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Defining the functional role of TBK1 using a novel zebrafish model in the context of amyotrophic lateral sclerosis

Grégoire Haouy

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PHD THESIS – SORBONNE UNIVERSITE
DOCTORAL SCHOOL « CERVEAU COGNITION COMPORTEMENT »



Defining the functional role of TBK1 using a novel zebrafish model in the context of amyotrophic lateral sclerosis.

Grégoire Haouy

A thesis submitted in partial fulfilment of the requirements for the PhD degree

Under the supervision of Doctor Edor Kabashi
“Translational research for neurological disorders”

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Summary

La sclérose latérale amyotrophique (SLA) est une pathologie caractérisée par une perte progressive des neurones moteurs. Ces neurones spécialisés conduisent l'information du cerveau vers les muscles permettant ainsi les mouvements. La perte de ces cellules induit donc une paralysie musculaire conduisant au décès des patients par défaillance respiratoire. A ce jour, aucun traitement efficace permettant le rétablissement des patients n'a été développé. Parmi les facteurs de risques génétiques associés au développement de la SLA chez les patients, ont été identifiées des mutations dans la séquence du gène *TBK1*. Le travail présenté ici est basé sur le développement d'un modèle d'étude chez le poisson zèbre associant une perte d'activité de la protéine Tbk1 avec les différentes marques cellulaires de la SLA. Ce travail a donc pour but d'identifier de nouvelles cibles thérapeutiques et par conséquent d'amener vers le développement de nouveaux traitements améliorant le pronostic des patients.

Amyotrophic lateral sclerosis (ALS) is a pathology characterized by the progressive loss of motor neurons. These specialized neurons conduct the information from the brain to the muscles in order to induce the movements. The loss of these cells therefore induces a muscular paralysis leading to patients' decease via respiratory failure. At this time, no treatments allowing patients recovering have been developed. Mutations in the sequence of the *tbk1* gene have been identified as risk factors in the development of ALS. The work presented in the following thesis is based on the development of a zebrafish model of study recapitulating the different cellular hallmarks of ALS associated to the loss of activity of TBK1 protein. This work is therefore aiming to identify new therapeutic target and consequently developed new treatments ameliorating patients' prognostic.

Abstract

La sclérose latérale amyotrophique (SLA) est une pathologie caractérisée par une neurodégénérescence progressive des neurones moteurs (NM) corticaux et spinaux induisant une paralysie musculaire conduisant au décès des patients par défaillance respiratoire. *TBK1* a été associé pour la première fois à la SLA en 2015. Etroitement lié à l'autophagie et à l'inflammation, deux mécanismes cellulaires associés à la pathophysiologie de la SLA, il est important de comprendre le rôle fonctionnel de *TBK1* dans le développement de cette pathologie. Due à sa ressemblance anatomique et neurochimique avec le système nerveux humain et à l'homologie élevée avec son orthologue *TBK1* humain, le poisson zèbre est aujourd'hui considéré comme un des meilleurs modèles pour l'étude de la SLA associée à *TBK1*. Afin d'identifier les mécanismes sous-jacents à la perte d'expression de *TBK1* dans la dégénérescence des NM *in vivo*, nous avons généré deux nouveaux modèles de perte d'expression de *tbk1* chez le poisson zèbre en utilisant deux approches : la réduction de l'expression via morpholinos (perte partielle d'expression – KD) et l'induction de mutations via la technologie CRISPR (perte totale d'expression – KO). Dans les 2 modèles nous avons identifié une diminution de la mobilité liée à la perte d'expression de *tbk1*, cependant le stade de développement du phénotype observé est différent en fonction du modèle : 2 jours post fécondation (jpf) dans le modèle KD et 5 jpf dans le modèle KO. De plus dans le modèle KD, la perte de capacité motrice est associée à une diminution dans le nombre de NM spinaux. Cette observation n'est pas reproduite dans le modèle KO et n'explique donc pas l'apparition d'un phénotype moteur. Enfin, à un stade plus avancé (9 jpf), nous avons observé une mortalité chez le modèle KO, mettant en évidence le rôle crucial de *TBK1* dans le développement du poisson zèbre. Afin d'identifier les dérèglements moléculaires responsables des phénotypes observés chez le modèle KO, nous avons effectué une analyse métabolique aux deux stades de développement d'intérêt (6 et 8 jpf). L'analyse métabolomique a révélé que la perte d'expression de *tbk1* était associée à une dérégulation du métabolisme du nicotinamide. En effet, l'incubation des larves avec un précurseur du NAD⁺ (métabolite central du métabolisme du nicotinamide) : le nicotinamide riboside (NR), a permis de restaurer la locomotion. Cette observation met en évidence que l'absence d'expression de *tbk1* chez le poisson zèbre altère les niveaux physiologiques de NAD⁺ ce qui induit à une plus grande échelle une perte de mobilité. Cependant l'incubation avec le NR n'a eu aucun impact sur la survie des larves. J'ai donc cherché quelle pourrait être la cause de la mort de ces larves en combinant analyses protéomique et métabolomique. Ces

