense, vesicle trafficking, Golgi function and traffic or secretory pathway (Kachaner *et al.*, 2012). It is also a well characterized autophagy receptor (Wild *et al.*, 2011) that possesses both UBA and LIR domains (Majcher *et al.*, 2015; Slowicka, Vereecke and van Loo, 2016) (**Fig i 23**). As previously mentioned in section **III.A.3) “Mitophagy”**, OPTN plays a key role in mitophagy. Among the other autophagy receptors that were found involved, only OPTN was described to be sufficient to trigger mitophagy and promoted autophagosome formation around mitochondria via its LIR domain (Wong and Holzbaur, 2014, 2015; Lazarou *et al.*, 2015). OPTN’s function in mitophagy is intimately linked to TBK1 phosphorylation of OPTN and formation of a TBK1-OPTN complex at the mitochondria (Heo *et al.*, 2015; Moore and Erika L. F. Holzbaur, 2016; Moore and Erika L.F. Holzbaur, 2016). TBK1 phosphorylation of

OPTN enhances its interaction with LC3 hereby promoting substrate clearance by selective autophagy (Wild *et al.*, 2011). Moreover, this TBK1-OPTN axis seem to play a role in another pathogenesis mechanism investigated in ALS, inflammation (Mankouri *et al.*, 2010; Gleason *et al.*, 2011; Weil, Laplantine and Génin, 2016). The LIR domain of OPTN may also play a role in this pathway as a deleterious artificial mutation of this region altered OPTN's inhibition of NF- κ B-mediated transcriptional activity (Maruyama *et al.*, 2010). Of note, OPTN may be one of the key actors in NF- κ B-mediated regulation of autophagy, relevant to ALS, Paget's disease of bone and cancer (Levine, Mizushima and Virgin, 2011; Trocoli and Djavaheri-Mergny, 2011; Rea *et al.*, 2014). OPTN involvement in autophagy also involves its interaction with p62/SQSTM1. Attachment of polyubiquitin chains to OPTN was reported to promote binding to p62/SQSTM1 and cargo recruitment to the autophagosome (Liu *et al.*, 2014). Similarly to p62, OPTN can target substrates for autophagy in a ubiquitin-independent manner as direct binding to mutant SOD1 was described (Wild *et al.*, 2011; Korac *et al.*, 2013).

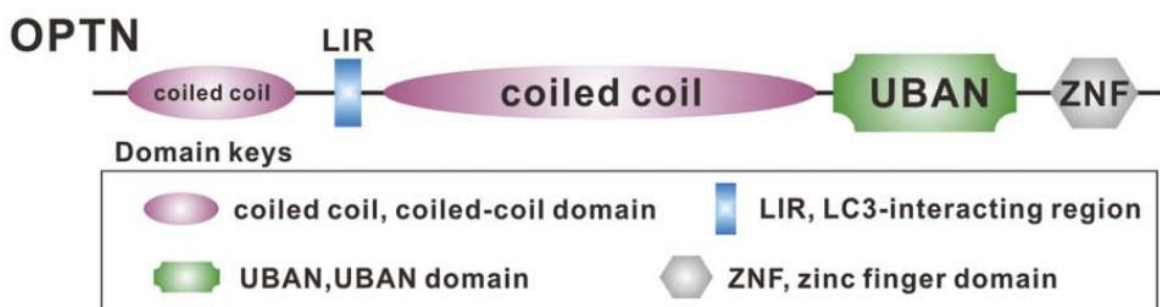


Figure i 23: The structure of OPTN; modified from F. Li et al, 2018.

OPTN was found in TDP-43-positive inclusions in some cases of sALS and in some mutant SOD1 patients (Maruyama *et al.*, 2010; Ling, Polymenidou and Cleveland, 2013).

Studies conducted on ALS patient cohorts indicate that 3% of fALS and around 1% of sALS are linked to OPTN mutations (Ling, Polymenidou and Cleveland, 2013) of various type but mostly target the UBA region (Maruyama *et al.*, 2010; Weishaupt *et al.*, 2013). Even if the exact pathogenesis is still unknown, these mutations may lead to a loss-of-function mechanism as they are linked to loss of ubiquitin-binding activity and seem to occur in a heterozygous manner indicating pathological haploinsufficiency (Slowicka, Vereecke and van Loo, 2016). In zebrafish, a morpholino-mediated knockdown of *OPTN* orthologue resulted in a motor axonopathy phenotype comparable with expression of mutant SOD1 (Korac *et al.*, 2013).

TANK-binding kinase 1:

The TBK1 protein is composed of four key domains: a Ser/Thr kinase domain at the N-terminal (KD), a ubiquitin-like domain (ULD) and two different coiled-coil domains CCD1 (notably allowing dimerization and binding to other proteins) and CCD2 (also called ABM for adaptor-binding motif) (Oakes et al, 2017) (**Fig i 24**).

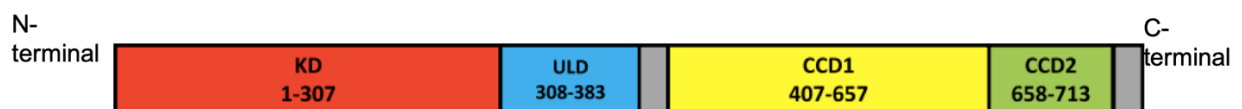


Figure i 24: The structure of TBK1; modified from Oakes et al, 2017.

It is a multifunctional protein and is involved in innate immunity, inflammation, nucleic acid sensing and autophagy (Pedros *et al.*, 2016; Zhang *et al.*, 2017). In order to be active, TBK1 needs to be recruited by adaptor proteins through CCD2-binding at discreet locations or in complexes and be phosphorylated by itself through dimerization (Hou *et al.*, 2011) or by other

kinases (Clark *et al.*, 2011). Autophosphorylation is required for proper TBK1 function and allows rapid activation of locally recruited TBK1 pools. But it is nonetheless structurally partially inhibited by TBK1's other domains to avoid aberrant and unwanted phosphorylation (G. Xu *et al.*, 2011; Ma *et al.*, 2012). Concerning its role in autophagy, TBK1 phosphorylates autophagy receptors NDP52, OPTN and p62 resulting in promotion of autophagic clearance by enhancing their ability to link LC3-II and ubiquitylated substrates (Wild *et al.*, 2011; Pilli *et al.*, 2012; Heo *et al.*, 2015; Lazarou *et al.*, 2015). In the case of mitophagy, as mentioned earlier, TBK1 is recruited at damaged mitochondria to phosphorylate OPTN and promote mitophagy by enhancing OPTN to bind ubiquitin. Interestingly, attachment of polyubiquitin chains on OPTN promotes the recruitment and retention of TBK1 at mitochondria which reflects a feed-forward amplification loop between TBK1 and OPTN promoting mitophagy (Heo *et al.*, 2015; Richter, Danielle A Sliter, *et al.*, 2016).

TBK1 was reported to colocalize with its upstream regulator Rab8b and with LC3 on autophagosomes. This mechanism was proposed to explain TBK1's role in autophagosome maturation as depletion of TBK1 altered the process in cells (Pilli *et al.*, 2012). The fact that TBK1 regulates dynein levels in cytoplasm and that phosphorylation of microtubule-binding protein CEP170 by TBK1 is required for proper microtubule activity, also implicates the kinase in autophagosome maturation, retrograde transport and fusion with lysosome along the microtubule network (Kimura, Noda and Yoshimori, 2008; Pillai *et al.*, 2015). Also related to autophagy, TBK1 activates SMCR8 allowing it to bind to C9orf72 which modulate mTOR and ULK1 activity in initiation of autophagy (Sellier *et al.*, 2016) as evoked earlier in C9orf72 overview in section **III.B.2) "Autophagy defects in ALS"**. TBK1 seem to share another, more direct link to mTOR as several studies report that TBK1 can directly bind and phosphorylate

mTOR and modulate mTORC1 activity (J. K. Kim *et al.*, 2013; Hasan *et al.*, 2017; Bodur *et al.*, 2018). However, these studies reported antagonist effects on mTORC1 signaling hence exposing a potential context-specific modulation of mTORC1 by TBK1 where environmental clues and localization of the TBK1-mTORC1 complex are believed to be crucial. In a cancerous context, TBK1 was reported to be able to physically bind and affect activity of mTORC1 regulatory elements such as Raptor, S6K and RagD which indicated direct role of TBK1 in mTOR signaling, localization and nutrient-sensing (Vaden *et al.*, 2017).

TBK1 mutations have been revealed in ALS-FTD patients. They are estimated to account for 4% of fALS, a very small portion of sALS (less than 0,1%) and around 1% of FTD cases (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015; Gijssels *et al.*, 2015). TBK1-ALS patients were reported to display classical TDP-43-positive inclusions and occasional colocalization with p62/SQSTM1 in these ubiquitin-positive inclusions in brain, motor neurons or glia (Freischmidt *et al.*, 2015; Pottier *et al.*, 2015; Van Mossevelde *et al.*, 2015). Once again, the presence of pathological ubiquitylated protein aggregates together with p62/SQSTM1 indicate TBK1 mutations may lead to ALS through impairments of the autophagic process. The reported mutations seem to indicate a loss-of-function mechanism for TBK1-mediated pathogenesis in ALS. Indeed, pathogenicity of Ser/Thr kinase domain mutations seem to be the result of haploinsufficiency (Le Ber *et al.*, 2013). Furthermore, another type of mutation that resulted in the truncation of the CCD2 of TBK1 lead to its inability to bind OPTN *in vitro*, which may be sufficient to cause ALS-FTD (Freischmidt *et al.*, 2015). However, the complex range of action of TBK1 and the relative importance of each of its domains is still unclear (Helgason, Phung and Dueber, 2013; Oakes, Davies and Collins, 2017). The data is missing to understand the precise mechanisms that link TBK1 loss-of-function to the development of ALS.

Both genetic and functional evidence have put the stress on autophagy and its defects in ALS research. Interestingly there appears to be a mechanistic convergence between autophagy and RNA metabolism which presents particular relevance for the disease.

IV) ALS: at a crossroad between autophagy and RNA metabolism

A) The links between autophagy and RNA metabolism

Genetic and histopathological evidence indicate that altered RNA metabolism and autophagy seem to be converging mechanisms in ALS (Ling, Polymenidou and Cleveland, 2013; Mandrioli *et al.*, 2019a). Indeed, as evoked in the previous sections, most of the identified genes in patients encode for proteins that are implicated in protein degradation systems such as autophagy or RNA-metabolism (Chia, Chiò and Traynor, 2018). Ubiquitin-positive inclusions containing aggregated and misfolded mutant or wild-type TDP-43, FUS (2 proteins widely involved in RNA metabolism) or SOD1 (that is also linked to RNA-metabolism defects when mutated) are the hallmark of ALS and a clear indicator of protein quality control failure (Klaips, Jayaraj and Hartl, 2018). Of note these inclusions can also be positive for proteins involved in protein clearance and autophagy (UBQLN2, VCP, p62, OPTN, CHMP2B, LC3...) (Ling,

Polymenidou and Cleveland, 2013). Moreover, C9orf72 is deeply involved in the autophagic process and its repeat expansion mutation, which is the major known genetic cause of ALS, is linked to RNA metabolism defects as described previously (see section **II.C.2) “RNA metabolism defects in ALS”**).

The two processes also converge at a crossroad homeostatic mechanism that is SG formation (Li *et al.*, 2013; Monahan, Shewmaker and Pandey, 2016). As previously mentioned, SG are membrane-less granules that form in the cytoplasm upon stress conditions via liquid-liquid phase separation and that are primarily composed of RNA-binding proteins and RNAs which translation is therefore postponed (Anderson and Kedersha, 2009). Numerous SG components are RNA-binding proteins such as TDP-43 (Colombrita *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010; Bentmann *et al.*, 2012), FUS (Sama *et al.*, 2013), ANG (Emara *et al.*, 2010; Thiyagarajan *et al.*, 2012), ATXN2 (Nihei, Ito and Suzuki, 2012), EWS, TAF15 (Andersson *et al.*, 2008; Blechingberg *et al.*, 2012) or TIA-1 (Kedersha *et al.*, 1999) that were found mutated in ALS patients (Chia, Chiò and Traynor, 2018). The dynamic of assembly and disassembly of SG is a key homeostatic cellular mechanism and SG that have incorporated RNA-binding proteins with disease-associated mutations have been reported to display slower disassembly kinetics and solid-like properties (Liu-Yesucevitz *et al.*, 2010; Molliex *et al.*, 2015; Patel *et al.*, 2015; Taylor, Brown and Cleveland, 2016; Mackenzie *et al.*, 2017). In vitro, such aberrant SG were observed to mature into ubiquitin-positive aggregates that contained phosphorylated TDP-43, p62/SQSTM1 or VCP giving rise to the hypothesis that ALS-hallmark protein inclusions may derive from aberrant SG (Neumann *et al.*, 2009; Zhang *et al.*, 2019). Furthermore, several autophagy-related ALS genes have been implicated in SG dynamics. More particularly, VCP has been implicated in SG disassembly, a process mediated via its upstream phosphorylation

by ULK1 (Wang *et al.*, 2019), but also in targeting aberrant SG to selective autophagy together with p62/SQSTM1 and C9orf72 (Buchan *et al.*, 2013; Chitiprolu *et al.*, 2018; Turakhiya *et al.*, 2018). Mutations in these genes have been reported to alter SG dynamics and potentially lead to their insolubility (Buchan *et al.*, 2013; Seguin *et al.*, 2014; Maharjan *et al.*, 2017; Chew *et al.*, 2019). Interestingly, SG have been shown to sequester mTORC1, master regulator of numerous cellular processes including autophagy, presumably to inhibit unnecessary induction of cell growth and prevent cell death during stress induction (Thedieck *et al.*, 2013; Wippich *et al.*, 2013).

B) TARDBP and autophagy

(1) Regulation of TDP-43 by autophagy

Autophagy related p62/SQSTM1, OPTN, VCP and LC3 colocalize with TDP-43 aggregates (Mizuno *et al.*, 2006; Hiji *et al.*, 2008; Ling, Polymenidou and Cleveland, 2013). Furthermore, ALS-related mutations in autophagy genes (such as VCP (J.-S. Ju *et al.*, 2009), UBQLN2 (Deng *et al.* 2011) or p62/SQSTM1 (Tanji *et al.*, 2012) among others) result in defective autophagy and general TDP-43 proteinopathy and aggregation. This suggest that autophagy is involved in degradation of TDP-43 pathological inclusions which is coherent with autophagy's role in the clearance process of protein aggregates called aggrephagy (Hyttinen *et al.*, 2014; Scotter *et al.*, 2014). More importantly, autophagy seems implicated in global turnover of both wild-type and mutant TDP-43 (Barmada *et al.*, 2014) and its induction, whether genetically (Gitcho *et al.*, 2009; J. S. Ju *et al.*, 2009; Ritson *et al.*, 2010; Brady *et al.*, 2011) or pharmacologically (I.-F. Wang *et al.*, 2012; Barmada *et al.*, 2014; Cheng, Lin and Shen, 2015; Crippa *et al.*, 2016), ameliorates TDP-43 aggregation and related toxicity. Autophagy

activation also seems to ameliorate clearance and toxicity of the truncated forms of TDP-43 (I.-F. Wang *et al.*, 2012; Zhou *et al.*, 2018). More globally, the UPS system and autophagy, which share common key molecular components such as p62/SQSTM1, OPTN, TBK1, UBQLN2 or VCP (Tanaka, 2013) as well as an implication in ALS (Mandrioli *et al.*, 2019b), seem to work collectively for the turnover of normal and pathological TDP-43 (Urushitani *et al.*, 2010; Wang *et al.*, 2010; Huang *et al.*, 2014; Scotter *et al.*, 2014). Thus, TDP-43 levels are regulated by these degradative pathways and keep in check the toxic effects of its altered levels (Ayala *et al.*, 2011; Avendaño-Vázquez *et al.*, 2012). Autophagy's control over TDP-43 levels may also be mediated by heat-shock proteins (Crippa, Carra, *et al.*, 2010; Crippa, Sau, *et al.*, 2010; Jinwal *et al.*, 2012; Crippa *et al.*, 2016).

(2) Regulation of autophagy by TDP-43

If autophagy is implicated in TDP-43 turnover and clearance of its pathological forms, TDP-43 appear in return to regulate autophagy (Budini *et al.*, 2017) (**Fig i 25**). Indeed, TDP-43 has been described to bind to *ATG7* mRNA (Bose *et al.* 2011). As a reminder, *ATG7* is essential for integration of the two ubiquitin-like conjugation systems (*ATG12-ATG5-ATG16L1* and *LC3/ATG8* family systems) into autophagosome membrane (Tanida *et al.*, 2004; Katahira *et al.*, 2007; Itakura and Mizushima, 2010; Hamasaki *et al.*, 2013; Walczak and Martens, 2013) (**Fig i 25**). Oligonucleotide-mediated knockdown of TDP-43 in cells resulted in a diminution of *atg7* mRNA and of its protein. The study reported that it lead to reduced levels of LC3-II but in an accumulation of p62/SQSTM1 and autophagosomes and concluded that autophagy thus appeared stimulated, in both control and pharmacologically-inhibited autophagy conditions (Bose *et al.* 2011). The RNA-binding protein also binds *raptor* mRNA, encoding for key mTORC1 subunit RAPTOR, as a similar depletion of TDP-43 *in vitro* caused reduced stability and level of

raptor mRNA and decrease levels of its protein (Xia *et al.*, 2016) (**Fig i 25**). This was associated with inhibited mTORC1 activity and accumulation of LC3-II, p62/SQSTM1 and autophagosomes. This increase in autophagy-related proteins upon depletion of TDP-43 could be explained by inhibition of mTORC1 activity, translocation of transcription factor TFEB to the nucleus and corresponding increased expression of autophagy and lysosomal biogenesis genes (Xia *et al.*, 2016). This study however concluded that autophagosome accumulation reflected failure of autophagy to go through completion. This might be explained by failure of autophagosome-lysosome fusion. Indeed, TDP-43 is able to bind *dynactin1* mRNA and its depletion caused reduced stability of the mRNA and thus potentially disrupt autophagosome trafficking and maturation (Xia *et al.*, 2016) (**Fig i 25**).

In addition, in a similar *in vitro* oligonucleotide-mediated knockdown model, TDP-43 loss-of-function was reported to cause abnormal p62/SQSTM1 accumulation through aberrant splicing of an ATG4b cryptic exon in both control and nutrient-deprived cells (Torres *et al.*, 2018). Interestingly, this cryptic exon was found augmented in ALS patient CNS tissues. Of note, direct binding of TDP-43 to *atg4b* mRNA could not be proved (Torres *et al.*, 2018). Interestingly, TDP-43 was reported to bind *optn* and *vcp* mRNAs in a large scale CLIP-seq based approach but no mechanistic conclusions was made from this analysis (Polymenidou *et al.*, 2011).