données ont montré une augmentation de marqueurs inflammatoires ainsi qu'une modification de l'expression des protéines responsables de la régulation de la mort cellulaire active (MCA) (apoptose ou nécroptose). Par conséquent, nous avons cherché à identifier laquelle de la MCA ou de l'inflammation était la cause primaire de ce phénotype. Pour répondre à cette question, j'ai incubé les larves avec un inhibiteur de la neuroinflammation : l'acide caféique. Cependant, cette prévention de la réaction neuroinflammatoire n'a eu aucune conséquence sur la survie des larves. Bien que cela n'élimine pas totalement le rôle de l'inflammation, cela suggère que le facteur induisant la mortalité dans notre population de larves pourrait être l'activation d'une MCA. Un futur criblage moléculaire permettant de prouver cette hypothèse et d'identifier quel mécanisme de MCA est responsable de la mortalité observée sera effectué dans la lignée de cette étude. Pour autant, ce travail apporte une meilleure compréhension de la cascade d'évènements menant de la perte d'expression de TBK1 au développement de la SLA.

Amyotrophic lateral sclerosis (ALS) is a neurological disease characterized by the progressive degeneration of both spinal and cortical motor neurons that leads to muscle paralysis and the subsequent death by respiratory failure. *TBK1* mutations potentially leading to haploinsufficiency were first described in ALS patients in 2015. Tightly linked with autophagy and inflammation, two cellular mechanisms reported to be dysregulated in ALS patients, it remains of major interest to understand the functional role of TBK1 in the development of this neurological condition. Due to its high anatomical and neurochemical resemblance with the human brain and the important homology with its orthologous *TBK1* human gene, the zebrafish is today considered as an excellent vertebrate model suitable for the study of *tbk1*-related ALS. In order to identify the subjacent mechanisms linked with *tbk1* loss of function and the subsequent degeneration of motor neurons *in vivo*, we have generated and characterized two new zebrafish models of TBK1 loss of function using two approaches; the first was via antisense morpholino oligonucleotide to generate a zebrafish model with partial loss of expression referred to as knockdown (KD). The second approach was to induce genomic mutations via the utilization of the CRISPR technology and to generate deletion mutant with loss of expression referred as the *tbk1* knockout (KO) model. In these two models, I report an impaired locomotor activity associated with the loss of *tbk1* expression. However, the motor phenotype occurs at various stages of development according to the approach used: 2 days post fertilization (dpf) in the KD model and 5 dpf in the KO model. Moreover, we associated the impaired locomotion in the KD model with a decrease in the number of spinal motor neurons indicating neurodegenerative features in this model. On the other hand, we did not observe any increased mortality of motor neurons in the KO model but we reported a high lethality upon *tbk1* complete deletion at 9dpf suggesting a crucial role for Tbk1 in the zebrafish development. In order to identify the subjacent molecular dysregulations responsible for the phenotypes observed in the KO model, we performed a metabolomics analysis at the two developmental stages of interest (6 and 8 dpf). The metabolomics analysis unveiled an association between *tbk1* loss and a severe dysregulation of the nicotinamide metabolism. The incubation of the larvae with a precursor of NAD⁺ (central metabolite of the nicotinamide metabolism): the nicotinamide riboside, permitted to restore the locomotion defects in the *tbk1* KO larvae. This observation revealed that *tbk1* reduced expression provoke an alteration of the physiological level of NAD⁺ which could

correlate with the loss of mobility. However, NR incubation was not sufficient to delay or to prolong increased mortality observed in the *tbk1* KO larvae.

To determine the cause of increased mortality observed in the *tbk1* KO model at 9 dpf, we combined the metabolomic analysis with proteomics to unravel altered pathways associated with *tbk1* KO. Increased inflammation markers and dysregulation of two programmed cell death (PCD) mechanisms, namely the necroptosis and the apoptosis were observed. To separate between these two hypotheses, we incubated the larvae with an inhibitor of the neuroinflammation process: caffeic acid. Caffeic acid treatment failed to reduce mortality in the *tbk1* KO model, thus suggesting that neuroinflammation may not play a crucial role in increased mortality observed. This suggests that the activation of programmed cell death pathways could be mainly responsible for the lethality observed in our KO model. A future pharmacological approach to reduce necroptosis and apoptosis activation will allow us to support this hypothesis, and to further identify which of the cell death pathways was involved in the *tbk1* KO model; thus, increasing our understanding of the cascade of events leading from the loss of *tbk1* expression to the development of the ALS and propose therapeutic avenues for this neurological disease.

INTRODUCTION

In this introduction, I will review the recent literature in the field of Amyotrophic Lateral Sclerosis (ALS) and one of the genetic causes for this neurodegenerative disorder, *TBK1* on which this thesis will focus. First, I will introduce the clinical features of ALS before discussing about the cellular hallmarks of this pathology. I will also review major cellular and animal disease models and their importance to a better understanding of pathogenic mechanisms of ALS. Secondly, I will review the recent studies on TBK1 functional role and its involvement in the pathogenicity of ALS. Finally, as part of the present work will be focusing on the metabolomics characterization of the novel *tbk1* KO zebrafish model that I developed during my doctoral work, I will review the emerging field of metabolic alterations both in patients and in genetic models of ALS.

Part I: Introduction to ALS

I. History and description of the disease:

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of both the upper and the lower motor neurons (MN). This is translated at a macroscopic scale by the progressive denervation and the atrophy of the skeletal muscles (except oculomotor and sphincter motor neurons) leading from paresis to complete paralysis and eventually death within 3 to 5 years after diagnosis, predominantly through respiratory failures (A. Chio *et al.*, 2009).

The term ALS was first mentioned in 1874 by the French neurologist Jean-Martin Charcot (1825-1893). However, it is important to mention that this first description was made possible by the work of many scientists prior to Charcot:

- Charles Bell (1774-1842), a Scottish anatomist and physiologist, published in 1824 "*Exposition of the natural system of the nerves*" where he describes for the first time two distinct roots in the spinal cord: 1) the anterior roots involved in the motor abilities 2) the posterior roots involved in the sensory abilities. He is therefore the first to mention that neurological disease could be purely motor related.
- François Amilcar-Arran (1817-1861), a French doctor, is the first to describe ALS in 1850 without being able to distinguish it from other neurodegenerative disease associated with a loss of motor functions. However, according to Arran, this disease

was only related to an alteration of the muscular system, without any neuronal involvement (F.A. Aran, 1850).

- Jean Cruveilhier (1791-1874), a French doctor and anatomist, in 1852, after performing a series of autopsies on three patients, observed an atrophy of the anterior roots of the cervical region. He therefore proposes to switch Aran's paradigm of an exclusively muscle related disease to a model reflecting a primary alteration of the spinal cord's anterior roots leading to a secondary muscle atrophy (J. Cruveilhier, 1852-1853).
- Jean-Martin Charcot was the first to observe an association between lateral sclerosis and grey matter of the spinal horn in 1868 (J.M Charcot, 1872-1873). He proposed in 1872 to split the heterogeneous group of muscular atrophies into two sub-classes: 1) the 'true spinal' muscular atrophies caused by the loss of anterior horn cells (corresponding to the progressive muscular atrophies described by Aran and Cruveilhier); 2) the 'false spinal' muscular atrophies caused by a secondary loss of anterior horn cells associated with the degeneration of the lateral fasciculi of the spinal cord (i.e the ALS). It is in 1874 that Charcot formalized the description of ALS in his lecture. This discovery was made possible for Charcot through the introduction of the microscopic analysis in opposition to the previous macroscopic observations performed by its precursors.

In addition, it is important to mention that although Charcot's name will always be associated with the disease, his discovery was only made possible by the findings of his two pupils and close collaborators Charles Bouchard (1837-1915) and Albert Gombault (1844-1904) whose name have been forgotten.

II. Clinical features of ALS-FTD

A. Epidemiology

Quantifying the worldwide incidence of ALS is complicated as the environment and the genetic background are greatly impacting the risk of contracting the pathology (B. Marin *et al.*, 2016). Therefore, although it is now accepted that the global incidence of ALS is approximately 1 to 2 cases per 100 000 persons per year and the prevalence is of 4-6 per 100 000 persons (L. Xu *et al.*, 2020; E.O. Talbott *et al.*, 2016; B. Marin *et al.*, 2016), it is more

relevant to study ALS epidemiology in a specific sub-population. Moreover, this bias is accentuated by the overrepresentation of populations with a European genetic background in the epidemiology studies published (more than 75% are focusing on European or North American populations) (L. Xu *et al.*, 2020).

Several risk factors have been identified to explain the influence of geography on the heterogeneity of ALS. Interestingly, it seems that the incidence is highly linked to the wealth of the population (Fig. 1) (4.94 in the western world in opposition to 2.15 in developing territories) (L. Xu *et al.*, 2020). This is possibly explained by a higher life expectancy, ALS being a late onset pathology. Still in this direction, although the onset of the disease occurs prevalently during adulthood, the median age of onset is highly variable according to the populations (E. Longinetti and F. Fang, 2019). For instance, a comparative study between Chinese and German population have shown a 10 years difference in the median age of onset (51 years in China vs 61 years in Germany) (J. Dorst *et al.*, 2019). Moreover, the survival time to ALS is also highly different according to the population studied (24 months in Europe in opposite to 48 months in central Asia) (L. Xu *et al.*, 2020; B. Marin *et al.*, 2016). However, the age of onset has been showed to directly impact the prognosis of the patients (W-M. Su *et al.*, 2022). Altogether, these observations suggest that population comparative epidemiology studies will always suffer from the lack of relevance of comparing various population.