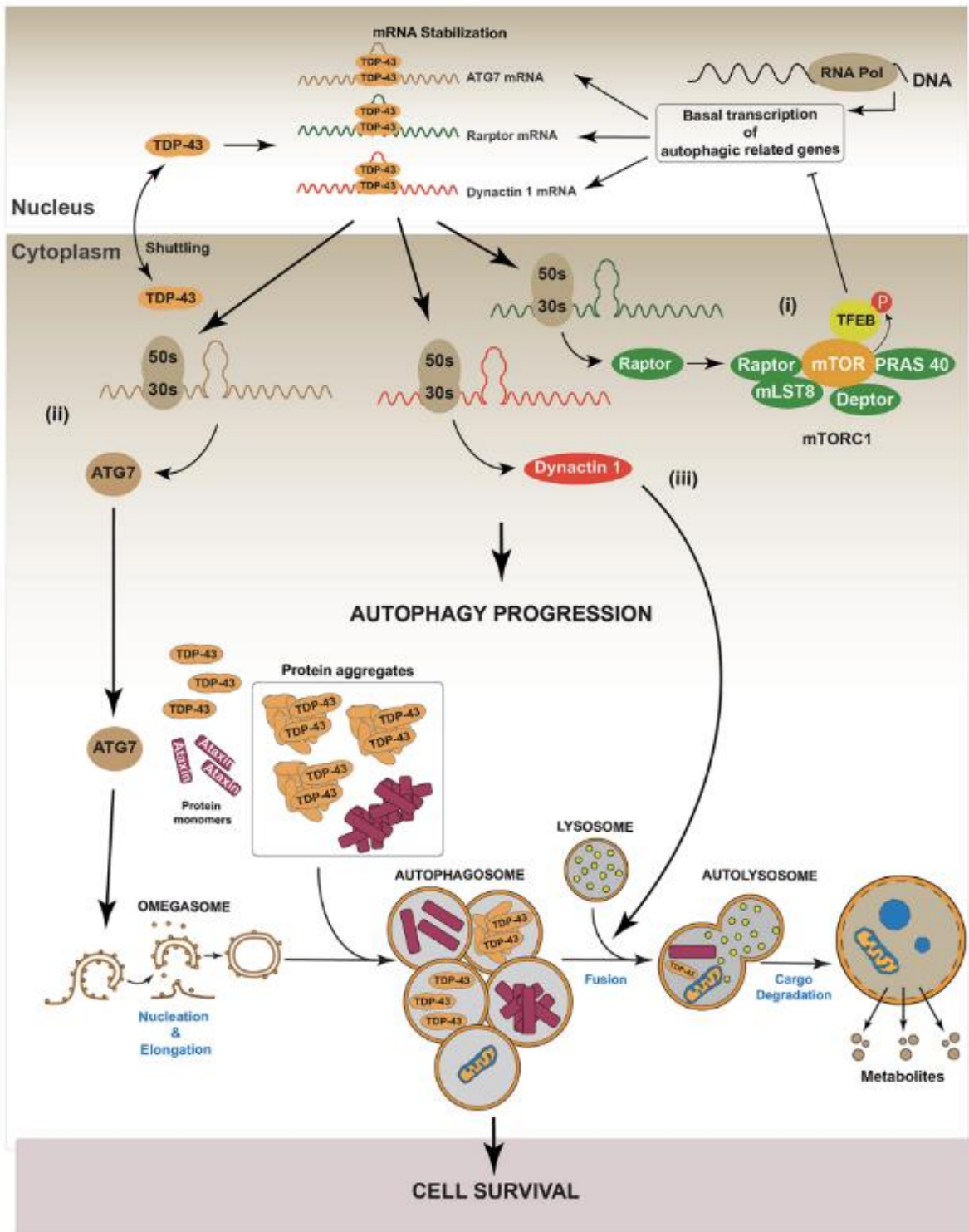


Figure i 25: Schematics of the modulation of autophagy by TDP-43; modified from Budini et al, 2017.

Overall, several cellular models tend to show that loss of TDP-43 function caused an accumulation of p62. However, as mentioned in section **III.A.4) “Measuring autophagy”**, conclusions on the end impact on autophagic flux is challenging and repression of autophagy-promoting ATG7, ATG4b and DCTN1 appears contradictory with RAPTOR-dependent inhibition of mTORC1 activity. Despite the unveiling of mechanistic interplays between ALS genes, one could argue that such *in vitro* models may not be relevant for conclusive understanding of TDP-43’s impact on autophagy, particularly in an ALS-relevant context. Indeed, there is limited evidence of TDP-43’s regulatory impact on autophagy *in vivo*. However, these *in vitro* observations potentially depict a model of feed-forward loop mechanism aggravating TDP-43 pathology. Through key interactions, TDP-43 is required for the autophagic process which, in turn, is responsible for TDP-43 turnover (Budini *et al.*, 2017). In a pathological context, TDP-43 cytoplasmic aggregation is thought to lead to TDP-43 nuclear depletion and loss-of-function (Budini *et al.*, 2015; De Conti *et al.*, 2015), which alters autophagy function thus aggravating TDP-43 proteinopathy and related toxicity.

C) Hypothesis and objectives of this thesis

TDP-43 proteinopathy remains an almost universal constant in ALS, a disease that displays great heterogeneity among patients and complexity in the potential pathological mechanisms reported in models. This contrast highlights the importance of deciphering TDP-43 normal and pathological function in the hope of understanding the complex profile of ALS. More particularly, unveiling new roles for TDP-43 in autophagy would strengthen the link between two major defective process in the disease and key homeostatic mechanisms, RNA

metabolism and proteinostasis. This thesis was aimed to further elucidate TDP-43's implication in the autophagic process through modulation of TDP-43 protein levels. Overexpression of TDP-43 will be performed to attempt reproducing some aspects of the proteinopathy and establish their effect on autophagy. However, modeling TDP-43 loss-of-function via knockdown will be our primary approach. Although not reproducing TDP-43 related pathological traits, this method allows to unveil potential disease modifiers, genetic interplays (Kabashi, Bercier, *et al.*, 2011) and identify new partners of action strengthening our understanding of TDP-43-mediated neurodegeneration. Furthermore, we hypothesize that TDP-43 may interact with key autophagy actors that were identified as genetic causes of ALS. This strategy will rely on the complementarity of our two models: the zebrafish, a vertebrate animal model that will help us unveil functional interplays; and SH-SY5Y cells, a simple human cellular model that will allow better characterization of the underlying biomolecular mechanisms. Because TDP-43 may interact with these novel partners via its RNA-binding function, potentially affecting the stability and dynamic of certain transcripts, we simultaneously started the development of techniques to visualize and track target RNAs in time through live-imaging.

Results and Discussion

l) An epistatic interaction between *tardbp* and *tbk1* in the zebrafish

A) Introduction

As previously discussed, emulating loss-of-function of TDP-43 via knockdown permitted identification of several of its RNA partners of interaction that are involved in autophagy. However, impact of binding to the two ALS-related *Optn* and *Vcp* has not been established on a mechanistical level (Polymenidou *et al.*, 2011) and interactions with *Raptor* (Xia *et al.*, 2016) and *Atg7* (Bose *et al.* 2011) were shown to lead to altered expression of several autophagy genes, and more specifically accumulation of autophagy receptor p62/SQSTM1, upon depletion of TDP-43 but also lead to contradictory conclusions on its overall impact on autophagy. Furthermore, the link between TDP-43 and TBK1, activator of both p62/SQSTM1 and OPTN (Wild *et al.*, 2011; Komatsu, Kageyama and Ichimura, 2012b; Pilli *et al.*, 2012; Matsumoto *et al.*, 2015; Richter, Danielle A Sliter, *et al.*, 2016), partner of interaction of RAPTOR (Vaden *et al.*, 2017), modulator of mTORC1 activity (J. K. Kim *et al.*, 2013; Hasan *et al.*, 2017; Bodur *et al.*, 2018) and recently associated with ALS/FTD (Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015; Gijssels *et al.*, 2015), has not been investigated.

The Kabashi lab has already shown the relevance of the zebrafish model and the use of morpholinos to study autophagy and its implication in ALS (Ciura *et al.*, 2013; Lattante *et al.*, 2015). Moreover, preliminary data that was obtained by our team indicate an epistatic interaction between TDP-43 and p62/SQSTM1. As previously described, AMO-mediated knockdown of p62/SQSTM1 leads to a locomotor deficit in zebrafish embryos (Lattante *et al.*,

2015) that can be ameliorated by co-injecting human wild-type *TARDBP* RNA. Interestingly, mutant G348C *TARDBP* RNA failed to rescue this phenotype (**Fig i 26**).

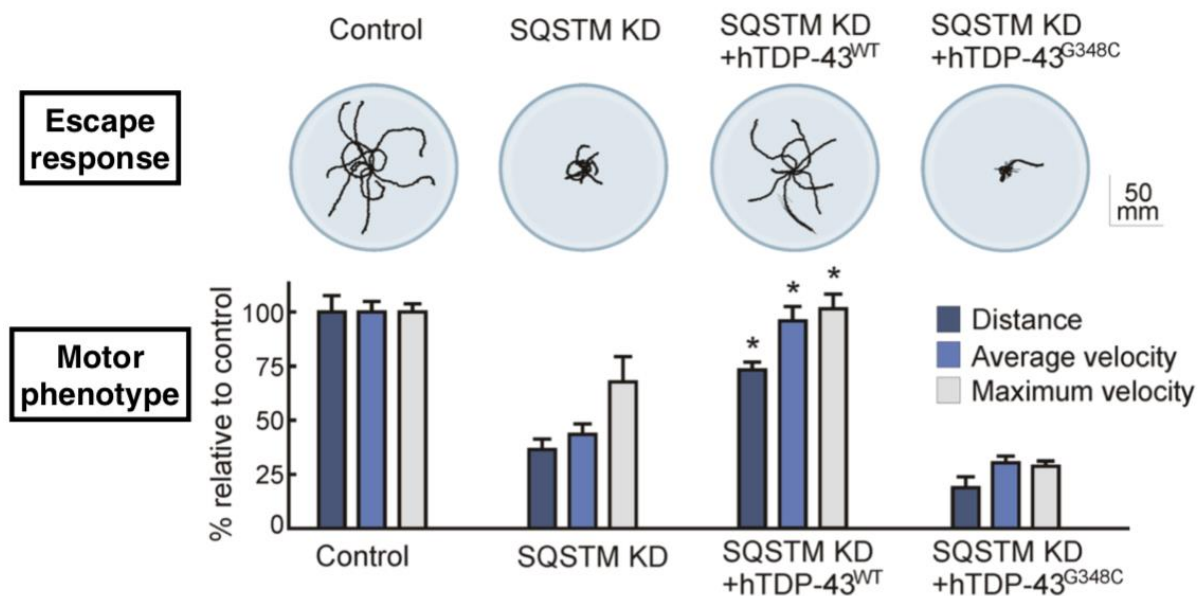


Figure i 26: *sqstm1*-AMO leads to motor deficits in zebrafish embryo that are ameliorated by human wild type *TARDBP* RNA.

In this study I used a zebrafish embryo model of morpholino-induced knockdown of TDP-43 that has already been well-established in our lab and efficiently leads to motor deficits (Kabashi *et al.*, 2009). We hypothesized that knockdown of *tardbp* in our zebrafish model would allow identification of altered expression of autophagy genes, with a particular interest for *p62/SQSTM1* and *TBK1*, and unveil novel interplays that would reinforce our knowledge of TDP-43's interaction with the autophagic process.

B) Transient knockdown of *tardbp* leads to decreased *tbk1* protein and RNA levels

Transient knockdown of *tardbp* was induced via injection of *tardbp*-AMO and fish displayed a locomotor deficit at 50hpf as previously established. Upon observation of this reproducible phenotype, embryos were collected to extract both proteins and RNAs in order to observe any quantifiable differences among expression of autophagy genes (**Fig 1**). *tardbp*-AMO fish display significantly reduced levels of p62/Sqstm1 and of its upstream activating kinase, Tbk1, as seen on immunoblotting (**Fig 1 A**) and resulting band quantification (**Fig 1 B**) as compared to control mismatch-injected fish. Indeed, p62/Sqstm1 levels are approximately depleted by half and only 35% of control Tbk1 levels remain in *tardbp*-AMO fish. At the RNA level, RT-qPCR analysis revealed a significant decrease in *tbk1* mRNAs (about 65% of mismatch levels). On the other hand, *p62/sqstm1* mRNA levels tend to be augmented (127% of mismatch levels) but not in a significant manner. However, *optn*, coding for another autophagy receptor and substrate of Tbk1, is greatly up-regulated upon depletion of TDP-43 as its RNA levels were doubled as compared to control fish (**Fig 1 C**). We next quantified transcripts that code for proteins involved in autophagy initiation. No significant differences were caused by knockdown of *tardbp*. This includes transcripts that were reported to be negatively affected by TDP-43 depletion *atg7* and *atg4b* (**Fig 1 D**). Hence, these first results are in contradiction with what was reported in cellular models (Bose, Huang and Shen, 2011; Xia *et al.*, 2016; Torres *et al.*, 2018) and highlight a decrease in both protein and RNA levels of *tbk1*.

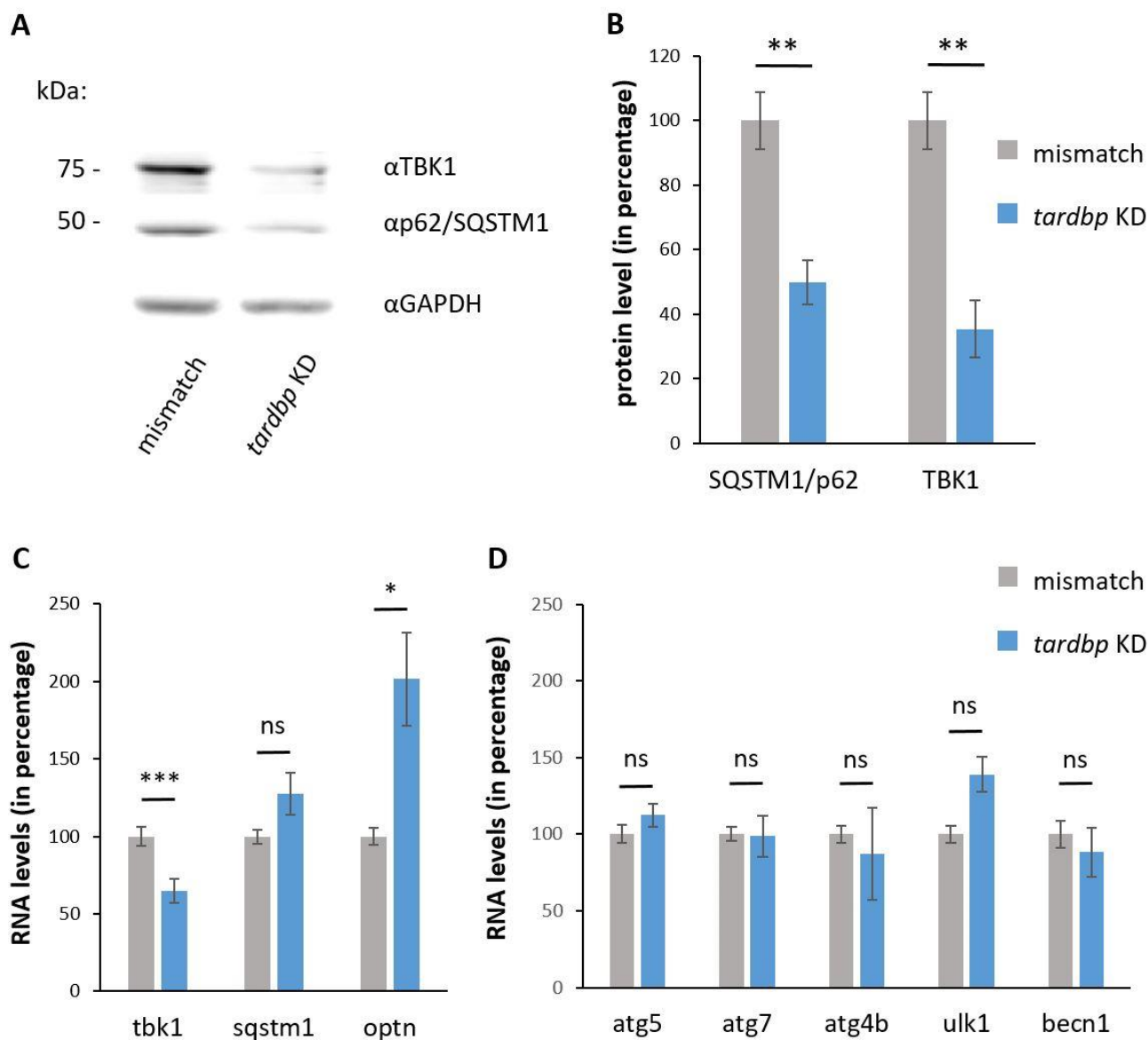


Figure 1: KD of *tardbp* disrupts expression of autophagy genes in zebrafish. (A) Immunoblotting and **(B)** its relative quantification showing that 50hpf *tardbp* AMO-injected fish embryos (in blue) have decreased protein levels of p62/sqstm1 and tbk1 as compared to control mismatch-injected fish (in grey). n=6 samples of 10-30 embryos each for both mismatch and *tardbp*-AMO. At the RNA level, **(C)** RT-qPCR results show *tardbp* AMO fish display a downregulation of *tbk1* and upregulated *optn* expression while **(D)** expression profile of genes involved in the initial steps of autophagy remains unchanged. n=12 samples of 10-30 embryos for each mismatch and *tardbp*-AMO. All results are mean \pm SEM. *P<0,05, **P<0,01, ***P<0,001 from Mann-Whitney's test.

C) Human *TBK1* RNA ameliorates *tardbp*-AMO-induced motor phenotype

As a response to these lowered *tbk1* RNA levels, human *TBK1* RNA was synthesized in vitro and coinjected with *tardbp*-AMO at the one-cell stage in zebrafish embryos of the wild-type AB and TL strains. At 50hpf, the motor phenotype of injected fish was assessed by recording and analysis of their touch-evoked escape response trajectory pattern. “*tardbp*-AMO + *TBK1* RNA” fish present swim trajectories that are greatly ameliorated and appear similar to all control conditions (“non injected”, “mismatch” and “mismatch + *TBK1* RNA”) as compared to sole injection of *tardbp*-AMO (**Fig 2 A**). This amelioration of *tardbp*-AMO-induced motor phenotype by human *TBK1* RNA is confirmed in terms of mean distance made by the fish: there is no significant difference between “*tardbp*-AMO + *TBK1* RNA” and control groups, unlike with “*tardbp*-AMO” (**Fig 2 B**). Of note, “*tardbp*-AMO + *TBK1* RNA” fish swim for an equivalent amount of time as control groups and significantly more than “*tardbp*-AMO” (**Fig 2 C**) but do not display a sensible amelioration of their speed (**Fig 2 D**). In other words, human *TBK1* RNA ameliorates *tardbp*-AMO-induced motor phenotype as seen in this partial rescue of their touch-evoked escape response swimming behavior.

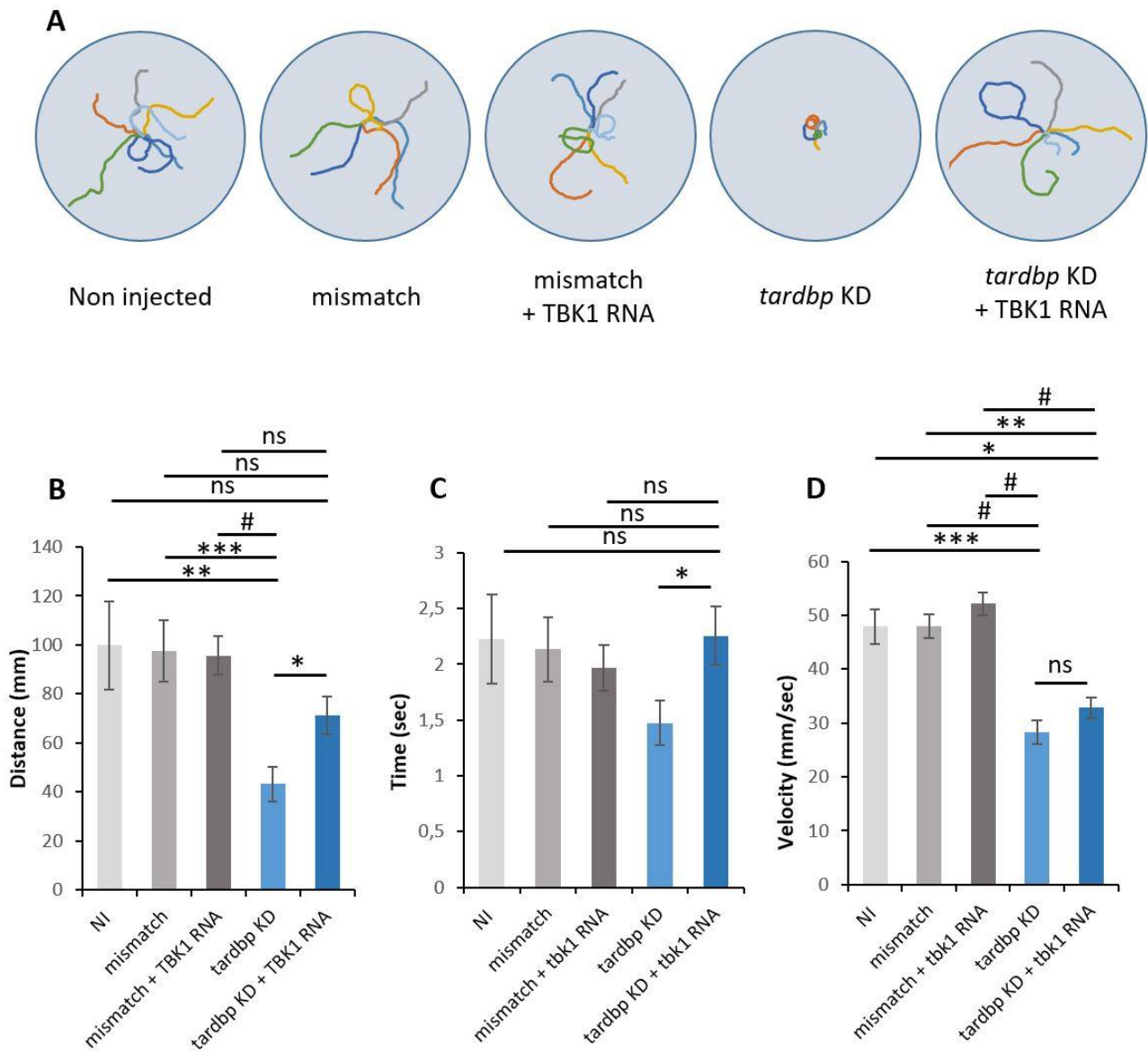


Figure 2: *tardbp* KD leads to altered motor phenotype that is partially rescued by human *TBK1* mRNA. (A) Graphic representation of injected 50hpf embryos escape trajectories in response to gentle touch of their tail (TEER test) and quantification of **(B)** distance, **(C)** time and **(D)** velocity parameters showing that co-expression of human *TBK1* RNAs significantly ameliorates the locomotor defects caused by *tardbp* KD. n=16 for non injected, 20 for mismatch, 30 for mismatch + TBK1 RNA, 32 for *tardbp*-AMO and 29 for *tardbp*-AMO + TBK1 RNA fish. All results are mean \pm SEM. *P<0,05, **P<0,01, ***P<0,001, #P<0,0001 from Kruskal-Wallis test followed by Dunn's posthoc analysis.