Another risk factor is gender, with males more likely to develop ALS (Fig1) (Z. R. Manjaly *et al.*, 2010; C. Ingre *et al.*, 2014) and exhibit both a higher incidence and prevalence (L. Xu *et al.*, 2020) with a male/female ratio located in between 1 and 2 (E. Longinetti and F. Fang, 2019; Z. R. Manjaly *et al.*, 2010). This sex ratio is inversely proportional to the age of onset (Z. R. Manjaly *et al.*, 2010). Therefore, it could be interesting to determine whether this ratio is increased in population characterized with an earlier onset of disease. In addition, it seems that gender could also impact the clinical subtypes of pathology, a study in a European population have reported a higher propensity for spinal-onset disease in male patients in opposition to female cases which are more associated with bulbar-onset pathologies (G. Logroscino *et al.*, 2010). Due to a lack of comprehension in the pathogenicity of ALS it is difficult to understand biologically the origin of this sex-related difference of incidence.

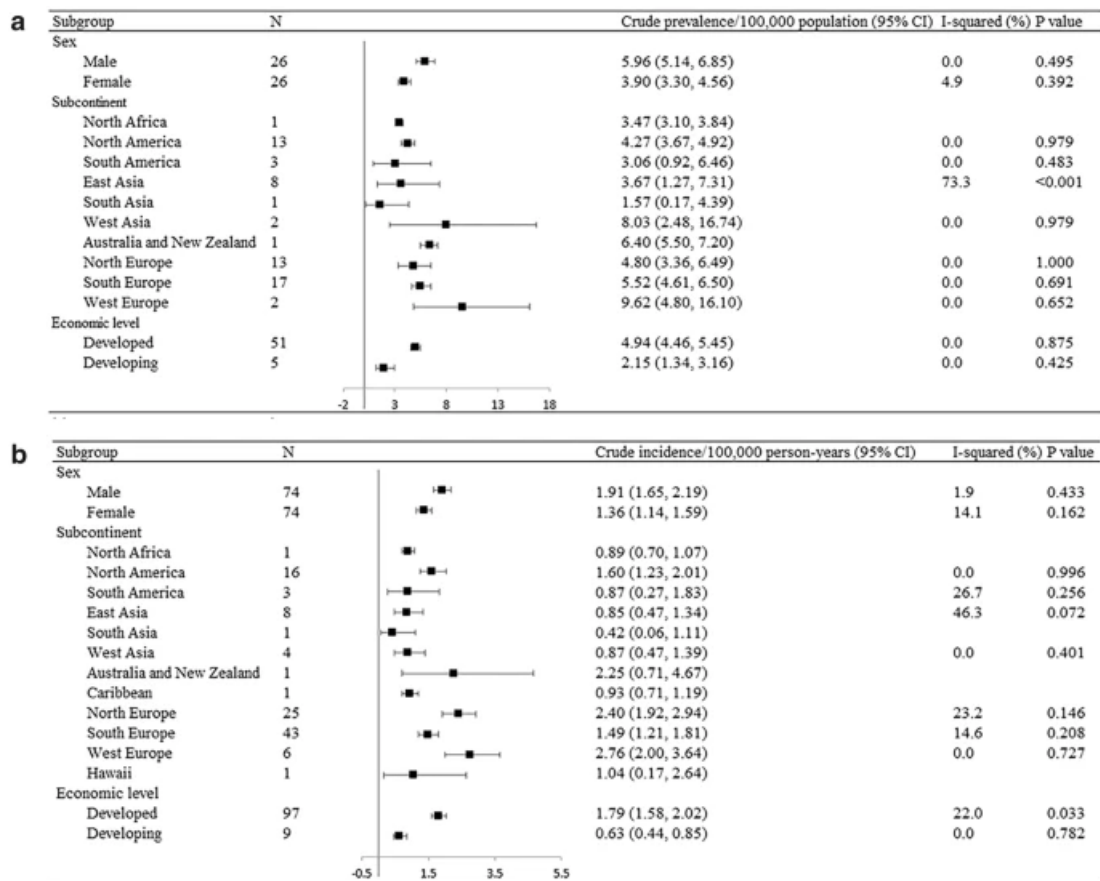


Figure 1: Illustration of the variation in the incidence and prevalence of ALS depending on sex, geographical population sets and wealth (derived from L. Xu et al., 2020)

B. Risk factors

1. Environmental risk factors

All these geographical/gender-based risk factors are even more difficult to elucidate as in most of the ALS cases (more than 80%), clinical features are thought to appear spontaneously within a generation without any first-degree relatives of the patients being affected. This feature leads to the creation of an over-simplified dichotomization of the pathology between the sporadic and the familial cases (which are hereditary passed through generations). The reality is different and ALS, similarly to other neurodegenerative diseases, is characterized by a multifactorial inheritance, thus triggered by a combination of dysregulated genes and environmental factors (O. Hardiman *et al.*, 2017). So far, 30 genes have been associated with a higher risk of contracting ALS but the real number is probably higher (S. Mathis *et al.*, 2019).

Several environmental factors have been associated with a higher risk of triggering the disease, further explaining the heterogeneity of the epidemiology. These risk factors are very diverse and can be linked to the lifestyle: dietary factors, exercise, body mass index, or smoking; to the professional occupation: exposition to metal or pesticides, electric shock or proximity with electromagnetic fields (B. Oskarsson *et al.*, 2015). Moreover, the risk of ALS was shown to be associated to specific health conditions related to viruses, metabolism alterations, or innate immune system dysregulation (A. Jawaid *et al.*, 2018; B. Oskarsson *et al.*, 2015). Interestingly, early life exposure to environmental cues has been reported to directly impact the innate immune response (M. Garand *et al.*, 2017; D.M. MacGillivray and T.R. Kollmann, 2014), further complexifying the role of environmental risks on the development of the disease.

Because investigating the impact of the risk factors on the development of the pathology is challenging and to this point there is no direct evidence linking an environmental trigger to ALS, the vast majority of the research has focused on the genetic part of the disease as discussed in the next section.

2. Genetic risk factors

Although most of the ALS cases appeared sporadically within a generation without a first degree relative being affected, approximately 10% of the cases are characterized by a familial history of the disease. As a partially hereditary disease, these cases are linked to one or several genomic mutations. Approximately 70% of the hereditary cases are associated with 4 genes out of the 30 identified: *Chromosome 9 open reading frame 72 (C9ORF72)*, *Superoxide dismutase 1 (SOD1)*, *Transactive response DNA-binding protein (TARDBP)* and *Fused in sarcoma (FUS)* (Fig 2) (A.M.G Ragagnin *et al.*, 2019; M. Ghasemi and R.H. Brown, 2018).

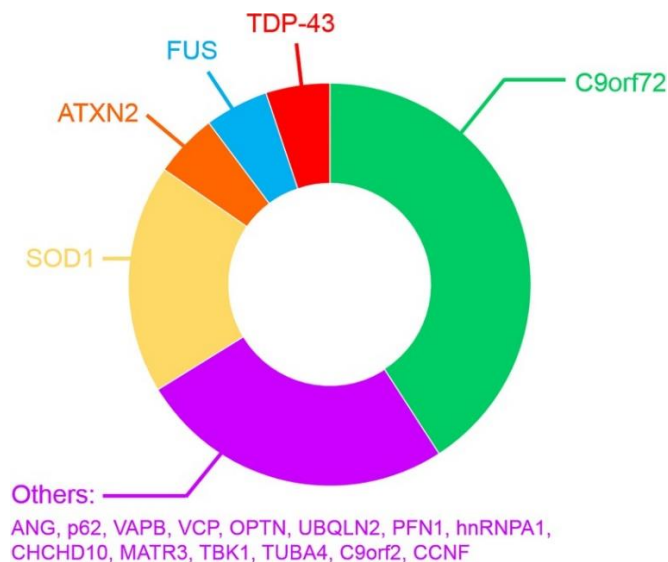


Figure 2: Circle chart illustrating the frequency of mutated genes in familial ALS patients (derived from A.M.G Ragagnin et al., 2019)

a. C9ORF72

In 2006, two studies were released identifying a locus on the chromosome 9 (*9p13.2–21.3*) in Scandinavian and Dutch set of patients as associated with the development of ALS (M. Morita *et al.*, 2006; C. Vance *et al.*, 2006). However, it is in only 5 years later, in 2011, that the presence of a massive GGGGCC hexanucleotide repeat expansion (G_4C_2) was identified within the first intron of the *C9ORF72* gene (M. Dejesus-Hernandez *et al.*, 2011; A.E. Renton *et al.*, 2011). Interestingly, healthy individuals present these G_4C_2 repeats, but at a lesser extend (under 30 copies) than patients with *C9ORF72* associated ALS that carry from 45 to several thousand G_4C_2 repeats (I. Gijselinck *et al.*, 2016). This discovery was a real breakthrough in the field of ALS, as it is the most frequent (around 30% of the cases) mutations found in ALS patients with a European genetic background (E. Majounie *et al.*, 2012). The role of *C9ORF72* G_4C_2 repeats in the development of *C9ORF72* related disease is still highly discussed as there is evidence of two deleterious consequences. First, this mutation induced a decrease in *C9ORF72* expression and the subsequent loss of function of its encoded protein (I. Gijselinck *et al.*, 2012). A decreased expression of *C9ORF72* has been associated with an impaired autophagic process and the presence of cytoplasmic aggregates (C.J. Donnelly *et al.*, 2013; C. Sellier *et al.*, 2016). This loss of function hypothesis is further confirmed in the zebrafish model where the reduced expression of the *C9ORF72* ortholog results in locomotor deficits (S. Ciura *et al.*, 2013). Secondly, the hexanucleotide repeats