D) Human *TBK1* RNA ameliorates *tardbp*-AMO-induced motor neuron axonopathy

I next wanted to see if this partial rescue of *tardbp*-AMO-induced motor phenotype by *TBK1* RNA was accompanied by an amelioration of its altered motor neuron morphology, which has been established by our lab. For this purpose, the transparency of zebrafish embryos was put at use and the same injections were performed in eggs from the *Hb9:GFP* transgenic line (for Homeobox gene Hb9) which selectively expresses a membrane-addressed fluorescent protein GFP in motor neurons allowing their imaging in live animals. As expected, “*tardbp*-AMO” fish display abnormal motoneuron morphology with aberrant branching and visible shortened axonal length in comparison with all control groups and “*tardbp*-AMO + *TBK1* RNA” fish, as seen in Caudal primary motor neurons in the presented confocal images (**Fig 3 A**). Quantification of the MN axonal length/spinal cord thickness ratio confirms that *TBK1* RNA significantly lead to a recuperation of axonal length to a level that is comparable with control groups (**Fig 3 B**). The fact that all groups present akin spinal cord thickness (**Fig 3 C**) indicate that there is minor neuronal developmental delay in these conditions.

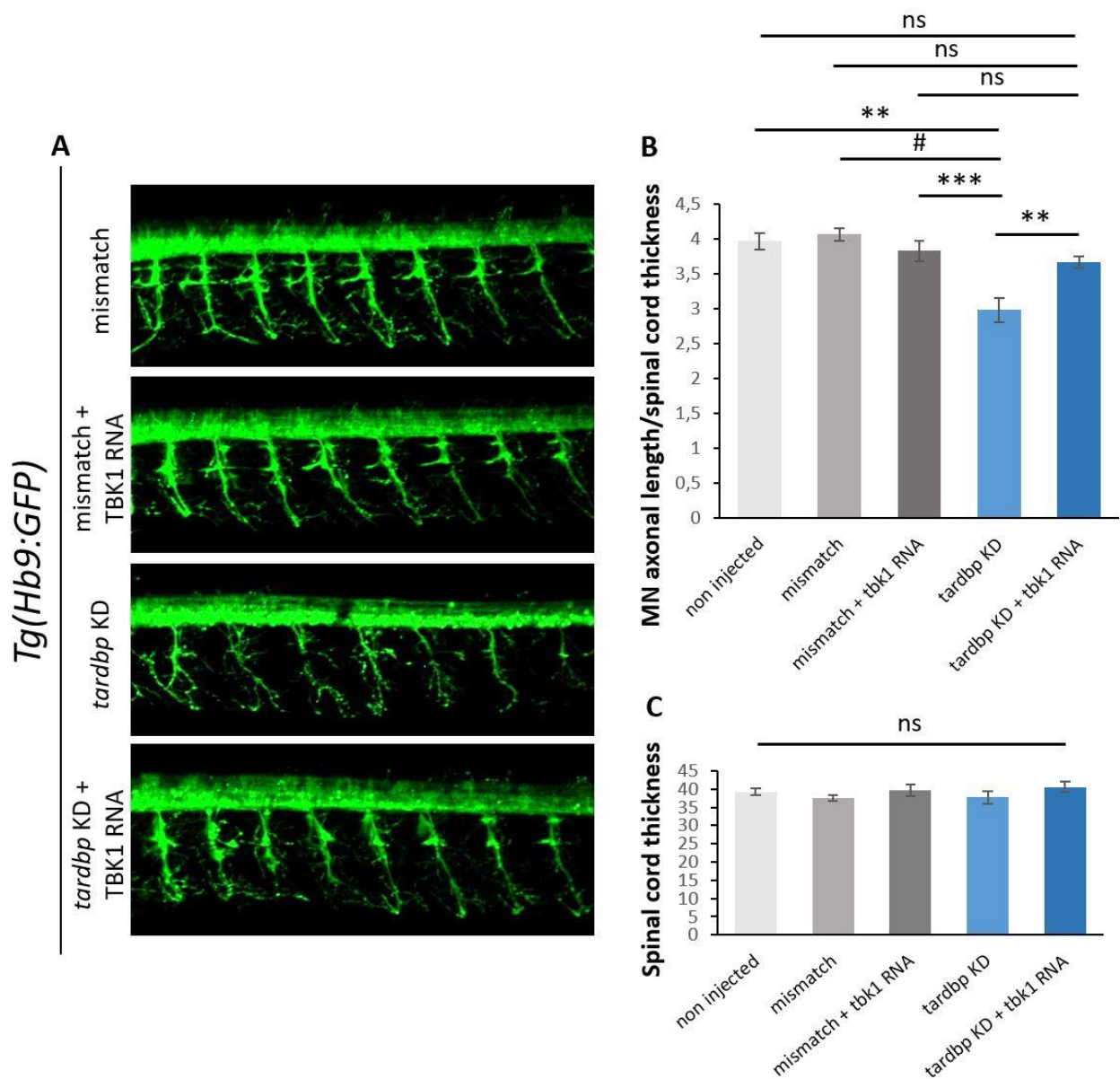


Figure 3: *tardbp* KD leads to axonopathy that is rescued by human *TBK1* mRNA. (A) Images of motor neuron (MN) projections of injected 50hpf embryos of the transgenic *Hb9:GFP* line and resulting **(B)** quantification of MN axonal length/spinal cord thickness ratio, showing that co-injection of human *TBK1* RNA ameliorates MN morphology alterations obtained through *tardbp* KD (n=24 neurons or 4 caudal primary neurons per fish on a total of 6 fish per condition). Of note, **(C)** quantification of spinal cord thickness indicate that MN axonal length of injected fish can be compared. All results are mean \pm SEM. *P<0,05, **P<0,01, ***P<0,001, #P<0,0001 from one-way ANOVA followed by Tukey's posthoc analysis.

E) Discussion

Overall, these data are the first evidence of a functional interplay between the two ALS-related genes *TARDBP* and *TBK1*. First, knockdown of *tardbp* in our zebrafish model leads to a decrease of both protein and RNA levels of *tbk1*. Yet, it is widely accepted that TBK1 pathology is based on a loss-of-function mechanism. Indeed, it was shown that sole haploinsufficiency of *TBK1* was sufficient to cause ALS which adds relevance to these findings. The other important information suggested by these results is that the effect of *tardbp*-knockdown appears to be, at least partly, mediated by *tbk1* in our model as *TBK1* mRNA partially rescues both the motor phenotype and axonopathy of *tardbp*-AMO fish. Even if morphological defects are clearly visible, the study could benefit from further quantification of the observed axonopathy and its amelioration, notably an analysis of axonal branching. The fact that human *TBK1* RNA was used strengthens a potential translation of this observed interplay to other models. However, the details of this interaction remain unknown. The decrease in *tbk1* mRNA levels upon depletion of TDP-43 may indicate a potential effect of the RNA-binding protein on the transcript.

Apart from TDP-43's interaction with TBK1, the effects obtained on other autophagy genes upon transient knockdown of *tardbp* are intriguing. Indeed, we report reduced protein levels of p62/SQSTM1 and no significant changes on the autophagy initiation transcripts such as *atg7* and *atg4b*. These results appear in total contradiction with what has been observed in human cell lines (Bose, Huang and Shen, 2011; Xia *et al.*, 2016; Torres *et al.*, 2018). Thus, it brings the question of the relevance of our animal model and of these cellular models to study TDP-43's normal function and implication in ALS. Of note, the fact that reduction of

p62/Sqstm1 was not accompanied by a decrease of its RNA levels in our model, but rather by a tendency for an increase, may suggests a compensatory mechanism to restore protein levels. It also reveals that TDP-43 loss-of-function may have a deleterious impact on *tbk1* mRNA quantity but not on *sqstm1* or *optn*. It however doesn't discard a perturbation on their dynamic, localization and translation.

Next, as TBK1 interacts and activates both p62/SQSTM1 and OPTN, it would be interesting to verify if *TBK1* RNA leads to restored p62/Sqstm1 protein levels in our model and if Optn protein levels follow the same trend in this model. Considering the importance of the TBK1/OPTN axis, the strong up-regulation of *optn* that we observe in *tardbp*-AMO fish appears worthy of further investigation. This would allow us to compare the impacts of TDP-43 depletion on both autophagy receptor and indicate if these impacts are Tbk1-dependent or solely due to *tardbp* modulation.

The zebrafish model has allowed the unveiling of another genetic interplay between *TARDBP* and *TBK1* with functional and phenotypic insights. However, complementary studies on a cellular model would help us detailing this interaction on the molecular level.

F) Materials and methods

Zebrafish Maintenance:

Adult and larval zebrafish (*Danio rerio*) were maintained both at the ICM (Institut du Cerveau et de la Moelle épinière, Paris) and Institut Imagine (Paris) fish facilities and bred according to the National and European Guidelines for Animal Welfare. Experiments were performed on wild-type and HB9:GFP transgenic lines from AB and TL strains.

Microinjections of oligonucleotides in zebrafish embryos:

Microinjection of both morpholinos and human RNA sequences were performed in wild-type or HB9:GFP zebrafish embryos at the one-cell stage using a PV 820 Pneumatic PicoPump injector (World Precision Instruments, USA) and heat-pulled borosilicate glass needles.

Antisense morpholino oligonucleotide (AMO) was synthesized by GeneTools (Philomath, USA) and targeted against the initial ATG of *tardbp* (AMO-*tardbp*: 5'-GTACATCTCGGCCATCTTTCCTCAG-3') zebrafish mRNA to realize transient knockdown of *tardbp*. A control morpholino (mismatch), containing a standard mismatch nucleotide sequence that do not bind anywhere in the zebrafish genome, was also used to assess the specific effects of AMOs on embryos. Microinjections were performed at 0.4 mM for AMO-*tardbp* and at an equal concentration for control Mismatch depending.

Human WT TBK1 RNA was synthesized from AgeI-linearised PCM6 TBK1 WT-myc&flag-tag plasmid (gifted by Dr. Nicolas Charlet-Berguerand) using T7 polymerase with the mMESSAGING Machine Kit (Ambion). WT and mutant G348C TARDBP RNAs were synthesized from NotI-linearized *pCS2 TDP-43 WT GU2* and *pCS2 TDP-43^{G348C} GX12* plasmids using SP6 polymerase with the mMESSAGING Machine kit. RNAs were purified through Trizol-chloroform extraction (TRIZOL Reagent from Sigma, USA) according to the manufacturer's protocol and resuspended in Milli-Q water. WT TBK1 RNA was used at 100nM

Final injection mixes were prepared by diluting AMOs and RNAs in injection buffer (*KCl 120Mm, Hepes 20Mm, pH 7,2 in Milli-Q water*) complemented with Fast green dye (Sigma-Aldrich, USA) at a final dilution of 0,05%.

Injected embryos were then raised at 28°C in an embryo medium called “blue water” (0.06 g/L aquarium salt (*Instant Ocean, Blacksburg, VA*) in Milli-Q water +0.01 mg/L methylene blue) until 50hpf and further experiments proceed. However, the embryos were manually dechorionated using fine forceps at 24 hpf.

Locomotion analysis of zebrafish embryos:

At 50 hpf, motor behavior of zebrafish embryos was assessed by performing a TEER test (Touch-evoked escape response). Embryos were touched lightly at the level of the tail with a tip and their flee pattern was scored as previously described (Ciura *et al.*, 2013; Lattante *et al.*, 2015). The motor responses were recorded with a Grasshopper 2 digital camera (Point Grey Research) at a rate of 30 frames/s and quantified using ImageJ. Of note, only embryos that appear morphologically normal are selected for assessment of motor phenotype.

Live-imaging of whole-mount zebrafish embryos and measurement of motor neuron axonal length:

At 50 hpf, injected embryos from the HB9:GFP line that appear morphologically normal were anesthetized in “blue water” supplemented with Tricaine (final concentration of 0,16 mg/mL) and embedded in agarose (1% *UltraPure LMP Agarose (Invitrogen, USA)* in “blue water” supplemented with tricaine) laying on their flank in a glass bottomed imaging dish. Motor neurons of the animals were visualized using Zeiss Spinning Disk (Carl Zeiss, Germany), a CSU-W1 head (Yokogawa, Japan), an ORCA-Flash 4.0 S-CMOS (C1144022CU) camera (Hamamatsu,

Japan) and through a X10 Plan Apo objective (Mitutowo, Japan). Images were taken using ZEN software from Zeiss and analysed using ImageJ software (Sun Microsystems, USA).

For each fish, four caudal primary motor neurons (CaP), located between the 5th and 8th somites were selected in order to measure axonal length. Distance was taken from the axonal root to their lowest point of projection down the ventral myotome, that is before they turn back up around the ventral musculature. Spinal cord thickness was measured between the first and second selected CaP. Six fish per condition were analysed.

Immunoblotting:

Protein extraction: Zebrafish embryos that appear morphologically normal were selected (50 hpf, 10-30 per tube) and put on ice for the rest on the protocol in order to anesthetize the embryos and inhibit protein degradation. The embryos are deyolked through gentle up and down pipetting in a deyolking buffer (EDTA 1mM, PMSF 0.3mM in Calcium-free Ringer solution). Excess yolk debris are discarded by two washes in Calcium-free Ringer solution. The deyolked embryos were homogenized on ice through sonication (Sonics Vibracell VC505) in Lysis Buffer (*Tris-HCl 50mM pH 7.5, NaCl 500mM, EDTA 5mM, Nonidet p-40 1%, Triton 0.5% supplemented with a cocktail of protease inhibitors (Complete mini, Roche, Switzerland)*). A 20 min centrifugation at 4°C and 14000 rpm was made to recover supernatant. Concentration of the protein extracts were established through BCA assay.

Western blot: 30µg of protein extract per condition was mixed with 5X SDS Loading buffer (*Bromophenol blue 0.25%, dithiothreitol 0.5M, glycerol 50%, Sodium dodecyl sulfate 10%, Tris-HCl (pH 6.8) 0.25M in Milli-Q water*) and denatured at 98°C during 7min in a dry bath. Samples were loaded in a 4-20% Mini-Protean TGX Gel (BioRad, USA) for electrophoresis. The

separated proteins were transferred to a nitrocellulose membrane (0,45µm, Life Sciences) and probed with the following antibodies:

- Anti-NAK/TBK1 (rabbit, ab109735) from Abcam, UK.
- Anti-SQSTM1/p62 (rabbit, PM045) from MBL International, USA.
- Anti-GAPDH (mouse, ab8245) from Abcam, UK.

Blots were incubated with corresponding fluorescent secondary antibodies and detection of protein bands was made using ODYSSEY CLx system (LI-COR Biosciences, USA). Analysis of bands was carried out using ImageJ software.

RNA extraction and qPCR:

Zebrafish embryos that appear morphologically normal were selected (50 hpf, 10-30 per tube) and put on ice for deyolking and washing as previously described. Fish were homogenized in TRIzol reagent (Sigma, USA) by rough up-and-down pipetting and TRIzol-Chloroform based RNA extraction was performed according to manufacturer's instructions. Resulting pellets of RNA were washed in ethanol and resuspended in sterilized Milli-Q water. RNA concentrations were determined using Lunatic system (Unchained Labs, USA).

First-strand cDNAs were obtained by reverse transcription of 1µg of total RNA per sample using High Capacity cDNA Reverse Transcription Kit (Roche, Switzerland) according to manufacturer's instructions. Quantitative-PCR amplification was performed with 2X SYBR Green (Bymake, USA). Primers were ordered from Sigma-Aldrich (USA) SYBR Green Primers Library and sequences are available upon request. Data were analyzed transforming raw Cq values into relative quantification data using the delta Cq method.

Statistical analysis:

All data values for the zebrafish experiments are represented as average standard error of mean (SEM) with significance determined using bilateral Mann-Whitney's, Kruskal-Wallis (followed by Dunn's posthoc analysis) and One-way ANOVA (followed by Tukey's posthoc analysis) tests. Analyses were performed using Excel (Microsoft, USA) and Prism 7.0 (Graph Pad, USA). Significance level was set at $p < 0.05$.

II) Antagonist effects of *TARDBP* modulation on key autophagy genes in SH-SY5Y cells

A) Introduction

Several models have highlighted that modulating TDP-43 levels, either via knockdown (Feiguin *et al.*, 2009; Kabashi *et al.*, 2009; Lu, Ferris and Gao, 2009; Li *et al.*, 2010) or overexpression (Ash *et al.*, 2010; Hanson *et al.*, 2010; Li *et al.*, 2010; Ritson *et al.*, 2010; Wils *et al.*, 2010; Igaz *et al.*, 2011), lead to toxicity thus showing the importance of maintaining the protein, which is also able to self-regulate via binding of its own mRNA (Ayala *et al.*, 2011; Polymenidou *et al.*, 2011; Tollervey *et al.*, 2011). Its ability to shuttle between nucleus and cytoplasm is essential to its normal function and both nuclear depletion as well as cytoplasmic mislocalization are characteristic traits of its proteinopathy and have been identified in toxic mechanisms (Lee, Lee and Trojanowski, 2012). TDP-43's complex role in RNA metabolism and

translation has been reported to be adaptative to stress-induction as TDP-43 is recruited to SG (Colombrita *et al.*, 2009; Liu-Yesucevitz *et al.*, 2010; Bentmann *et al.*, 2012) and to stalled ribosome within SG via its RNA-binding function upon oxidative-stress induction and contributes to cell survival (Higashi *et al.*, 2013).

Interestingly, TDP-43's adaptability to the autophagic context is poorly known and more particularly its potential changes in interactome have not been explored. Our previously evoked *tardbp*-knockdown zebrafish model was successful in identifying a novel interplay with *tbk1*, yet, it presented a contradictory profile of expression among autophagy related genes as compared to *TARDBP*-KD cellular models that unveiled interaction with *RAPTOR* and *ATG7* mRNAs (Bose, Huang and Shen, 2011; Xia *et al.*, 2016). We hypothesize that drug-mediated modulation of autophagy may help us understand the inconsistency of these data and provide us with a better grasp of TDP-43's normal function and consequences of its modulation.