were shown to undergo a non-AUG translation leading to the expression of dipeptide repeat proteins (DPR) which are forming cytoplasmic aggregates (K. Mori *et al.*, 2013). Interestingly, the accumulation of these DPRs does not appear to impact the efficiency of the autophagy or the proteasome (S. Leskela *et al.*, 2021). Therefore, the actual paradigm concerning *C9ORF72* associated disease due to expanded G₄C₂ hexanucleotide repeats is both a loss and a gain of function mutation, inducing the generation of cytoplasmic aggregates and preventing their clearance (Y. Shi *et al.*, 2018; A. Pal *et al.*, 2021; J. Smeyers *et al.*, 2021).

b. SOD1

Mutations in the *SOD1* gene were the first identified mutations associated with the development of ALS in 1993 (D.R. Rosen *et al.*, 1993). This discovery still remains as one of the biggest breakthroughs in the study of the ALS as it allowed the comprehension of many features of the disease through the design and generation of many *in vivo* model of the pathology. The murine model *SOD1*_{G93A} is still the most used in-vivo model in the ALS research field (M.E. Gurney *et al.*, 1994). Since 1993, more than 180 variants have been identified along the sequence of *SOD1* in the context of ALS, and *SOD1* mutations are still the most common known genetic cause of ALS in the Asian population (approximately 30%) and the second in the European genetic background population (Z-Y. Zou *et al.*, 2017). *SOD1* protein is involved in the clearance of deleterious reactive oxygen species (ROS) through their conversion into oxygen and hydrogen peroxide species (J. Azadmanesh and G.E.O. Borgstahl, 2018). Although *SOD1* mutations have been associated with oxidative stress (R.T. Ferrante *et al.*, 1997), the cascade of events linking *SOD1* mutations to MN degeneration is unclear. Large body of evidence indicates that it is not the actual loss of *SOD1* activity that is responsible for these alterations but rather a gain of toxicity induced by a conformational change in the protein and subsequent cytoplasmic aggregation (Y. Hayashi *et al.*, 2016). Evidences from *in vitro* and *in vivo* model support the hypothesis of a prion-like propagation mechanism in which a misfolded mutant or wild-type *SOD1* protein transmits its aberrant misfolding properties to native proteins inducing the formation of toxic cytoplasmic aggregates (C. Münch *et al.*, 2011; J.I. Ayers *et al.*, 2016; M. Polymeridou and D.W. Cleveland, 2011).

c. TARDBP

TARDBP mutations were first associated with ALS in 2008 (E. Kabashi *et al.*, 2008). *TARDBP* is the third most common known genomic mutation associated with ALS in European genetic background population with 4.2% of the cases concerned (and the fourth in the Asian population: 1.5% of the cases) (S. Mathis *et al.*, 2019). *TARDBP* encodes a nuclear ribonucleoprotein, TAR DNA binding protein 43 (TDP-43), which is involved in the regulation of RNA processing and gene transcription (T.J. Cohen *et al.*, 2011). Mutation in the *TARDBP* sequence is known to induce misfolding of TDP-43 inducing its cytoplasmic mislocalization and its aggregation (T. Arai *et al.*, 2006; M. Neumann *et al.*, 2006), which is the main pathological hallmark of ALS. Indeed, neuronal TDP-43 aggregates are found in approximately 97% of the cases (E.M. Scotter *et al.*, 2015), with these ubiquitinated inclusion bodies representing the most prevalent marker of the disease. However, TDP-43 involvement on the pathophysiology is not restricted to the aggregation of the misfolded proteins. Indeed, severe dysregulations in the RNA metabolism have been associated with the development of ALS. This includes: RNA mis-localization, altered splicing, and defect in transcription (S. Reber *et al.*, 2016; C. Lagier-Tourenne *et al.*, 2012; E.S. Arnold *et al.*, 2013; A. Ratti and E. Buratti, 2016). As TDP-43 is involved in the regulation of RNA processing and gene transcription (T.J. Cohen *et al.*, 2011), this suggests a two-step pathology induced by both the loss of function of TDP-43 in the nucleus and a toxic gain function in the cytoplasm through their aggregation. Moreover, the aggregated form of TDP43 is characterized by an abnormal phosphorylation and it has been proved that the TDP-43 phosphorylated form disseminates in a prion-like behaviour along the motor pathways similarly to what observed in SOD1 misfolded proteins (H. Braak *et al.*, 2013; E-M. Hock and M. Polymeridou, 2016).