In this set of experiments, I used the human neuroblastoma SH-SY5Y cell line to further investigate the TDP-43/TBK1 interaction but also focus on p62/SQSTM1 as results in similar cellular models and in our zebrafish model reveal antagonist yet significant effects on this key autophagy protein after knockdown of *TARDBP*. Overexpression of TDP-43 has also been performed to observe a potential effect on p62/SQSTM1.

B) Knockdown of *TARDBP* leads to altered expression of key autophagy genes

Transient knockdown of *TARDBP* was performed on SH-SY5Y cells via transfection of a specifically targeted siRNA-*TARDBP*. 48h after transfection, we analyzed protein and RNA levels of several autophagy genes and compared them to siRNA-Control cells. Immunoblotting

reveals that siRNA-*TARDBP* is efficient in reducing TDP-43 levels. Interestingly it correlates with bands of higher intensity for both p62/SQSTM1 and TBK1 as compared to control (**Fig 4 A**). A significant depletion of TDP-43 (about 38% of control levels) and increase in p62/SQSTM1 (141% of control levels) was observed in these cell lines. Mean levels of TBK1 are at 155% of control but this increase is not significant (**Fig 4 B**). Quantification of RNA levels reveal that *TBK1* isn't affected by TDP-43 depletion contrarily to what we reported in zebrafish. Transcript levels of autophagy receptors *p62/SQSTM1* and *OPTN* are increased in a significant manner (respectively 124% and 114% of control levels) (**Fig 4 C**). I next assessed the effect of siRNA-*TARDBP* on autophagy initiation genes. As previously reported in similar cellular models, quantity of *RAPTOR* and *ATG7* transcripts is significantly decreased upon TDP-43 depletion as well as for *ULK1*, *BECN1* and *ATG5* (**Fig 4 D**). Single-molecule Fluorescent in-situ hybridization (smFISH) was performed and confirms the upregulation of *p62/SQSTM1* in siRNA-*TARDBP* cells as compared with siRNA-Control (**Fig 4 E**). Indeed, number of *SQSTM1* mRNA puncta appear augmented when depleting TDP-43. siRNA-*SQSTM1* treated cells served as a control of the technique to evaluate the semi-quantitative aspect of smFISH. However augmented, no apparent mislocalization of *SQSTM1* mRNA is visible in siRNA-*TARDBP* cells (**Fig 4 E**). Altogether, the profile of expression of autophagy genes appears comparable to what has been described in similar models but in opposition with our previous data in zebrafish. More importantly, *TBK1* protein and RNA levels are not negatively affected by knockdown of *TARDBP* unlike in the zebrafish model indicating mechanistic differences.

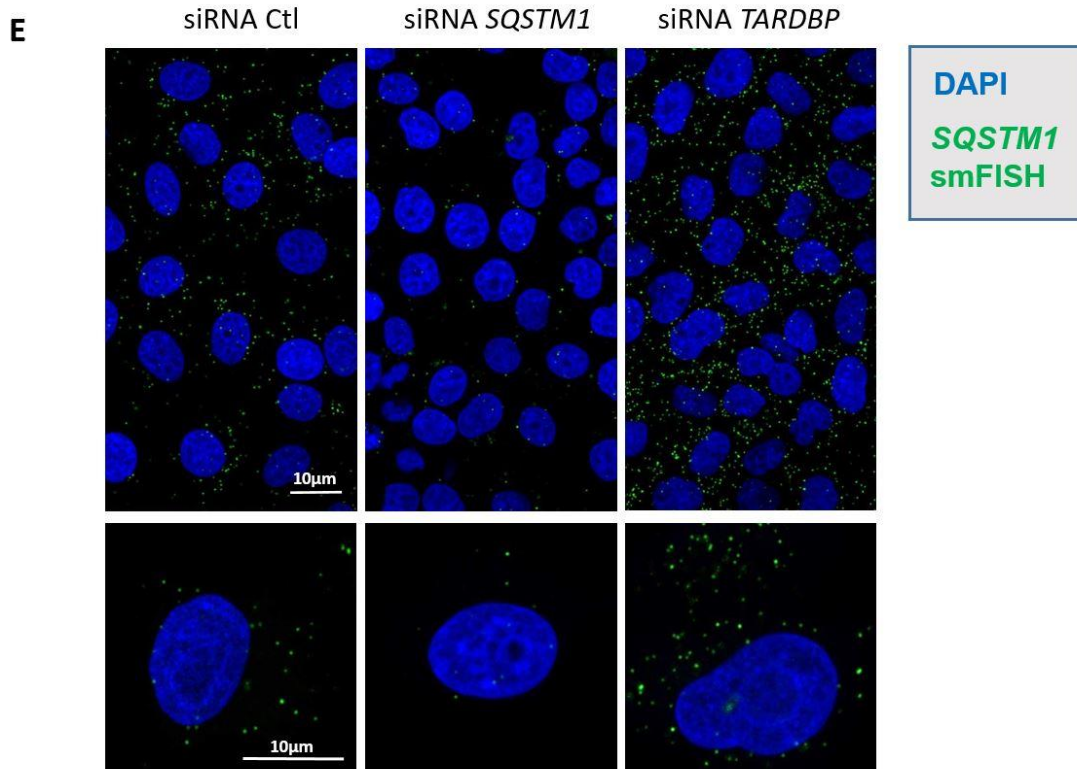
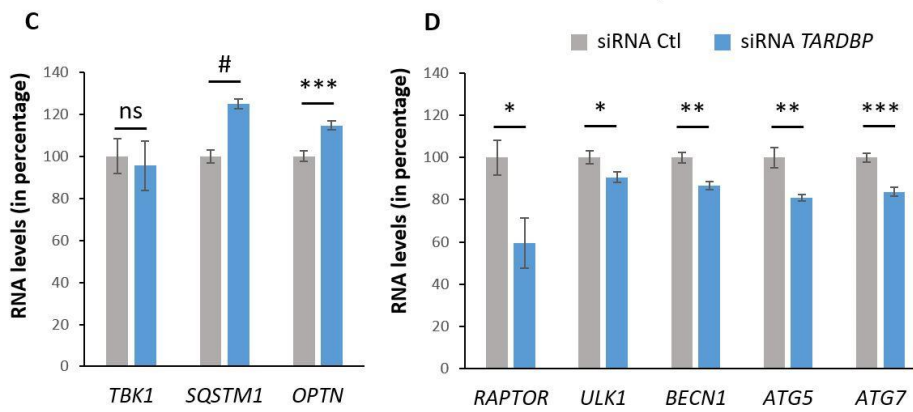
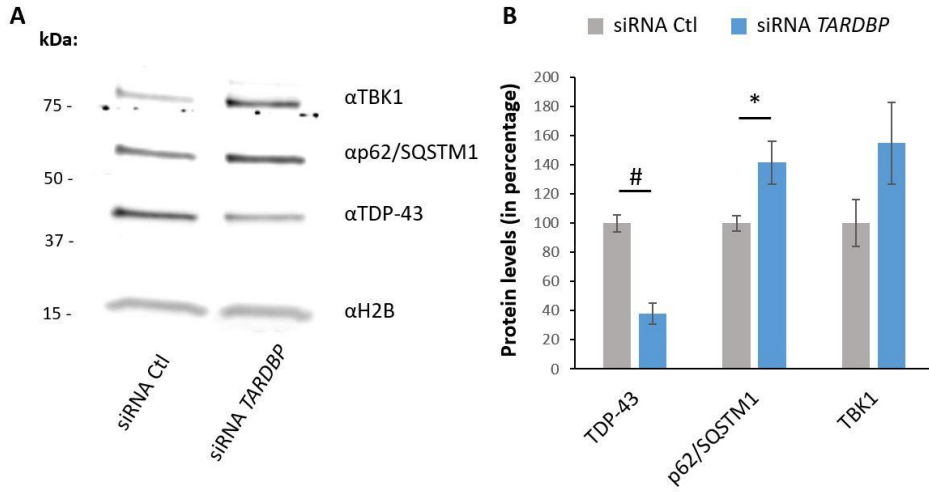


Figure 4: KD of TARDBP leads to altered expression of autophagy genes in SH-SY5Y cells. (A) Immunoblotting and **(B)** its relative quantification showing that siRNA-mediated knockdown of *TARDBP* (in blue) leads to increased protein levels of p62/SQSTM1 and a strong tendency of increase in TBK1 as compared to control siRNA (in grey). At the RNA level, **(C)** RT-qPCR results show that siRNA *TARDBP* transfected cells don't display *TBK1* downregulation but upregulated expression of autophagy receptors *p62/SQSTM1* and *OPTN* while **(D)** expression profile of genes involved in the initial steps of autophagy are globally decreased. n=6 for each siRNA-Control and siRNA-TARDBP conditions. All results are mean \pm SEM. *P<0,05, **P<0,01, ***P<0,001, #P<0,0001 from unpaired Student t test. (E) Images of smFISH labeling *SQSTM1* RNA confirming siRNA *TARDBP* seem to increase *SQSTM1* RNA levels.

C) Drug-induced modulation of autophagy leads to antagonist effects of TDP-43 depletion on p62/SQSTM1 and TBK1

In order to understand the differences between zebrafish and cell lines, two potent autophagy modulating drugs have been used in addition to siRNA treatments. Bafilomycin B1 (Baf B1) is an inhibitor of autophagosome fusion with the lysosome and degradation often used to help estimate autophagic flux. Torin 1 is an inhibitor of mTOR signaling and thus activator of autophagy induction. In Baf B1 or Torin 1 conditions, siRNA-*TARDBP* still shows significant efficiency in depleting TDP-43 as seen on immunoblotting (**Fig 5 A**) and its relative quantification (**Fig 5 B**). When we look at the sole effect of drugs on siRNA-Control levels, we can see that Baf B1 induces a great augmentation of protein levels (285% of control levels) that suggests accumulation of p62/SQSTM1 in undegraded autophagosomes, as expected (**Fig 5 C**). As for TBK1, sole effect of drug treatment doesn't affect protein levels in a significant manner (**Fig 5 E**).

Concerning the effect of siRNA-*TARDBP* within each treatment, the increase in p62/SQSTM1 and TBK1 we observe in untreated cells is abolished in Torin 1 cells (**Fig 5 D and**

F). In fact, it is the only condition where band intensity appear decreased for p62/SQSTM1 and TBK1 (**Fig 5 A**) and respective mean quantification are at 90% and 81% of control levels (**Fig 5 D and F**). Those decreases are however not significant. Yet, they highlight different impacts of TDP-43 depletion in untreated and Torin 1 cells. Hence, TDP-43 seems to act in different manners depending on modulation of autophagy.

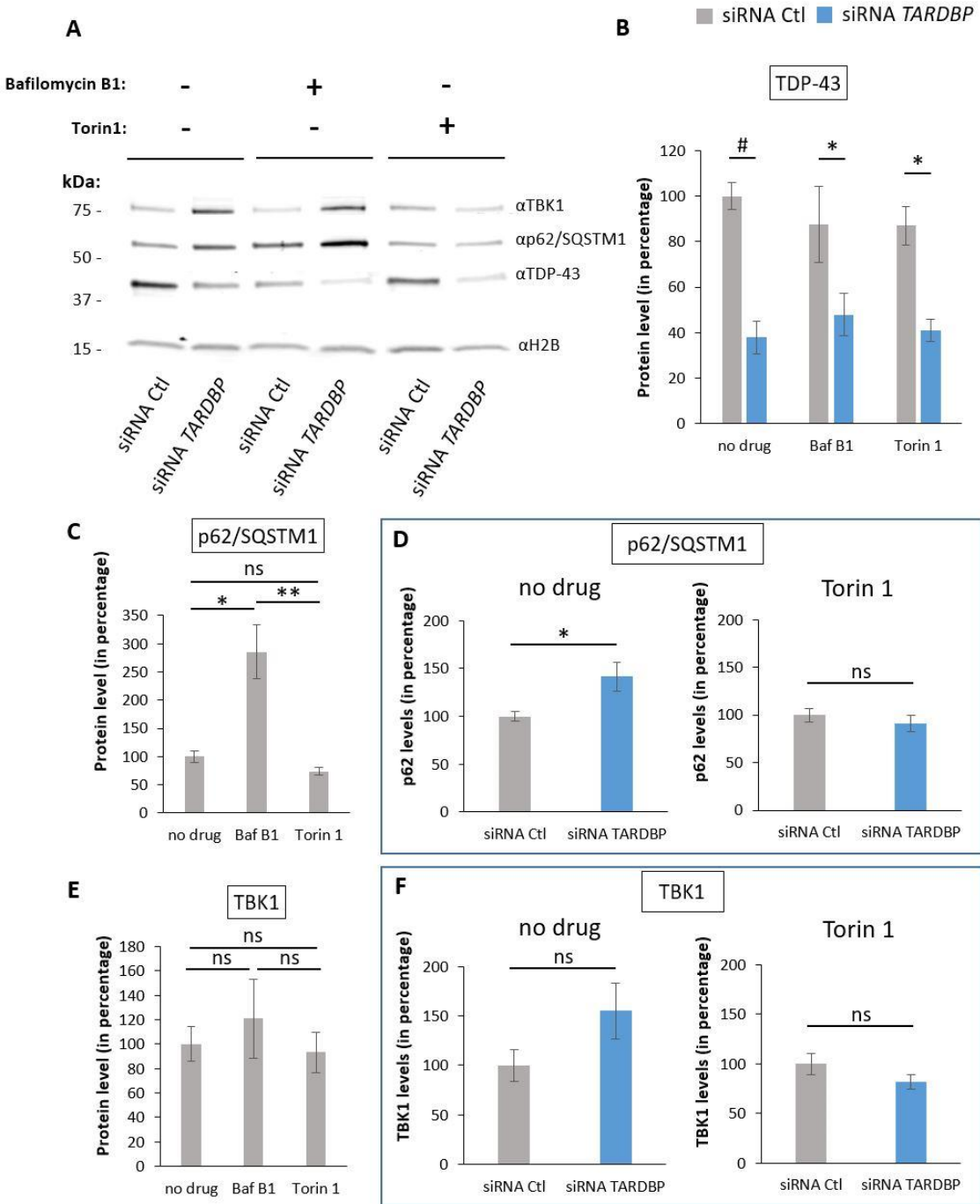


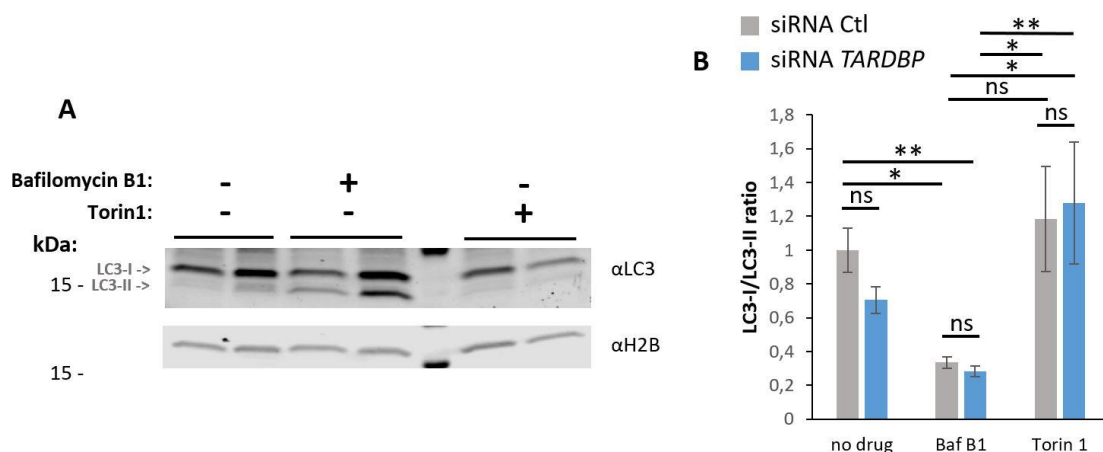
Figure 5: Drug-induced modulation of autophagy leads to antagonist effects of *TARDBP* KD on ALS-related autophagy protein levels in SH-SY5Y cells. (A) Immunoblotting and relative quantifications of **(B)** TDP-43, that confirms knockdown, and of autophagy-related **(C and D)** p62/SQSTM1 (n=12 for each siRNA Control and siRNA-*TARDBP* in untreated cells and n=6 in Bafilomycin B1 and Torin 1-treated cells) and **(E and F)** TBK1 protein levels (n=6 for each siRNA-Control and siRNA-*TARDBP* treatment in all drug conditions) which show different effects of siRNA-*TARDBP* in Torin 1-treated cells as compared to untreated and Bafilomycin B1 cells. All results are mean \pm SEM. *P<0,05, **P<0,01, #P<0,0001 from two-way ANOVA followed by Sidak's posthoc analysis **(B)**, Kruskal-Wallis test followed by Dunn's posthoc analysis **(C and E)** and Mann-Whitney's test **(D and F)**.

D) siRNA-*TARDBP* may differentially impact autophagosome dynamics and correlates with a nuclear depletion of TDP-43

Immunoblotting of the LC3 protein reveals its two forms LC3-I and LC3-II, that are respectively unintegrated and integrated into autophagosome membrane. As expected, LC3-II is augmented after Baf B1 treatment and Torin 1 leads to reduced LC3-II as compared to control cells **(Fig 6 A)**. As LC3 immunoblotting is generally difficult to interpret due to different affinity of the antibody against each form, we calculated a relative LC3-I/LC3-II band intensity ratio normalized on what is obtained for untreated siRNA-Control cells. When this ratio goes down, it translates a shift of balance towards LC3-II and thus accumulation of autophagosomes, as logically observed with Baf B1 treatment **(Fig 6 B)**. When it goes up, it shows a shift towards LC3-I it indicates that fewer LC3 are integrated in autophagosome like in Torin 1 cells **(Fig 6 B)**. This could either depict a reduction of autophagosome formation or improved degradation (autophagic flux). The ratio confirms that depleting TDP-43 could impact autophagosome formation in a different way depending on modulation of autophagy. Indeed, in control and Baf B1 treated cells, the ratios show respective mean drops of 30% and 16% from their siRNA-Control while it is not the case with Torin 1 treatment (8% increase from

siRNA-Control) (**Fig 6 B**). This data may indicate that depletion of TDP-43 promotes autophagosome accumulation in control and Baf B1 cells but not in Torin 1 cells.

We next performed immunofluorescence to visualize another marker of autophagosome p62/SQSTM1 as well as TDP-43 depending on the same treatments. A plasmid expressing GFP has been used in combination with siRNAs to identify which cells have been transfected. Concerning p62/SQSTM1, the accumulation of the autophagy receptor into puncta of higher intensity may reveal the formation of autophagosome. p62/SQSTM1 puncta are particularly visible in cells treated with Baf B1, a condition where accumulation of autophagosome is indeed highly expected (**Fig 6 C**). Within untreated cells, more p62/SQSTM1 puncta can be observed upon TDP-43-depletion versus siRNA-Control. In Baf B1 and Torin 1 cells, any conclusion concerning the effect of TDP-43 depletion on autophagosome formation is difficult, contrarily to untreated cells. There is variability in the number and distribution of puncta (**Fig 6 C**). In parallel, TDP-43 immunofluorescence reveals a comparable effect of *TARDBP* knockdown in both drug-treated and untreated cells. Indeed, while cytoplasmic signal seems rather unaffected, TDP-43 is not as strongly located in the nucleus in siRNA-*TARDBP* cells as compared to siRNA-Control. Nuclear TDP-43 seems specifically depleted and up to a point where TDP-43 appears homogenously localized between nucleus and cytoplasm (**Fig 6 C**).