d. FUS

FUS was first associated with ALS in 2009 (T.J. Kwiatkowski *et al.*, 2009; C. Vance *et al.*, 2009). *FUS* mutations are the fourth most common known genomic alterations associated with ALS in European genetic background population with around 2.8% of the ALS patients being concerned (and the third in the Asian population: 6.4% of the cases) (S. Mathis *et al.*, 2019). *FUS*, the encoded protein, is a nuclear RNA-binding protein involved in the RNA biogenesis and function (S. Dini Modigliani *et al.*, 2014), a transcription factor (L. Yang *et al.*, 2014), and is involved in the response to DNA damage (W-Y. Wang *et al.*, 2013). Most of the *FUS* ALS-associated mutations are located in its nuclear localization sequence, therefore

inducing its cytoplasmic mislocalization and aggregation (J. Gal *et al.*, 2011). Once more, the *FUS*-induced pathology could be both induced by the loss of function of the encoded protein and by a toxic gain of function through its aggregation. Similarly, to SOD1 and TDP-43 proteins, there's evidence of a prion-like behaviour spreading of misfolded FUS along the motor tracts (J. Shorter, 2017; M. McAlary *et al.*, 2019; M. Polymenidou and D.W. Cleveland, 2011).

The identification of the genetic causes of this pathology has constituted the foundation of the majority of the current ALS research as it allowed the generation of a variety of cellular and animal models.

C. Symptoms and diagnosis:

1. Motor symptoms

ALS is characterized by the degeneration of both the upper and the lower MN. At the clinical scale, this is translated by a progressive muscular weakness accompanied by cramps, fasciculations and muscle stiffness. Every voluntary muscle appears to be affected with the exception of the ocular and the sphincter muscles (M. Ahmadi *et al.*, 2010; M. Carvalho *et al.*, 1995). However, the clinical features of ALS are rather heterogenous and differ from one patient to another.

These differences have led to the creation of two subclasses: spinal onset in upper and lower limbs observed in roughly two-thirds of the patients, and on the other hand, bulbar onset observed in patients presenting first dysarthria or dysphagia sometimes accompanied with dysphonia, or reduced mouth closure or chewing problems (P. Masrori and P. Van Damme, 2020).

2. Extra-motor symptoms:

In addition to muscular symptoms, up to 50% of the patients can present extra-motor cognitive and behavioural impairments. Fronto-temporal dementia (FTD) designed a group of neurodegenerative diseases characterized by the progressive loss of neurons in the frontal and temporal lobes, inducing a change in the behaviour, a decrease in the language proficiency and the executive function (J. Bang *et al.*, 2015). FTD is considered as the third cause of dementia worldwide (J.J. Young *et al.*, 2018; J. Bang *et al.*, 2015). In the last two

decades, more and more patients that were primary diagnosed with ALS have been exhibiting FTD signs suggesting clinical overlap between these two disorders. It is now considered that 13% of the ALS patients will develop a form of FTD in the course of the disease (G.M. Ringholz *et al.*, 2005; J. Phukan *et al.*, 2012). Furthermore, both of these pathologies are characterized by the presence of cellular inclusions in neurons prior to their death, with TDP-43 positive inclusions, a pathological hallmark for an important percentage of FTD cases (I.R.A Mackenzie and R. Rademakers, 2010; S-C. Ling *et al.*, 2013). The development of genomic screening further strengthened the link between these two diseases. Indeed, several genes associated with ALS, such as *FUS*, *TARDBP*, *C9orf72*, or *TBK1* (R. Ferrari *et al.*, 2013; B. Borroni *et al.*, 2009; S.J. Sha *et al.*, 2012; I. Gijselinck *et al.*, 2015) were found mutated in FTD patients. However, it is unclear why certain genes seem to be more associated with FTD and others with ALS. For instance, *TBK1* mutations were shown to be associated with approximately 0.4-4% of the total ALS cases whereas they are much more frequent in patients presenting an ALS-FTD comorbidity (I. Le Ber *et al.*, 2015). Nowadays, due to their genetic and pathological overlap, these two diseases are considered to represent the two ends of a clinical, neuropathological and genetic single spectrum disorder, often termed ALS-FTD (F.B. Gao *et al.*, 2017; S-C. Ling *et al.*, 2013; D. De Silva *et al.*, 2016).