C

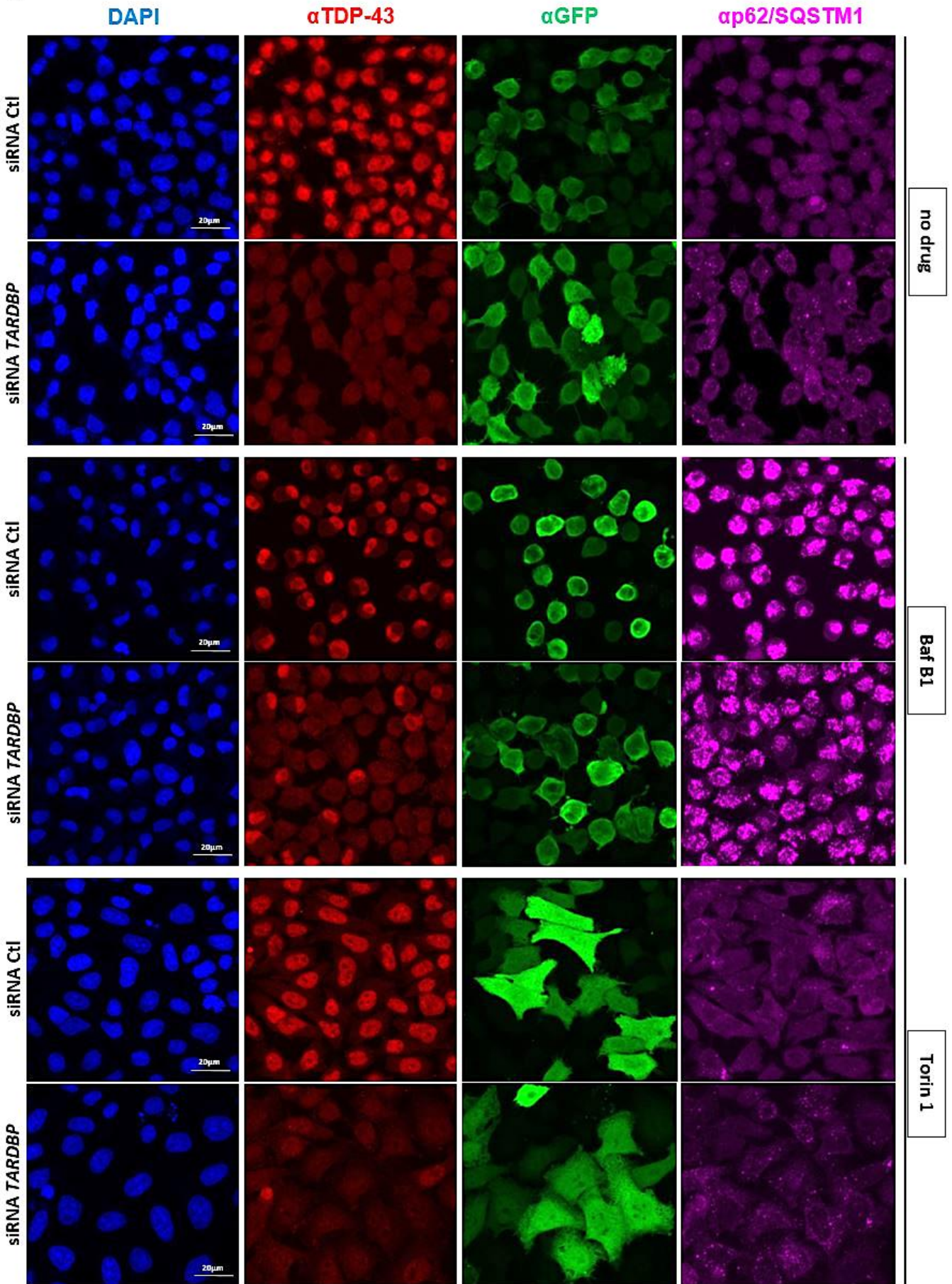
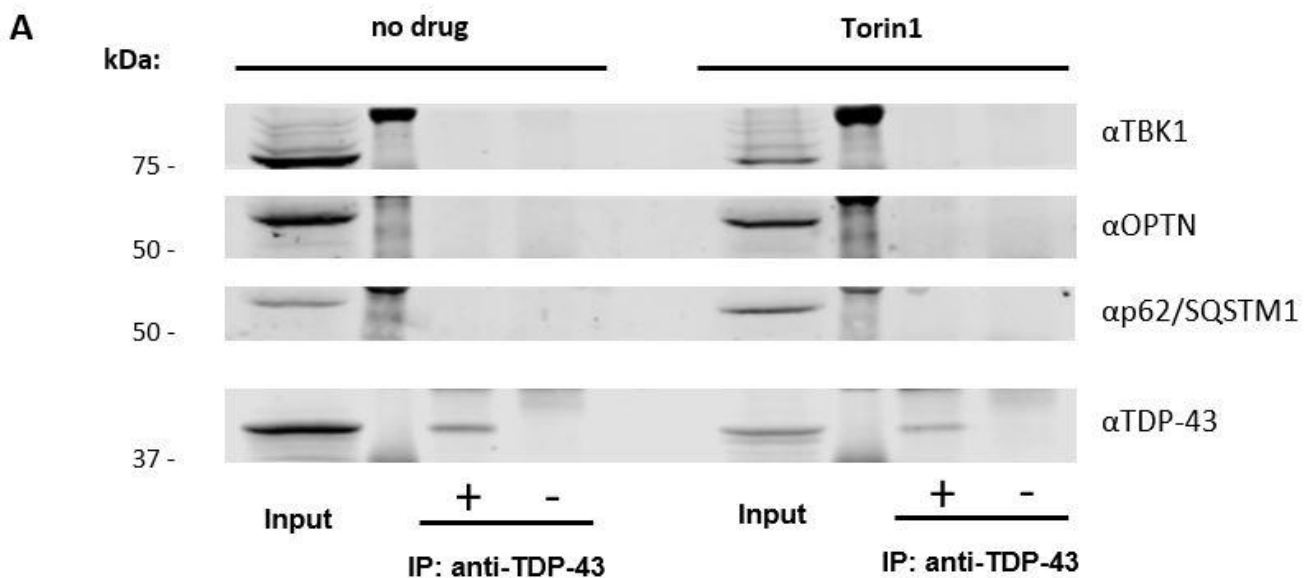


Figure 6: Pharmacological modulation of autophagy leads to antagonist effects of *TARDBP* KD on autophagosome markers in SH-SY5Y cells. (A) Immunoblotting of LC3-I and LC3-II, **(B)** relative quantification of LC3-I/LC3-II ratio and **(C)** immunofluorescence in SH-SY5Y cells showing siRNA-mediated TDP-43 depletion may promote autophagosome accumulation in control and Bafilomycin B1 conditions but not upon Torin 1 treatment. n=6 for each siRNA-Control and siRNA-*TARDBP* in all control, Bafilomycin B1 and Torin 1-treated cells. All results are mean \pm SEM. *P<0,05, **P<0,01 from Kruskal-Wallis test followed by Dunn's posthoc analysis.

E) Drug-induced modulation of autophagy leads to conditional binding of TDP-43 to autophagy-related transcripts.

After observing such antagonist effects of TDP-43 depletion depending on autophagy modulation, we focused on Torin 1 and untreated cells and performed an immunoprecipitation of TDP-43. We aimed to identify any potential protein or RNA interactors among our autophagy genes of interest that could explain the phenomenon. As immunoblotting of the precipitated proteins in both control and Torin 1 cells reveal, neither TBK1, OPTN or p62/SQSTM1 seem to bind to TDP-43 at the protein level. The membrane does confirm that immunoprecipitation was efficient as TDP-43 itself is detectable (**Fig 7 A**). Regarding RNA immunoprecipitation, we confirm that the experiment was well performed as strong binding to positive control *TARDBP* RNA is obtained in all conditions (**Fig 7 B**). Detection of target transcript corresponds to a relative quantification that is superior to 1 unit, which is the level of detection of this particular RNA via RT-qPCR in a control immunoprecipitation with α IgG antibody. We report for the first time a binding of TDP-43 to the two ALS-related *TBK1* and *p62/SQSTM1* mRNAs. These interactions seem to be close to inexistent in control cells but promoted by autophagy activation via Torin 1 treatment (**Fig 7 C and D**). In an opposite

manner, binding of TDP-43 to *OPTN* and *RAPTOR* mRNAs is logically detected in control cells, as it was already reported, but is completely inhibited in Torin 1 cells (**Fig 7 E and F**). Logically, the significant decrease in total *RAPTOR* mRNA that we reported upon depletion of TDP-43 in untreated cells, isn't present when cells are treated with Torin 1. This confirms the loss of binding of TDP-43 to the transcript in this condition. This loss of binding does not appear to be caused by a rarefication of the transcript as *RAPTOR* levels are comparable in both siRNA-Control untreated and Torin 1 cells (**Fig 7 G**). The conditional binding of TDP-43 to these key RNAs depending on autophagy modulation is consistent with the observed antagonist effects of *TARDBP* KD on autophagy genes and may explain these mechanistically.



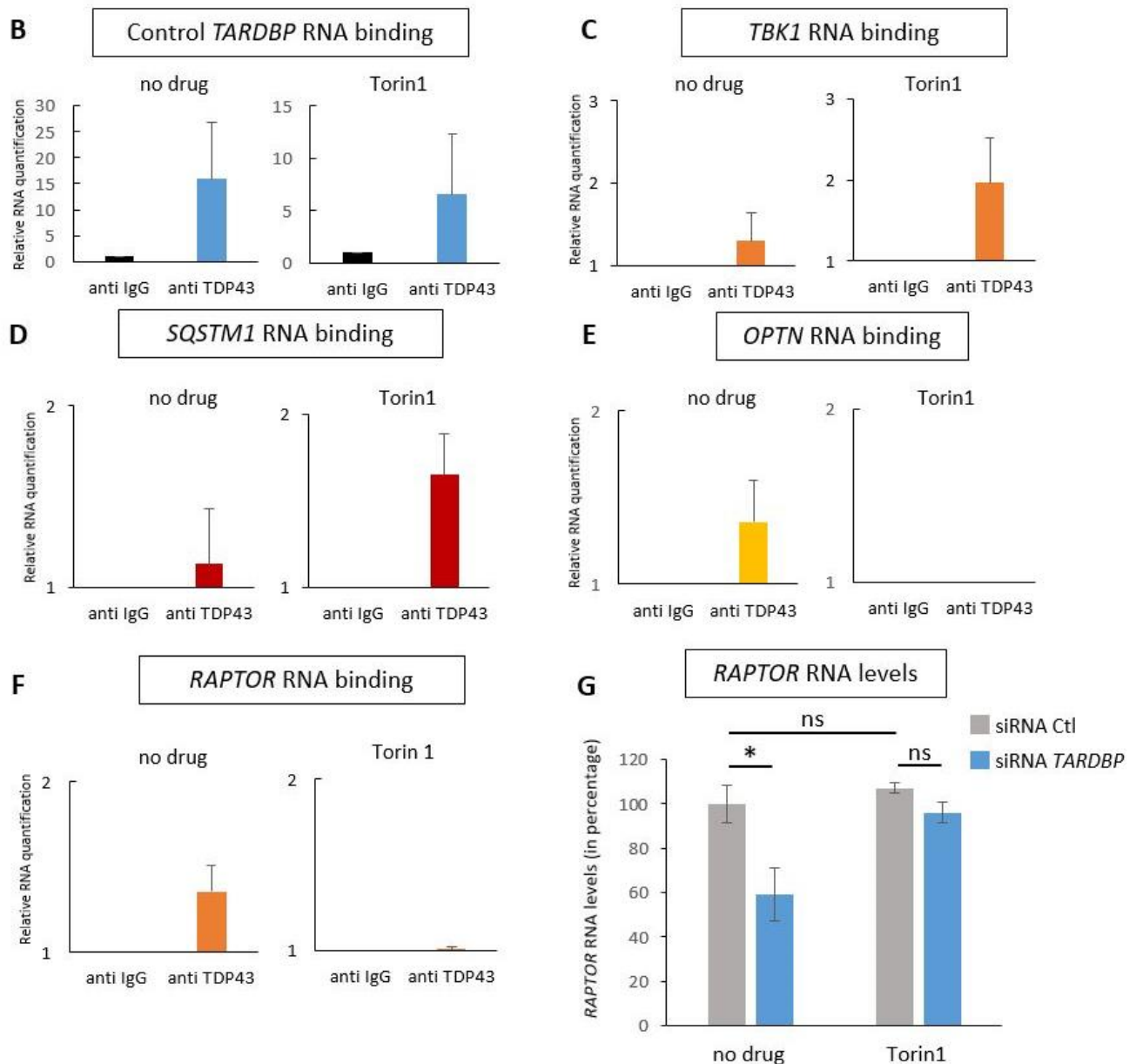


Figure 7: Conditional binding of TDP-43 to key autophagy transcripts in response to drug-induced autophagy modulation in SH-SY5Y cells. (A) Immunoblotting after immunoprecipitation of TDP-43 showing no direct interaction with p62/SQSTM1, TBK1 or OPTN proteins. RNA immunoprecipitation of TDP-43 confirming control binding to **(B)** *TARDBP* RNA and revealing that Torin 1 treatment promotes binding of TDP-43 to novel ALS-related mRNAs **(C)** *TBK1* and **(D)** *SQSTM1* but inhibits binding to **(E)** *OPTN* and **(F)** *RAPTOR* mRNAs. n=3 for each control and Torin 1-treated cells. **(E)** RT-qPCR results showing Torin 1 treatment suppresses the decrease of *RAPTOR* mRNA levels induced by *TARDBP* KD. n=6 for each siRNA-Control and siRNA-*TARDBP* in control and Torin 1-treated cells. All results are mean \pm SEM. *P<0,05, from two-way ANOVA test followed by Tukey's posthoc analysis.

F) Gradual overexpression of *TARDBP* leads to appearance of ALS-related truncation and mislocalization of TDP-43

To further investigate the impact of TDP-43 modulation on key autophagy actor p62/SQSTM1, we transiently overexpressed the wild-type and untagged RNA-binding protein in SH-SY5Y. We used a starting concentration of 0,5µg/mL to extract proteins and perform western immunoblotting. The blotting membrane reveals a band of higher intensity for TDP-43, thus indicating overexpression, but also for p62/SQSTM1 as compared to control levels obtained with transfection of the control vector *pCS2 empty* (**Fig 8 A**). They respectively show mean increase of 68% and 32% as compared to control levels. However, these results are not significant as great variability was observed among replicates (**Fig 8 B**). At this same concentration, smFISH targeting *SQSTM1* mRNA was performed and indicate that transcript levels are also increased upon overexpression of TDP-43. Each individual punctum reflects the presence of a single *SQSTM1* mRNA. They are visible in higher amounts with *pCS2+ TDP-43*. They however do not appear to be abnormally distributed in cells (**Fig 8 C**).

To understand the variability observed in the previous conditions, I performed a gradient of overexpression of *TARDBP* extending going from 0,25 up to 5µg/mL. Results of protein quantification were normalized to mean TDP-43 and p62/SQSTM1 levels acquired in both minimum and maximum concentrations of *pCS2 empty* of each replicate. TDP-43 levels appear gradually augmented throughout the range of increasing *pCS2+ TDP-43* concentration until it reaches a maximum of 151% of control levels. Nevertheless, this augmentation is not proportional to plasmid concentration and seem to plateau rapidly. Furthermore, the lowest concentration interestingly tends to display lower TDP-43 levels than mean of controls. These

aspects of TDP-overexpression could be explained by TDP-43 ability to self-regulate which would buffer our exogenous modulation of its levels. The first concentrations that display augmented TDP-43 levels are 0,5 and 1 $\mu\text{g}/\text{mL}$ and are accompanied by a mean increase in p62/SQSTM1 of 20% and 18% (**Fig 8 D**). However, in higher concentrations of *pCS2+ TDP-43*, this dynamic of increasing p62/SQSTM1 appears reversed. It gradually diminishes until final plasmid concentration is reached and p62/SQSTM1 levels are diminished by 14% as compared to control levels (**Fig 8 D**). However, variability is still strong and none of these results are significant.

We next tried to pinpoint events happening along this range of *pCS2+ TDP-43* concentrations that would help us understand the peculiar dynamics at play. Immunoblotting against the C-terminal part of TDP-43 reveals the appearance of ALS-related pathological C-terminal fragments of TDP-43 namely TDP-35 and TDP-25 upon overexpression of the wild-type protein. These pathological truncated forms appear as soon as 0,5 $\mu\text{g}/\text{mL}$ of plasmid and in, what seems to be, a dose-dependent manner (**Fig 8 E**). We then performed an immunofluorescence to localize TDP-43 in certain selected concentrations of *pCS2+ TDP-43*. The protein is normally distributed and thus predominantly nuclear in control 5 $\mu\text{g}/\text{mL}$ of *pCS2 empty* and minimum *pCS2+ TDP-43* concentration of 0,25 $\mu\text{g}/\text{mL}$. However, from 1 $\mu\text{g}/\text{mL}$ to final 5 $\mu\text{g}/\text{mL}$ of *pCS2+ TDP-43*, TDP-43 is gradually and massively mislocalized into the cytoplasm of SH-SY5Y cells. This corresponds to another histopathological trait of TDP-43 proteinopathy in ALS (**Fig 8 F**). Hence, expression of *p62-SQSTM1* seem to fluctuate upon appearance of TDP-43 truncated forms and its mislocalization.

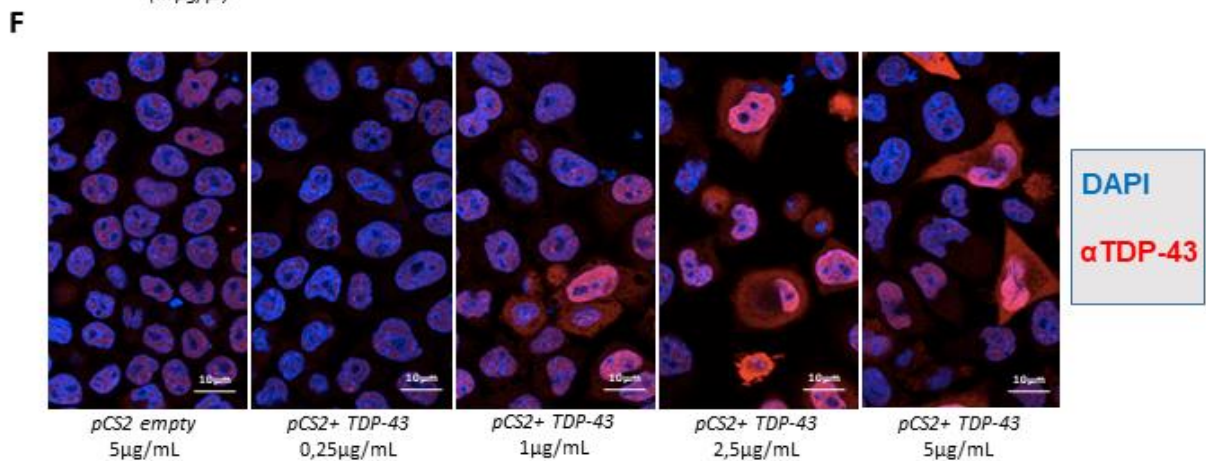
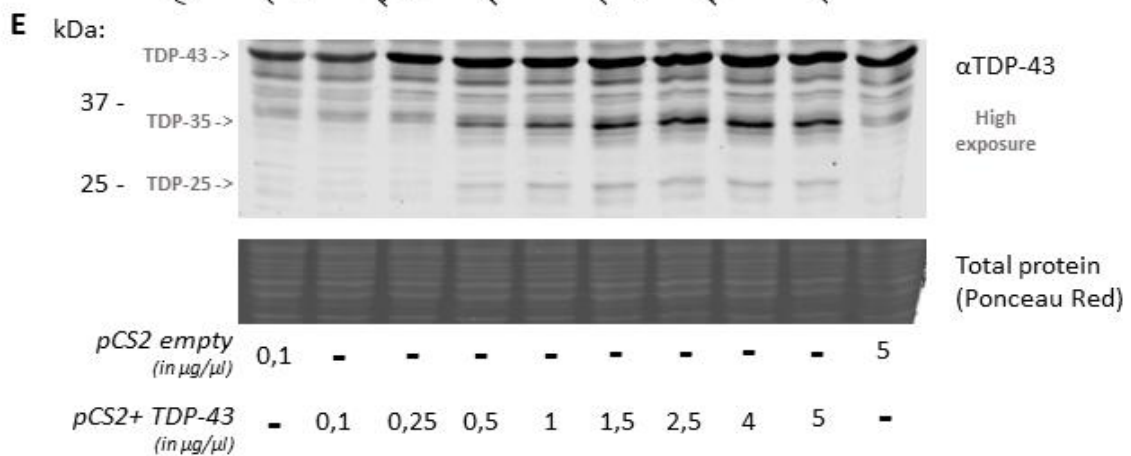
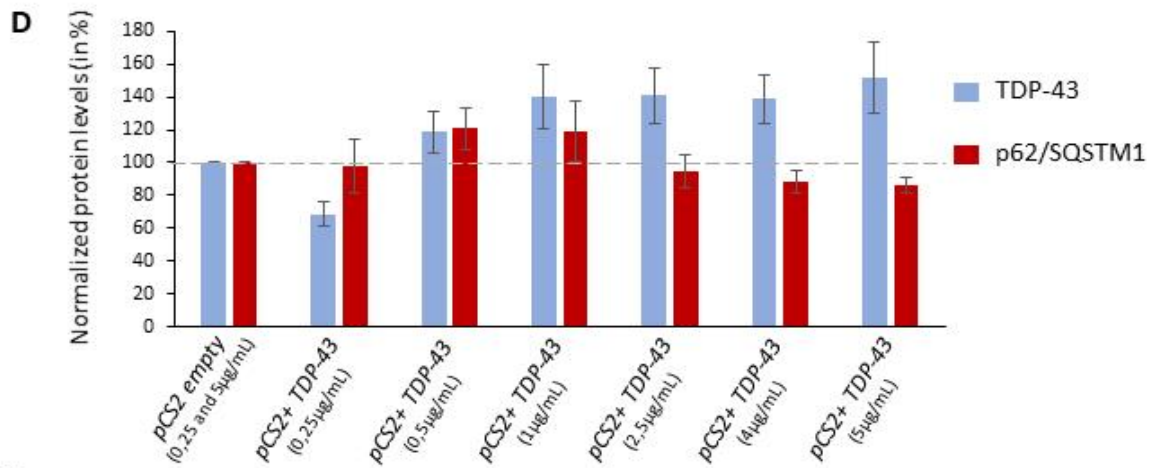
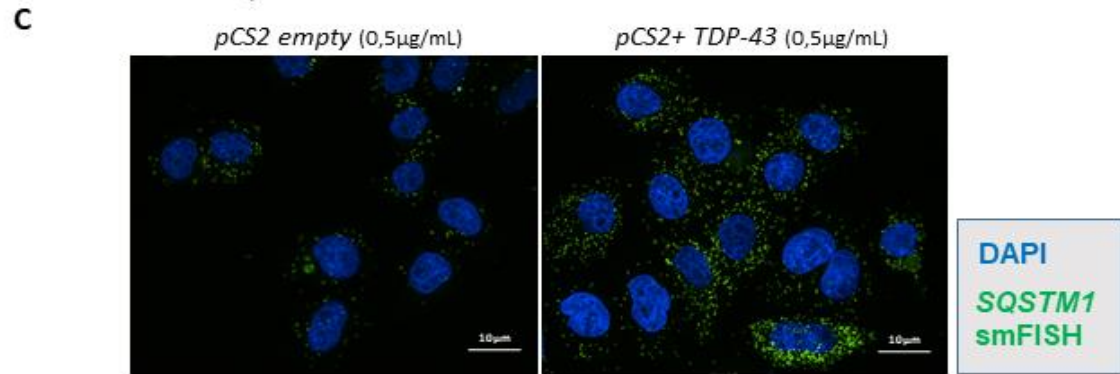
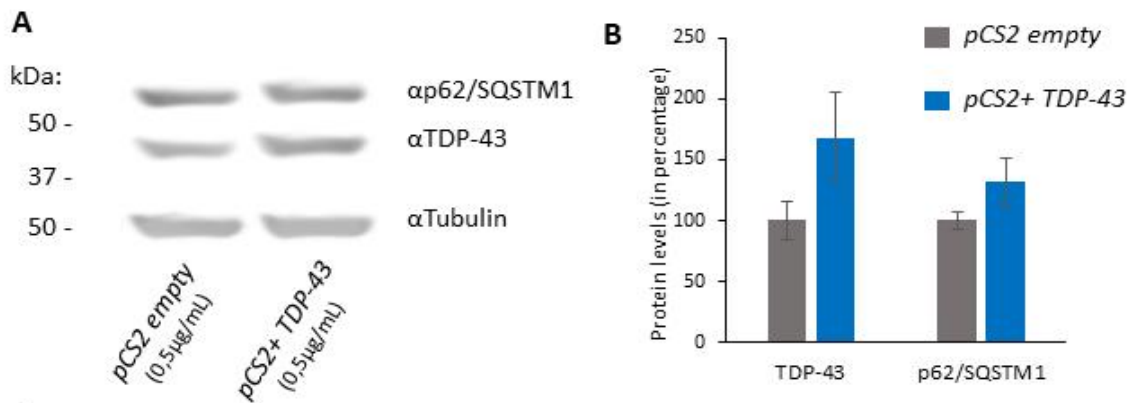


Figure 8: *TARDBP* overexpression leads to appearance of cleaved forms and mislocalization of TDP-43 in a dose-dependent manner in SH-SY5Y cells. (A) Immunoblotting and **(B)** relative quantification of TDP-43 and p62/SQSTM1 protein levels and **(C)** images of smFISH labeling *SQSTM1* RNA showing that overexpression of *pCS2+ TDP-43 GU2* at a concentration of 0,5µg/mL could lead to increased protein and RNA levels of *p62/SQSTM1*. **(D)** Normalized quantification of TDP-43 and p62/SQSTM1, **(E)** immunoblotting and **(F)** immunofluorescence of TDP-43 throughout a gradient of increasing *pCS2+ TDP-43 GU2* expression revealing the appearance of cleaved forms and mislocalization of TDP-43 in a dose-dependent manner. n=10 for 0,5µg/mL of *pCS2+ TDP-43 GU2* and n=6 for the rest. All results are mean ± SEM.

G) Discussion

Altogether, these data are the first report of: 1) antagonist effects of *TARDBP* knockdown on autophagy genes depending on activation of autophagy; 2) binding of TDP-43 to ALS-related *TBK1* and *p62/SQSTM1* mRNAs; 3) conditional binding of TDP-43 to key target mRNAs (namely *OPTN*, *RAPTOR*, *TBK1* and *p62/SQSTM1*). Furthermore, siRNA-mediated knockdown of *TARDBP* lead to nuclear depletion of TDP-43 while overexpression lead to its mislocalization in the cytoplasm, as reported previously (Casella *et al.*, 2016). However, this last study did not report the appearance of TDP-43 C-terminal fragments we observed upon overexpression of *TARDBP*. Each of these ALS-related pathological traits could affect *p62/SQSTM1* and other key autophagy genes in different ways. Overall, this unveils new links between TDP-43 and autophagy and bring details to TDP-43's complex normal function and possible contribution to ALS pathological mechanisms. However, each of these novel reports remain incomplete and would greatly benefit from further investigation.

First of all, several of the results presented here show strong tendencies but still lack statistical significance. It appears essential to repeat experiments even more to gain in robustness and confirm our data. However, these tendencies all appear to follow a common

dynamic that is consistent with the proposed mechanisms. For example, the antagonist effects that were witnessed upon TDP-43 depletion are not isolated phenomena, they are shared by multiple actors, namely p62/SQSTM1, TBK1 and LC3. They also correlate with the conditional binding of TDP-43 to *OPTN*, *RAPTOR*, *TBK1* and *p62/SQSTM1* mRNAs. Moreover, our data on untreated cells concerning the profile of expression of autophagy initiation genes, the increase in p62/SQSTM1 and in LC3 proteins in response to TDP-43 depletion as well as binding of TDP-43 to *RAPTOR* and *OPTN* mRNAs are all coherent with the previous reports on similar cellular models by other teams (Bose, Huang and Shen, 2011; Polymenidou *et al.*, 2011; Xia *et al.*, 2016). In fact, sole binding to *RAPTOR* mRNA was described to be the cause of these changes (Xia *et al.*, 2016). Its loss in Torin 1 treated cells could, on its own, logically explain their disappearance. That is why the exact effect of all conditional bindings we report here should be further detailed. In order to do so, an analysis of the effect of *TARDBP* knockdown on the stability of each of these transcripts remains to be conducted.

The fact that we report conditional binding of TDP-43 to key RNA targets puts in perspective our current knowledge of TDP-43's RNA interactome. It also highlights a novel aspect of TDP-43's ability to adapt to cellular context that should be further explored to better understand its normal function and implication in pathogenesis. Based on the results of this thesis, I hypothesize that TDP-43 is involved in autophagy modulation through its conditional binding to autophagy genes. TDP-43 binds *RAPTOR* mRNA and feeds mTORC1 inhibitory control over the degradative pathway, which defines basal autophagy in cell lines. Torin 1 treatment leads to the binding of TDP-43 to *TBK1* and *SQSTM1* mRNAs thus shifting the equilibrium towards the promotion of autophagy. This may fuel the activity of the p62/TBK1 axis, which is key for cargo engulfment into autophagosome and feeds the autophagic process. **(Fig H 1)**. The role of *OPTN* in this potential mechanism needs further investigation.

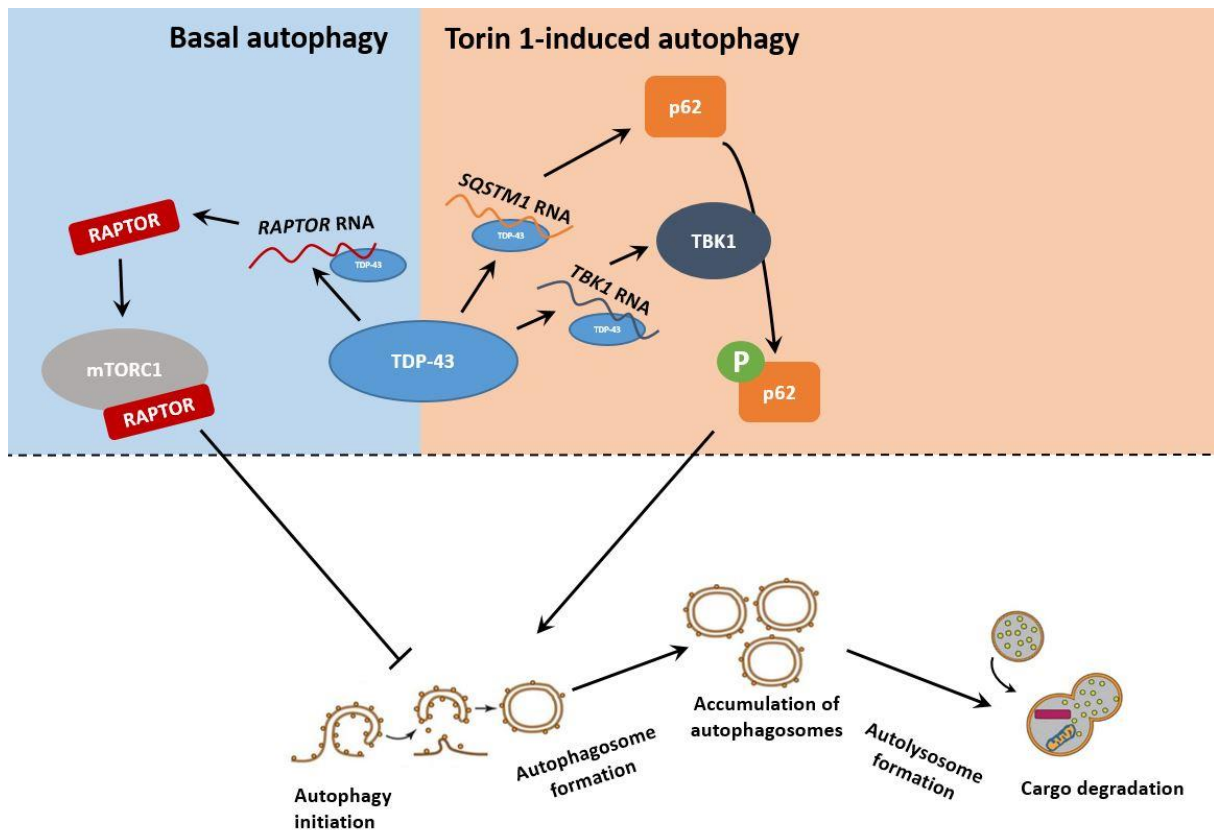


Figure Hypothesis 1: Proposed mechanism for TDP-43 conditional regulation of autophagy

The mechanism through which Torin 1 induces such changes in TDP-43's RNA-binding function is unknown but may be particularly relevant to ALS as several autophagy modulating drugs are currently being tested for their therapeutic power in clinical trials. In the future, the effects observed upon Torin 1 treatment should be compared to other autophagy induction methods. Interestingly, Torin 1 treated cells appeared larger and morphologically different from the rounder untreated and Baf B1 cells. As mTOR is involved in cell growth, Torin 1 may be responsible for this change of aspect.

Furthermore, clarifying the mechanisms at play, especially the conditional binding of TDP-43 to autophagy-related mRNAs and its consequences, may help us understand the fluctuation in *p62/SQSTM1* expression that seemed to accompany *TARDBP* overexpression. This set of experiments needs further investigation and precision as results were inconclusive

and displayed variability. However, as TDP-43 levels are gradually augmenting, we observe an intriguing sequence of correlating events. First the appearance truncated and mislocalized TDP-43 with a potential increase in *p62/SQSTM1* RNA and protein levels. Then the progressive aggravation of these TDP-43 pathological traits with a decrease in *p62/SQSTM1*. As mislocalized and truncated TDP-43 are known substrates of autophagy, we hypothesize that their appearance may first induce an autophagic response that we now suggest has the potential to condition TDP-43's RNA partners of interaction. By extent, this could then influence autophagy genes in different manners via further alteration of TDP-43 normal function. To verify this, the loss-of-TDP-43-RNA-binding-function, even if suggested by the progressive truncation and sequestration of TDP-43 in cytoplasm, has to be demonstrated in this TARDBP-overexpression model.

Overall, both knockdown and overexpression of TARDBP cause ALS-related TDP-43 defects. Indeed, siRNA *TARDBP* leads to nuclear depletion of TDP-43 while gradual overexpression caused its progressive cleavage and cytoplasmic mislocalization. In that sense, considering the challenge that is TDP-43 modeling, emulating such ALS-related hallmarks is encouraging. These ALS-related traits are known to affect TDP-43's normal function including its RNA-binding function which requires N-terminal RRM and correct nucleus/cytoplasm shuttling. The key to linking all of these results may reside in understanding the exact consequences of such alterations of TDP-43 to key RNA targets. For this purpose, the development of reliable RNA tracking techniques could be determinant.

H) Materials and methods

Cell culture, transfection and drug treatment:

The human cells SH-SY5Y were grown in DMEM Media-GlutaMAX™ (Dulbecco's Modified Eagle medium; Gibco®, Life technologies Paisley, UK) supplemented with 10% fetal bovine serum (FBS; SIGMA, USA) and 1% of penicillin/streptomycin solution (10.000U/mL; pen/strep; Gibco). Cells were seeded at an approximate density of 3×10^5 cells in COSTAR 12 well Cell Culture Plate (3512, Corning, USA) and transfected the next morning with the following siRNAs and/or plasmids using Lipofectamine™ 2000 (Invitrogen™, Life technologies Paisley, UK) according to manufacturer's instructions.

- siGENOME SMART pool siRNA Human TARDBP M-012394-01-0005 (Dharmacon, USA) at a final concentration of 50 nM for transient knockdown of TDP-43.
- siGENOME SMART pool siRNA Human SQSTM1 M-010230-00-0005 (Dharmacon, USA) at a final concentration of 50 nM for transient knockdown of p62/SQSTM1.
- siGENOME non-targeting siRNA #2 D-001210-02-05 (Dharmacon, USA) at a final concentration of 50 nM for control siRNA transfection.
- *pCS2+ GFP* plasmid at a final concentration of 800ng/mL for siRNA transfection control in immunochemistry experiments.
- *pCS2+ TDP-43 GU2* at a range of concentration going from 100 ng/mL to 5µg/mL to overexpress wild-type and untagged human TDP-43.
- *pCS2+ empty* at a range of concentration going from 100 ng/mL to 5µg/mL for *pCS2+ TDP-43 GU2* transfection control.

Transfection mixes are made in Opti-MeM Reduced Serum Medium (ThermoFischer Scientific, USA) and added to DMEM Media-GlutaMAX™, 10% FBS to reach desired final concentrations. Transfection medium is left to incubate cells during 6 hours and changed back to DMEM Media-GlutaMAX™, 10% FBS, 1% pen/strep.

If required, cells are treated with autophagy modulating drugs at 24 hours after transfection. Medium is changed for new DMEM Media-GlutaMAX™, 10% FBS, 1% pen/strep supplemented with Torin1 or Bafilomycin B1 at final concentrations of 250nM and 100nM respectively, as previously described (Kaizuka *et al.*, 2016). As stock solution of Torin1 and Bafilomycin B1 are made in DMSO (D8418; Sigma Aldrich, USA), control conditions are incubated with vehicle DMEM Media-GlutaMAX™, 10% FBS, 1% pen/strep supplemented with a corresponding quantity of DMSO.

After 48 hours of transfection, cells are recuperated for Immunohistochemistry, Immunoblotting, RNA extraction or Immunoprecipitation protocols that will be described hereafter.

Immunohistochemistry (IHC):

IHC were performed on 10mm-diameter cover glasses that were placed before cell were seeded as previously described. After transfection protocol was carried out, cover glasses are recuperated and put in agitation at room temperature for the following steps:

- 1) 5min wash in PBS 1X
- 2) 20min fixation in a 4% formaldehyde solution (252549; Sigma Aldrich, USA; stock at 37% diluted in PBS 1X)
- 3) three 5min washes in PBS 1X
- 4) 10min permeabilization in PBS 1X supplemented with 0.1% Triton (from stock Triton X-100, Sigma-Aldrich, USA)
- 5) three 5min washes in PBS 1X

6) 1 hour blocking in Blocking Buffer (PBS Triton 0.1% supplemented with 5% Bovine Serum Albumine (A2153; Sigma-Aldrich, USA))

Cover glasses are then incubated overnight at 4°C in agitation with the following antibodies diluted at desired concentrations in Blocking Buffer:

- Anti-TDP-43 C-terminal (rabbit, 12892-1-AP) from Proteintech, USA.
- Anti-SQSTM1 (mouse, sc-28359) from Santa Cruz Biotechnologies, USA.
- Anti-GFP (chicken, GTX13970) from GeneTex, USA.

The next day, protocol continued with the following steps in agitation and at room temperature:

- 1) three 5min washes in PBS 1X
- 2) 1-hour incubation with appropriate fluorophore-conjugated secondary antibodies diluted into Blocking Buffer at a 1/500 ratio from stock solutions (Alexa Fluor series from ThermoFischer, USA). For this incubation and the rest of the protocol, cover glasses are carefully kept in the dark
- 3) three 5min washes in PBS 1X
- 4) 10min incubation in PBS 1X supplemented with DAPI diluted at 1/1000 from stock solution (D3571; Invitrogen™, Life technologies Paisley, UK)
- 5) A series of 5 min washes in PBS 1X, PBS Triton 0.1%, PBS 1X and in Milli-Q water.

Cover glasses were then mounted on Microscope Slides (J1800AMNZ; ThermoFischer, USA) in VECTASHIELD Mounting Medium (H-1000; Vector Laboratories, USA) and sealed with standard transparent nail polish.

Single molecule Fluorescent in Situ Hybridization:

smFISH was performed on previously described cover glasses using a ViewRNA ISH Cell Assay Kit (QVC0001; ThermoFischer, USA) and according to manufacturer's instructions. In order to specifically detect human sqstm1 mRNAs, SQSTM1 Alexa Fluor 488 ViewRNA Cell Probe Set (VA4-3084431-VC; ThermoFischer, USA) was used. Cover glasses were mounted on Microscope slides as previously described.

Fluorescent microscopy:

Immunocytochemistry or smFISH performed on SH-SY5Y cells were visualized using Leica SP8 STED system and both PMT and Hybrid detectors (Leica, Germany) through a 63X HC PL APO objective from Leica. Images were taken with LAS X software from Leica and analysed using ImageJ software (Sun Microsystems, USA).

Immunoblotting:

Protein extraction: Cells at full confluence in Falcon® 100mm Cell Culture Dish (353003, Corning, USA) are briefly washed in PBS 1X, put in Lysis Buffer (*Tris-HCl 50mM pH 7.5, NaCl 500mM, EDTA 5mM, Nonidet p-40 1%, Triton 0.5% supplemented with a cocktail of protease inhibitors* (Complete mini, Roche, Switzerland)) recuperated using a scrapper in tubes. They were then homogenized on ice through sonication (Sonics Vibracell VC505) in Lysis Buffer (*Tris-HCl 50mM pH 7.5, NaCl 500mM, EDTA 5mM, Nonidet p-40 1%, Triton 0.5% supplemented with a cocktail of protease inhibitors* (Complete mini, Roche, Switzerland)). A

20 min centrifugation at 4°C and 14000 rpm was made to recuperate supernatant. Concentration of the protein extracts were established through BCA assay.

Western blot: 30µg of protein extract per condition was mixed with 5X SDS Loading buffer (*Bromophenol blue 0.25%, dithiothreitol 0.5M, glycerol 50%, Sodium dodecyl sulfate 10%, Tris-Cl (pH 6.8) 0.25M in Milli-Q water*) and denatured at 98°C during 7min in a dry bath. Samples were loaded in a 4-20% Mini-Protean TGX Gel (BioRad, USA) for electrophoresis. The separated proteins were transferred to a nitrocellulose membrane (0,45µm, Life Sciences) and probed with the following antibodies:

- Anti-NAK/TBK1 (rabbit, ab109735) from Abcam, UK.
- Anti-SQSTM1/p62 (rabbit, PM045) from MBL International, USA.
- Anti-TDP-43 C-terminal (rabbit, 12892-1-AP) from Proteintech, USA.
- Anti-Optineurin C-term (rabbit, #100000) from Cayman Chemical, USA.
- Anti-LC3B (rabbit, ab51520) from Abcam, UK.
- Anti-histone H2B (A-6) (mouse, sc-515808) from Santa Cruz Biotechnologies, USA.
- Anti-Tubulin (mouse, T5168) from Sigma-Aldrich, USA.
- Anti-GAPDH (mouse, ab8245) from Abcam, UK.

Blots were incubated with corresponding fluorescent secondary antibodies and detection of protein bands was made using ODYSSEY CLx system (LI-COR Biosciences, USA). Analysis of bands was carried out using ImageJ software.

RNA extraction and RT-qPCR:

Cells are briefly washed in PBS 1X, put in TRIzol reagent (Sigma-Aldrich, USA) and recuperated using a scrapper in tubes. Cells are homogenized by up-and-down pipetting and TRIzol-Chloroform based RNA extraction was performed according to manufacturer's instructions. Resulting pellets of RNA were washed in ethanol and resuspended in sterilized Milli-Q water. RNA concentrations were determined using Lunatic system (Unchained Labs, USA).

First-strand cDNAs were obtained by reverse transcription of 1µg of total RNA per sample using High Capacity cDNA Reverse Transcription Kit (Roche, Switzerland) according to manufacturer's instructions. Quantitative-PCR amplification was performed with 2X SYBR Green (Bymake, USA). Primers were ordered from Sigma-Aldrich (USA) SYBR Green Primers Library and sequences are available upon request. Data were analyzed transforming raw Cq values into relative quantification data using the delta Cq method.

RNA immunoprecipitation:

RNA immunoprecipitation was carried out using EZ-Magna RIP RNA-binding Protein Immunoprecipitation Kit (Merck Millipore, USA) and according to manufacturer's instructions and with positive control anti-SNRNP70 antibody (rabbit, CS203216) and negative control anti-IgG antibody (rabbit, PP64B) both included in Merck Millipore's kit and anti-TDP-43 C-terminal antibody (rabbit, 12892-1-AP from Proteintech, USA). TRIzol-Chloroform based RNA extraction was performed according to manufacturer's instructions. Resulting pellets of RNA were washed in ethanol and resuspended in sterilized Milli-Q water. First-strand cDNAs were obtained by reverse transcription of immunoprecipitated RNAs using High Capacity cDNA Reverse Transcription Kit (Roche, Switzerland) according to manufacturer's instructions. Quantitative-PCR amplification was performed with 2X SYBR Green (Bymake, USA). Primers

were ordered from Sigma-Aldrich (USA) SYBR Green Primers Library and sequences are available upon request. Data were analyzed transforming raw Cq values into relative quantification data (by comparing to negative control anti-IgG data) using the delta Cq method.

Statistical analysis:

All data values are represented as average standard error of mean (SEM) with significance determined using bilateral Student t-test, Mann-Whitney's, Kruskal-Wallis (followed by Dunn's posthoc analysis), one-way ANOVA (followed by Tukey's) and two-way ANOVA (followed by Sidak's posthoc analysis) tests. Analyses were performed using Excel (Microsoft, USA) and Prism 7.0 (Graph Pad, USA). Significance level was set at $p < 0.05$.

III) The development of novel live-imaging RNA tracking techniques

A) Introduction

The field of RNA research has benefited from great biotechnological advances that allowed RNA quantification, sequencing and identification of targets of RNA-binding proteins on a high-throughput scale thus opening the transcriptomic era (Schena *et al.*, 1995; Niranjankumari *et al.*, 2002; Mortazavi *et al.*, 2008; Jiang *et al.*, 2015). However, these techniques do not give detailed information on transcript localization, a key parameter to understand normal metabolism of an RNA and its potential implication in a pathological mechanism.

Historically, *in-situ* hybridization has been used to localize specific mRNAs (Bauman et al. 1980; Poulsen et al. 1993). The technique relies on the binding of complementary anti-sense-RNA probes that are coupled with a detection system to a target transcript in a fixed tissue. However, in its empiric form, the technique does not allow detection of transcripts with sufficient precision to observe individualized RNA molecules and thus their exact location in the cell. More recently, the technique has been updated and is able to detect RNAs at the single molecule level (single molecule or smFISH) by multiplying the number of fluorescent probes that bind the specific RNA to ameliorate signal-to-noise ratio (Femino *et al.*, 1998). We have successfully used a variation of smFISH, based on a system of high signal amplification (F. Wang *et al.*, 2012), to detect and quantify *p62/SQSTM1* mRNAs in SH-SY5Y cells as described in sections II **“Antagonist effects of TARDBP modulation on key autophagy genes in SH-SY5Y cells”** of **Results and Discussion**.

The technique is however restricted to fixed tissues which can lead to biochemical alterations and cannot reveal the subcellular dynamic of a target transcript in time through live-imaging. Several strategies relying on different biomolecular systems have been investigated to solve this problem. We have selected two of the techniques that appeared the most promising: the MS2 system and a modified CRISPR/Cas9 system (George *et al.*, 2018).

The MS2 system comes from the bacteriophage and takes advantage of a simple interaction. The MS2 coat protein (MCP) has high binding affinity towards unique bacteriophage RNA hairpin structures also called MBS (MCP binding site) (Peabody, 1993; Stockley *et al.*, 2016). In order to detect a specific RNA sequence in another organism, the MCP protein is fused to a fluorescent protein such as GFP, and the target RNA has to integrate several MBS sequences. Moreover, the MCP protein is tagged with a nuclear localization signal

(NLS) that confines it to the nucleus until it binds a target nucleic acid and can be detected in the cytoplasm. This ensures that visualization of MCP-GFP corresponds to an MCP/MBS complex and not to an MCP that did not bind the RNA sequence of interest. In other words, the system requires the transfection of both an exogenous target RNA/MBS sequence and a fusion MCP-GFP protein (**Fig i 27**).

The system has first been used in yeast (Sheth and Parker, 2003) but was then adapted to *Drosophila* (Forrest and Gavis, 2003), cell culture (with noteworthy ALS-related studies on preferential recruitment of certain RNAs to granules containing mutant FUS) (Sama *et al.*, 2013; Yasuda *et al.*, 2013) and more recently to zebrafish (Campbell *et al.*, 2015; Sun *et al.*, 2018). Our strategy to develop the system in zebrafish is based on this last study by Campbell *et al.* 2015.

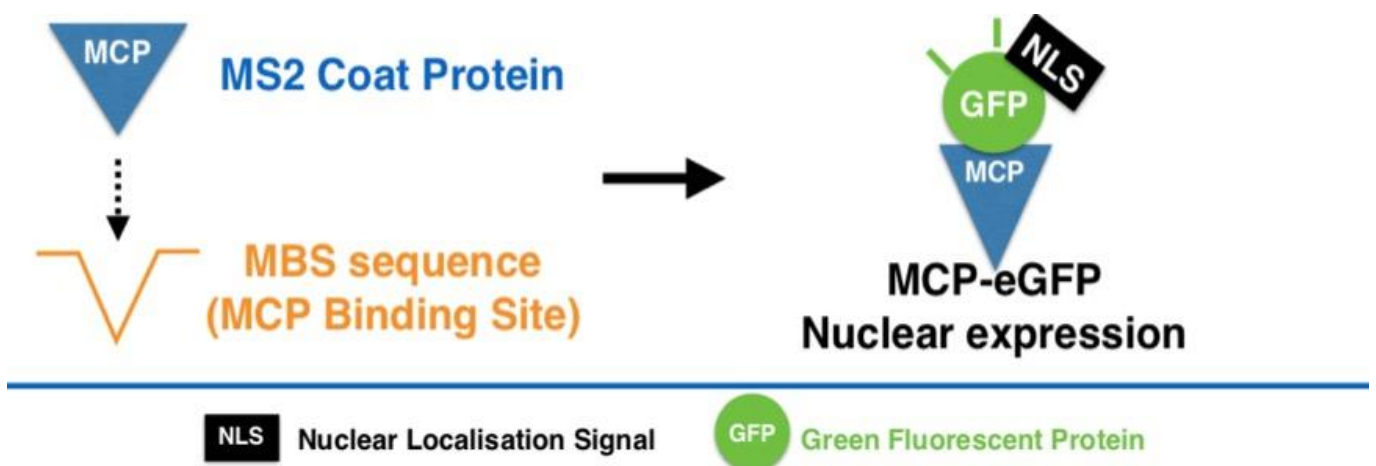


Figure i 27: Principle of the RNA tracking MS2 system derived from the bacteriophage

The other technique in development is based on the CRISPR/Cas9 system. As a reminder, the original high precision genome editing system is based on the cleavage of genomic DNA by the Cas9 enzyme on two conditions: 1) its recruitment at a precise locus by

a single guide RNA (sgRNA) that is complementary to the target strand; 2) the presence of a PAM (protospacer adjacent motif) on the opposite non-target strand (Doudna and Charpentier, 2014). In this modified RNA tracking system, the Cas9 is unable to cleave the target RNA as it is enzymatically inactive. This deadCas9 (dCas9) still binds to the transcript of interest through its recruitment by an sgRNA. However, because RNA is single-stranded, the PAM is now integrated into another complementary oligonucleotide called PAMmer (Nelles *et al.*, 2016). The dCas9 associates with the sgRNA and PAMmer in a complex with the targeted RNA with high affinity. In a similar manner to the MS2 system, dCas9 is fused to a fluorescent protein, for detection, and an NLS, for sequestration into the nucleus until it binds to a transcript and is transported (**Fig i 28**).

Contrarily to MS2, the RNA tracking CRISPR/Cas9 system has not been used in other models than live cell culture (Nelles *et al.*, 2016). Furthermore, the design of an adequate PAMmer can be complex. However, this strategy presents the advantage of targeting endogenous transcripts (George *et al.*, 2018).

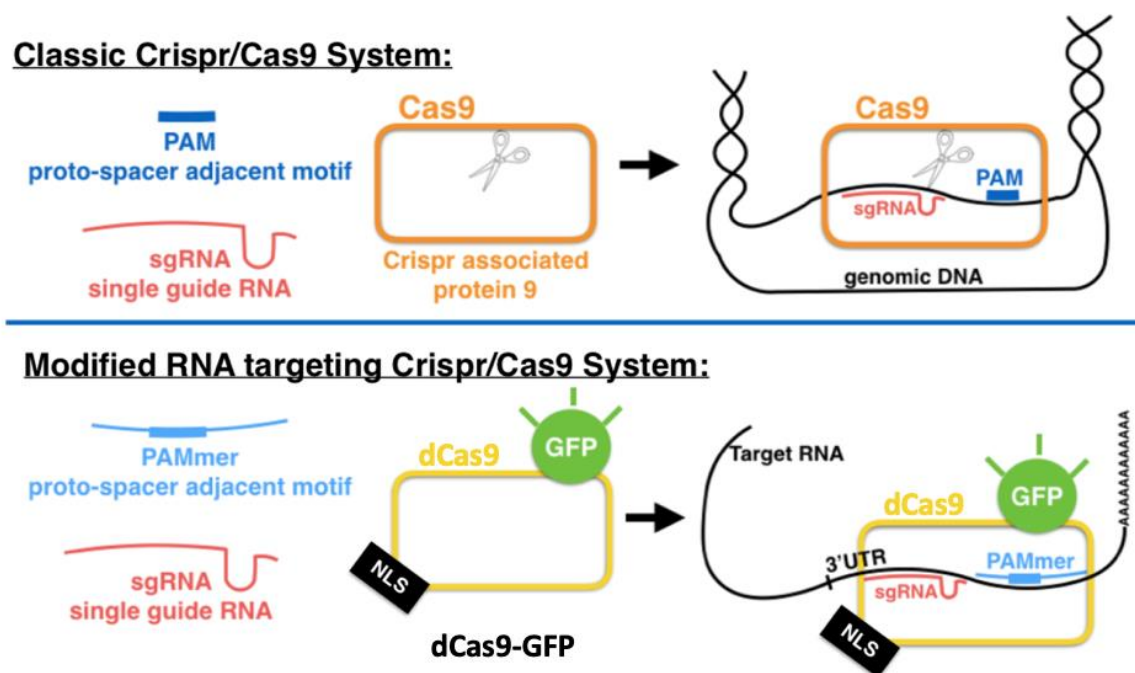


Figure i 28: Principle of the RNA tracking CRISPR/Cas9 system

B) Expression of MCP-GFP and RNA MBS sequences in the zebrafish embryo

Our strategy is based on the generation of two stable transgenic zebrafish lines expressing the MCP-GFP (**Fig 9 A**) through Tol2-mediated transgenesis. One expressing the protein under the *βactin* promoter while the other uses the inducible *Hsp70* promoter. Tg(*Hsp70:MCP-GFP*) fish do not express MCP unless a 1 hour 37°C heat shock is performed. The embryos however express GFP in their lens as a reporter of transgenesis (white arrow of **Fig 9 B**), that can be easily detected in 72hpf fish. On the other hand, the *βactin:MCP-GFP* line expresses the bacteriophage protein in a ubiquitous manner as soon as 24hpf (**Fig 9 B**). Indeed, MCP-GFP is strongly expressed and is visible in all regions of the animal as seen in selected images of expression of the fusion protein in somitic muscles, midbrain and forebrain (**Fig 9 C**). A more detailed observation of the expression pattern of Tg(*βactin:MCP-GFP*) fish show that the MCP protein appears to be exclusively nuclear, as expected. However, MCP-GFP is not distributed homogeneously within the nucleus. Round structures display higher signal intensity (white arrows of **Fig 9 D**). This particular trait was not evoked in the study of reference from which the constructs were obtained (**Fig 9 D**).

The *βactin:MCP-GFP* line was chosen to go further in the development of the MS2 system as we attempt to express RNA MBS sequences. More precisely, our construct is aimed at expressing an RNA mCherry sequence that is flanked with 24 repeats of MBS (*mCherry-24xMBS*) in order to allow identification of cells expressing the transcript and optimize binding of the MCP protein (**Fig 9 A**). Overall, several techniques of transient expression were tested including one-cell stage injections of circular plasmid, linear plasmid or *in-vitro* synthesized RNA containing the construct. The yield of transfection of these methods revealed to be rather

low as few cells display mCherry fluorescence. However, injection of the full circular plasmid was the most efficient method. Confocal microscopy on live 24hpf embryos reveal that *mCherry-24xMBS* can be expressed in various type of cells including somitic muscle cells, epidermal cells (respectively the elongated rectangular cell and the round cells indicated by white arrows in upper panels of **Fig 9 E**) and neuronal cells (white arrow of the lower panels of **Fig 9 E**). Based on its location and morphology, this neuron that presents long longitudinal projections along the animal may be a Lateralis afferent neuron which is known to project its axon dorsally to Mauthner cells in the hindbrain. Interestingly, these mCherry expressing cells display MCP-GFP in their cytoplasm (better seen at blue and red arrows of upper panels of **Fig 9 E**). This export of MCP-GFP outside the nucleus indicate the successful formation of a complex with RNA *MBS* repeats that is able to be transported and detected throughout the cell.

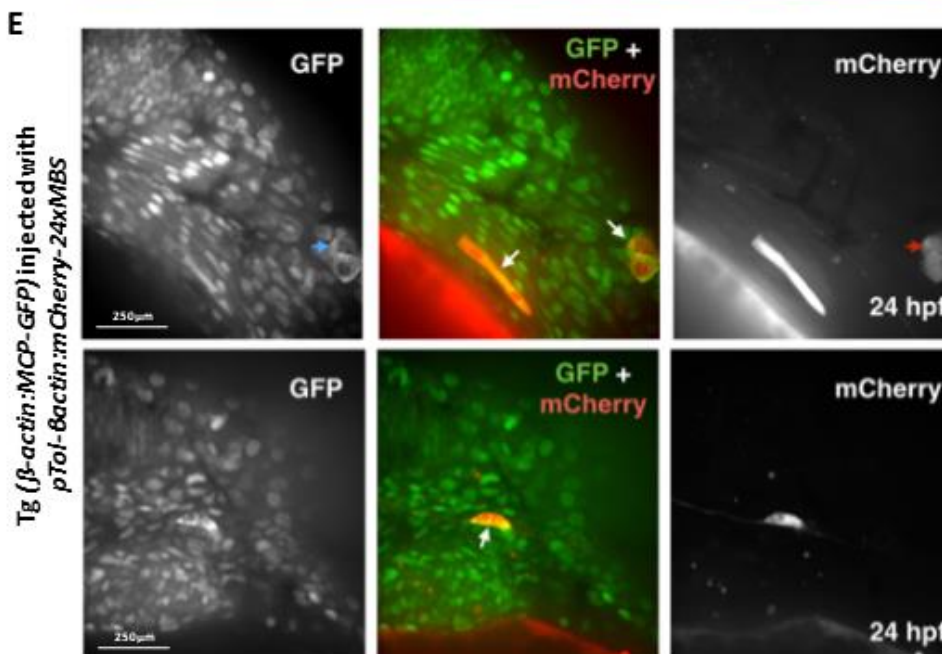
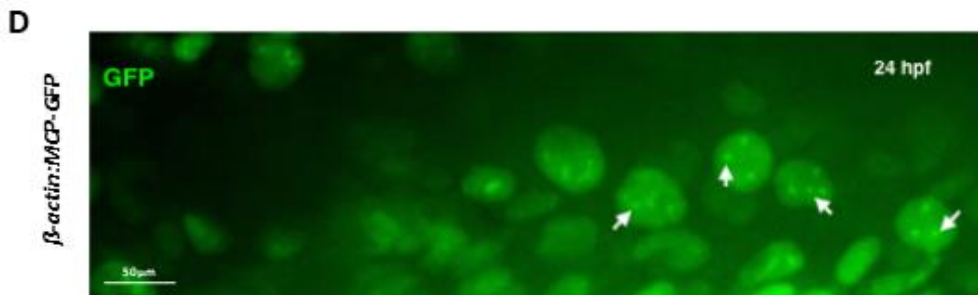
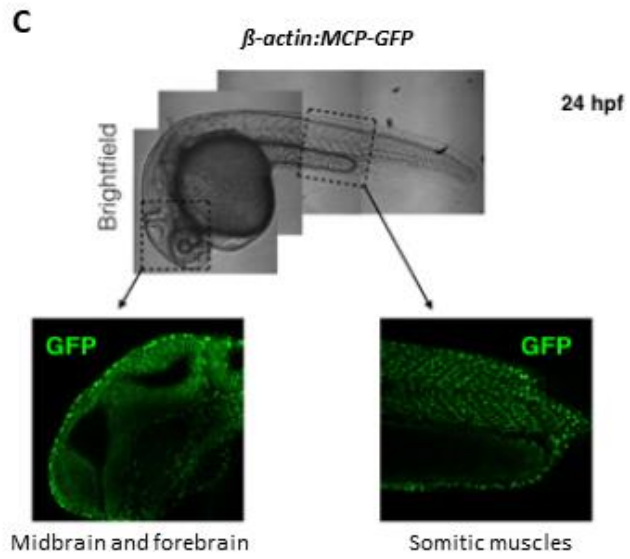
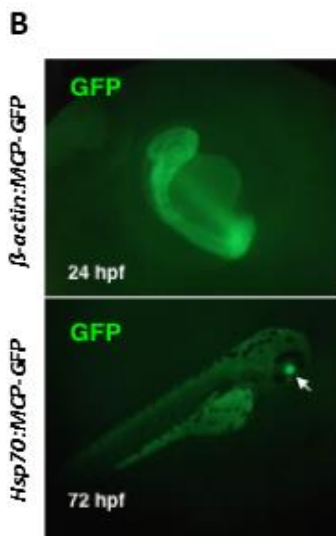
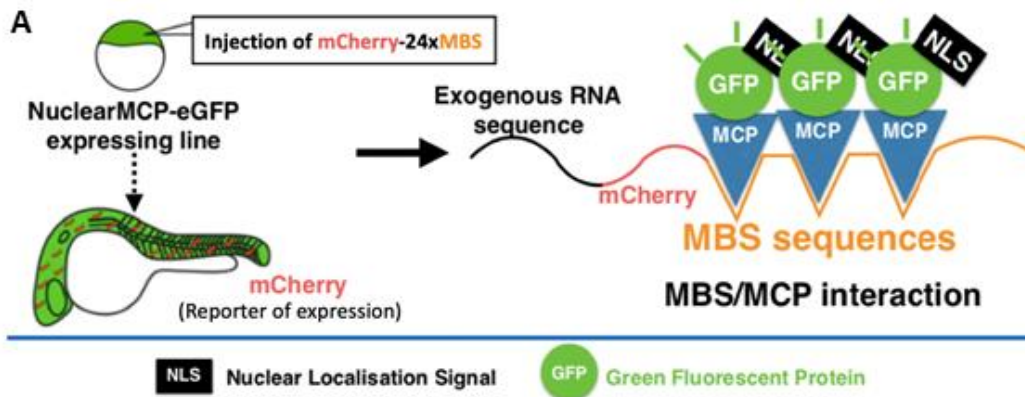


Figure 9: Expression of MCP-GFP and MBS RNA sequences from the MS2 system in the zebrafish embryo. Pattern of expression of GFP as seen **(A)** at the macroscopic and **(B and C)** microscopic levels in β -actin:MCP-GFP and Hsp70:MCP-GFP zebrafish transgenic lines. Tg(Hsp70:MCP-GFP) does not express the MCP-GFP protein in the absence of heat-shock but displays its fluorescent transgene reporter in the lens of the embryo at 72hpf (white arrow of **A**), as expected. Tg(β -actin:MCP-GFP) shows strong, ubiquitous expression of MCP-GFP as early as 24hpf that appear strictly nuclear and particularly stronger in acute points within the nucleus (white arrows). **(D)** Confocal microscopy performed on live 24hpf Tg(β -actin:MCP-GFP) embryos injected with *pTol- β actin:mCherry-24xMBS* at the one-cell stage. The transient expression of MBS RNA sequences is visible in MCP-GFP expressing cells of various subtype and morphology (white arrows) and appear to trigger delocalization of MCP-GFP in the cytoplasm (blue and red arrows) thus indicating formation of the MCP/MBS complex that can migrate in the cytoplasm.

C) Expression of dCas9-GFP in SH-SY5Y cells

Concerning the development of the RNA tracking CRISPR/Cas9 system, the *pCS2 deadCas9-GFP* plasmid was transfected in SH-SY5Y cells for transient expression of the fusion dCas9-GFP protein. In live-imaging, transfected cells express the protein in their nucleus as expected. However, similarly to the MCP-GFP protein in the zebrafish, the pattern is not homogeneous within nuclei as cells present round structures of stronger intensity (red arrow of **Fig 10**). These structures are interestingly inversely complementary to the staining pattern obtained with DAPI. This suggests dCas9-GFP is predominantly located at nucleoli. The selected image also shows the presence of a cell presenting an unexpected localization of dCas9-GFP in the cytoplasm (white arrow of **Fig 10**). Such phenomenon was rare among transfected cells but could lead to misinterpretation in the next steps towards RNA tracking. Overall, the yield of transfection was low and was not ameliorated through transfection of *in-vitro* synthesized RNA of the dCas9-GFP construct.

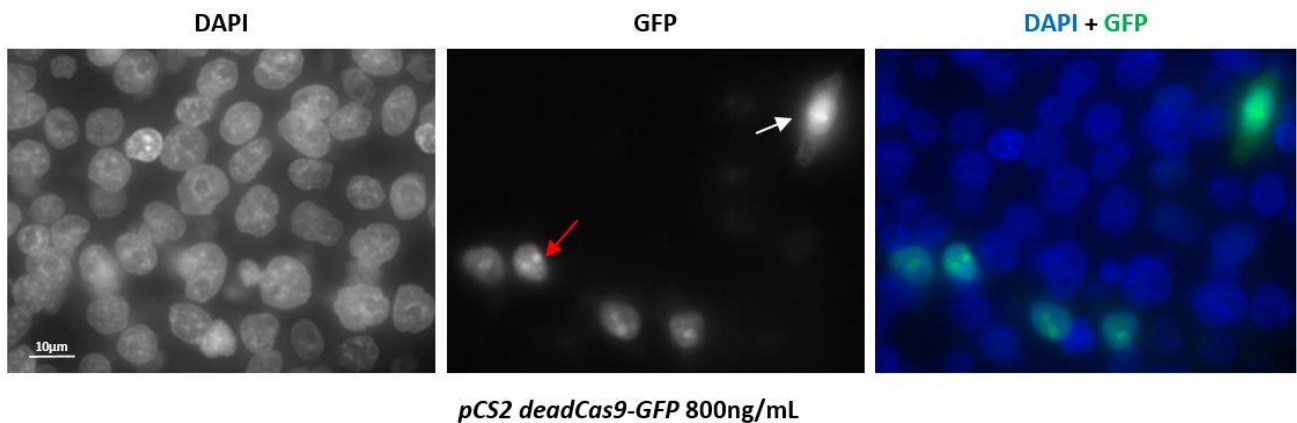


Figure 10: Expression of deadCas9-GFP from the RNA Crispr/Cas9 system in SH-SY5Y cells. (A) Fluorescent microscopy performed on SH-SY5Y cells transfected with *pCS2 deadCas9-GFP* revealing transient expression of the deadCas9 protein in the nucleus, as expected, but also acute point of stronger intensity within the nucleus (red arrow) and occasional abnormal cytoplasmic GFP signal (white arrow).

D) Discussion

We were successfully able to express MCP-GFP and RNA *MBS* sequences in the zebrafish and dCas9-GFP in SH-SY5Y cells on our way to developing the two respective RNA-tracking systems MS2 and CRISPR/Cas9. Two promising strategies that could help us elucidate RNA metabolism defects in the context of ALS.

Concerning the MS2 system in the zebrafish, while expression of MCP-GFP in our stable transgenic lines was successful, the yield of transfection of MBS repeats was unsatisfactory and may compromise tracking of RNA targets into cells of great relevance for ALS such as motor neurons. Indeed, the random expression of the two functional components of the MS2 system into such cells seems unlikely concerning their reduced number. The Tol2 gateway-

compatible system could be used in order to ameliorate transfection efficiency of *MBS* repeats.

The development of the RNA tracking CRISPR/Cas9 was also attempted in the zebrafish. The dCas9-GFP construct was integrated into another vector that used the Gal4/UAS expression system. Expression of dCas9-GFP in embryos was not achieved as injection of the plasmid caused unwanted toxicity. Such a strategy could allow us to express dCas9-GFP in a specific cell population like motor-neurons. In SH-SY5Y cells, transfection should be optimized as few cells expressed dCas9-GFP. Furthermore, the occasional aberrant cytoplasmic localization of the protein could be greatly problematic for the tracking of RNAs. One could also argue that such undifferentiated cells might be less relevant than cells presenting more complex morphological features like differentiated neurons, in which precise subcellular localization may be of greater interest. By comparing dCas9-GFP localization to DAPI staining, and as MCP-GFP presented similar heterogeneous localization in nuclei, we hypothesize that both proteins are preferentially located in nucleoli which is where ribosomal RNAs are transcribed. Such a pattern was not reported in neither of the studies from which our systems are based.

Both RNA tracking systems present technical limitations that may interfere with the specific requirements of our projects. It is important to note that neither strategies allow localization of target RNAs within the nucleus. Yet, TDP-43 is known to be particularly located and participating in RNA-metabolism in the nucleus. Furthermore, depending on the targeted regions of the RNAs of interest, the formation of either RNA tracking complex may compete and disturb the interaction with endogenous RNA-binding protein such as TDP-43. For the *MS2*, one could argue the introduction of an exogenous RNA sequence may exhibit unwanted

effects or even fail to mimic transport of the endogenous target. Finally, the design of adequate exogenous RNA sequences for the MS2 system and of PAMmers for the CRISPR/Cas9 system is complex and requires scrupulous testing and validation through comparison of RNA localization with *in-situ* techniques.

Nevertheless, tracking RNAs in live tissues and analyzing their dynamic in time through the development of these systems appears greatly promising. Of note, updated version of these systems or alternative strategies should be explored. For example, an alternative Cas13a-based system (that do not require PAMmers but solely sgRNAs) has recently been reported (Abudayyeh *et al.*, 2017). Our team has also explored an *in-vivo* compatible RNA-labeling technique that uses anti-sense probes called “molecular-beacons” (Catrina, Marras and Bratu, 2012; Zhao *et al.*, 2016). This long-established but recently optimized system presents the advantage of not requiring any transfection. However, designing a set of probes that guaranties specificity for the autophagy transcripts of interest appears challenging for the moment. Future updates of the probe-design algorithms may solve this issue in the near future.

E) Material and method

Zebrafish Maintenance:

Adult and larval zebrafish (*Danio rerio*) were maintained both at the ICM (Institut du Cerveau et de la Moelle épinière, Paris) and Institut Imagine (Paris) fish facilities and bred according to the National and European Guidelines for Animal Welfare. Experiments were performed on wild-type fish from AB and TL strains.

MCP stable transgenic lines:

Plasmid transfection was performed using the Tol2 Transposase system via microinjection in wild-type zebrafish embryos at the one-cell stage using a PV 820 Pneumatic PicoPump injector (World Precision Instruments, USA) and heat-pulled borosilicate glass needles.

Both *pTol- β -actin:NLS-tdMCP-eGFP* and *pTol-Hsp70:NLS-tdMCP-eGFP* plasmids (gifted by Dr. Marlow; Albert Einstein College of Medicine, USA) were linearized through AgeI digestion and coinjected with Tol2 Transposase RNA (synthesized from Tol2 transposase kit optimized for zebrafish) at a final concentration of 25ng/ μ l each.

Final injection mixes were prepared by diluting plasmid and RNA in Injection Buffer (*KCl 120Mm, Hepes 20Mm, pH 7,2 in Milli-Q water*) complemented with Fast green dye (Sigma-Aldrich, USA) at a final dilution of 0,05%.

Injected embryos were then raised at 28°C in an embryo medium called “blue water” (*0.06 g/L aquarium salt (Instant Ocean, Blacksburg, VA) in Milli-Q water +0.01 mg/L methylene blue*). GFP-expressing embryos were selected at 5dpf and raised to generate founders.

Transient expression of MBS RNA sequences:

mCherry-24xMBS RNA sequences have been expressed by direct microinjection of the *pTol- β -actin:mCherry-24xMBS* plasmid (gifted by Dr. Marlow; Albert Einstein College of Medicine, USA) in *β -actin:NLS-tdMCP-eGFP* zebrafish embryos at the one-cell stage using a PV 820

Pneumatic PicoPump injector (World Precision Instruments, USA) and heat-pulled borosilicate glass needles. Final plasmid concentration of 50ng/ μ l was obtained by dilution in Injection Buffer.

Live-imaging of zebrafish embryos:

For gross detection of fluorescence expression, 24hpf β -actin:NLS-tdMCP-eGFP or 72hpf *HSP70*:NLS-tdMCP-eGFP embryos were anesthetized in Blue water supplemented with Tricaine (0,16 mg/mL) and pictures were taken directly in the medium under a fluorescent stereomicroscope (Zeiss, Germany) coupled with a Grasshopper 2 digital camera (Point Grey Research).

Expression of fluorescence at the cellular level was visualized in 24hpf β -actin:NLS-tdMCP-eGFP zebrafish embryos using Leica SP8 STED system and both PMT and Hybrid detectors (Leica, Germany) through 10X, 20X or 40X HC PL APO objectives from Leica. Images were taken with LAS X software from Leica. Prior to confocal imaging, embryos that appear morphologically normal were anesthetized in blue water with 1X Tricaine and embedded in agarose (1% UltraPure LMP Agarose (Invitrogen, USA) in "blue water" supplemented with tricaine 25X) laying on their flank in a glass bottomed imaging dish.

All pictures were analysed using ImageJ software (Sun Microsystems, USA).

Cell culture and transfection:

The human cells SH-SY5Y were grown in DMEM Media-GlutaMAX™ (Dulbecco's Modified Eagle medium; Gibco®, Life technologies Paisley, UK) supplemented with 10% fetal bovine serum (FBS; SIGMA, USA) and 1% of penicillin/streptomycin solution (10.000U/mL;pen/strep;

Gibco). Cells were seeded at an approximate density of 3×10^5 cells in COSTAR 12 well Cell Culture Plate (3512, Corning, USA) and transfected the next morning with *pCS2 deadCas9-GFP* or control *pCS2+ GFP* plasmids at a final concentration of 800ng/mL using Lipofectamine™ 2000 (Invitrogen™, Life technologies Paisley, UK) according to manufacturer's instructions.

To obtain the *pCS2 deadCas9-GFP* plasmid, double digestion of *5UAS:deadCas9-GFP* vector (synthesized on command by GenScript, USA) by EcoRI/XhoI was performed and loaded in an electrophoresis gel in order to recuperate the deadCas9-GFP construct through band selection and DNA purification using QIAquick gel extraction kit (28 706; Qiagen, Germany) according to manufacturer's instructions. The construct was ligated into EcoRI/XhoI digested host *pCS2+ empty* vector using T4 DNA Ligase (M0202S; New England BioLabs, USA) according to manufacturer's instructions.

Transfection mixes are made in Opti-MeM Reduced Serum Medium (ThermoFischer Scientific, USA) and added to DMEM Media-GlutaMAX™, 10% FBS to reach desired final concentrations. Transfection medium is left to incubate cells for 6 hours and changed back to DMEM Media-GlutaMAX™, 10% FBS, 1% pen/strep.

Live-imaging of SH-SY5Y cells:

24 hours after transfection, medium was changed for 1X PBS and cells are visualized directly into their 12 well Cell Culture Plate using an inverted Apotome.2 system and an Imager.M2 stand (Carl Zeiss, Germany) via a 40X objective from Zeiss. Images were taken using ZEN software from Zeiss and analysed using ImageJ software (Sun Microsystems, USA).

IV) Conclusion and future directions

As we conclude this thesis, it appears important to put in perspective the results obtained throughout the different projects that were presented. Associating the studies conducted on the zebrafish and SH-SY5Y cells highlights key conclusions strengthening TDP-43's interaction with autophagy.

First, I characterized a novel link between ALS-associated TDP-43 and TBK1. Indeed, TDP-43 could bind to *TBK1* mRNA in cell lines. By extent, this could justify the fact that depletion of TDP-43 caused a downregulation of *tbk1*, an event that mediated the appearance of motor deficits and axonopathy in our zebrafish model. This data corroborates with the fact that TBK1 loss-of-function was reported to be sufficient to cause ALS and ties it with TDP-43. The second conclusion of this thesis is that TDP-43 may conditionally bind to its RNA targets depending on cellular context. More specifically, Torin 1-induced activation of autophagy inhibited binding to known RNA targets *RAPTOR* and *OPTN* but also promoted binding to novel targets *p62/SQSTM1* and *TBK1*. Finally, TDP-43 could impact autophagy in opposite manners depending on the cellular context. Indeed, I described that drug-induced modulation of autophagy lead to antagonist effects of TDP-43-depletion on the profile of expression of autophagy genes and autophagosome markers. Results were less conclusive when overexpressing *TARDBP* but a potential comparable impact on autophagy appears worthy of investigation. I hypothesize that these complex mechanisms could be due to the combination of several phenomena: 1) TDP-43 antagonist impacts are the result of its adaptive RNA-binding

function to autophagic context (as illustrated by our description of TDP-43 conditional binding to autophagy transcripts); 2) Truncated and mislocalized TDP-43 induce autophagy and, by extent, force TDP-43 RNA-binding function to adapt to the context; 3) Modulation of TDP-43 causes altered TDP-43 RNA-binding function notably through the appearance of ALS-related TDP-43 pathological traits in both our knockdown (nuclear depletion) or overexpression (truncation and mislocalization) models.

These assumptions could be verified by determining: 1) the subcellular localization for the binding of TDP-43 to key autophagy transcripts; 2) the outcome of TDP-43 modulation on those specific RNAs; 3) the consequences of emulated TDP-43 pathologic traits on the autophagic context and loss of RNA-binding function. In this context, the development of RNA tracking strategies that we detailed in this thesis could help tackle these questions. Meanwhile, more traditional RNA studies using RT-qPCR, smFISH and nuclear RNA immunoprecipitation experiments as well as a better characterization of autophagic context are already under way in order to find the final piece of the puzzle.

Besides highlighting novel aspects of TDP-43's interaction with autophagy, this study more globally unveils a new aspect of TDP-43 role in normal physiology and pathology. Pinpointing that TDP-43 function, and more specifically its interactome, may adapt to cellular context, puts in perspective the vast amount of data the community has collected on the protein. More than ever, the relevance of the model we use to the pathology of ALS should be questioned. For example, TDP-43-depletion without any modulation of autophagy in our cellular model, which was in line with the results obtained in recent studies on similar cells, presented data that was in total contradiction with our zebrafish model (Bose, Huang and

Shen, 2011; Xia *et al.*, 2016; Torres *et al.*, 2018). This raises the question of the relevance of these models to ALS pathology. Of note, as both zebrafish embryos and Torin 1 treated cells displayed a similar profile of expression of autophagy genes, the differences are likely to be explained by the fact that embryos display important basal autophagy levels. Autophagy is indeed crucial for the development. More than the model of the study, the relevance of the physiological or pathological context within each model has to be examined. This foreshadows a complexification of the already convoluted field of research around TDP-43. Up to now, reproducing all of TDP-43 pathological traits, that are present in the vast majority of ALS patients, has been more than challenging in animal or cellular models. A better understanding of the importance of the context in which TDP-43 proteinopathy is being introduced could potentially help with the matter. It appears crucial to bear in mind that ALS is a late-onset pathology of highly specialized cells that are motor neurons. Finally, as the disease is defined by a heterogeneous clinical presentation, understanding the potential plasticity of TDP-43 function could be insightful.

These results concerning TDP-43 could also fit into other areas of research. Indeed, TBK1, p62/SQSTM1 and OPTN are also involved in the inflammatory pathway (Mankouri *et al.*, 2010; Gleason *et al.*, 2011; Komatsu, Kageyama and Ichimura, 2012a; Rea *et al.*, 2013; Bitto *et al.*, 2014; Weil, Laplantine and Génin, 2016), another investigated mechanism in neurodegeneration and ALS (Corcia *et al.*, 2013; Brites and Vaz, 2014; Endo, Komine and Yamanaka, 2016). Moreover, these molecular actors are implicated in tumorigenesis (Trocoli and Djavaheri-Mergny, 2011; Komatsu, Kageyama and Ichimura, 2012b; Moscat, Karin and Diaz-Meco, 2016). This thesis reports that TDP-43 is able to conditionally bind to *RAPTOR* or *TBK1* transcripts, which interaction at the protein level is of great relevance in cancer research

(Vaden *et al.*, 2017; Bodur *et al.*, 2018). Therefore, exploring the incidence of these novel interactions that implicate TDP-43 to such pathological mechanisms could be instructive.

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