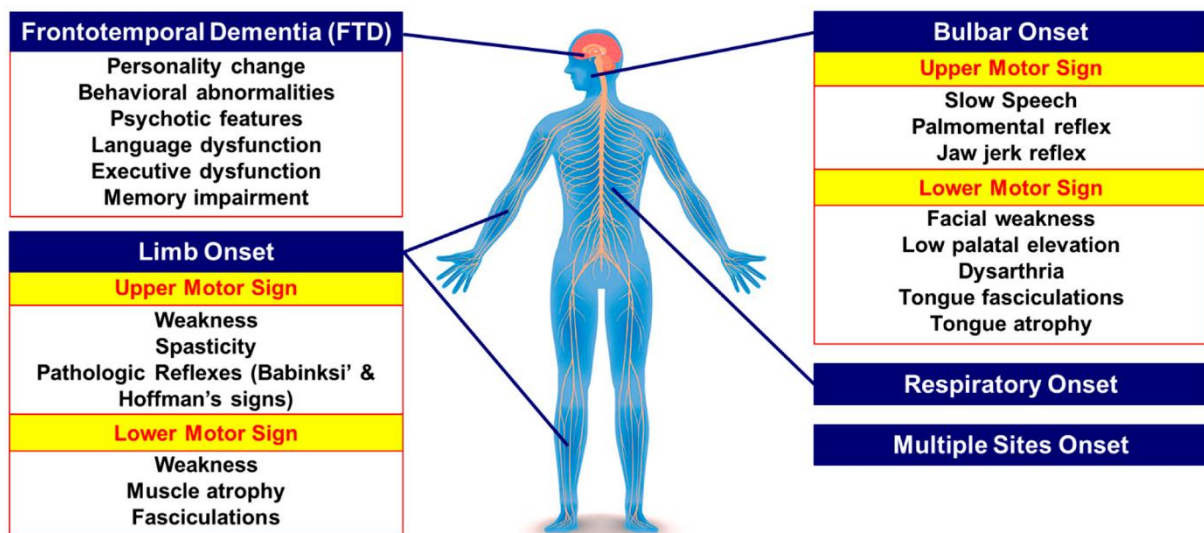


Figure 3: Illustration of the symptoms induced by the different forms of pathologies of the ALS-FTD spectrum (derived from M. Ghasemi *et al.*, 2021)

3. Diagnosis

Due to the heterogeneity of symptoms, identifying ALS can be challenging and misdiagnosis often occurs. Because of the urgent need of a precise diagnosis criteria, the World Federation of Neurology Subcommittee on Motor Neuron Disease gathered in 1990 in El Escorial, Spain, to design and write a list of universal criteria that would help the diagnosis of the disease: The El Escorial criteria (B.R. Brooks *et al.*, 1994). These criteria are based on a clinical, electrophysiological and neuropathologic surveillance to assess the evidence of lower or upper MN degeneration and the monitoring of the course of the disease with the spreading of neurodegeneration to other areas of the central nervous system (CNS). Together with these diagnostic inclusion criteria, exclusion criteria were also designed to distinguish the pathology from others.

As the El Escorial criteria based on the identification of neurodegeneration, when a patient is diagnosed with ALS, the disease course is already well advanced (B.R. Brooks *et al.*, 1994). Therefore, a change of paradigm and a revised set El Escorial criteria in the close future is conceivable. With the great advances in the technology of metabolites measurement and the identification of dysregulated biomarkers, diagnosis could occur a lot earlier. The most promising biomarker is the presence of neurofilaments (NF) (both light and heavy chains), quantifying their levels in the cerebro-spinal liquid (CSF) or in the blood could help diagnosed patients at earlier stages of the disease (Z. Xu *et al.*, 2016). In addition to help diagnosis, NF have also been reported to reflect accurately the disease progression (Y-N. Zhou *et al.*, 2021), and are now used in clinical trials to investigate the impact of a treatment on the course of the pathology (T. Miller *et al.*, 2022).

Amongst these biomarkers under investigation we can also cite, the elevated level in glucose originated from a hypermetabolism associated with the disease (T.W. Tefera *et al.*, 2021) in the brain, spinal cord, muscles or in circulating fluids. Unfortunately, the quest for metabolites that are prevalent in ALS patients has not been entirely successful with no real specific metabolic signature for this disease as of yet (see Part III - metabolites dysregulation in ALS).

D. Treatment:

Despite approximately 200 clinical trials, only a few compounds have been identified to have a positive impact on ALS patients.

1. Riluzole:

Riluzole is a glutamate inhibitor that has shown modest but beneficial effect in the late stages of disease, prolonging the survival by 2-3 months (J. Wokke, 1996; T. Fang *et al.*, 2018; U. Saitoh and Y. Takahashi, 2020). Glutamate excitotoxicity has been shown to play an important role in ALS pathogenicity (E. Foran and D. Trotti, 2009). Indeed, patients exhibit higher level of glutamate in the CNS (O. Spreux-Varoquaux *et al.*, 2002). Glutamate is used in the brain as a neurotransmitter, transmitting excitatory signals between neurons, however, it is known that an increased firing rate is detrimental for neurons (Y. Zhou and N.C. Danbolt, 2014). It is still unclear how riluzole blocks the glutamatergic neurotransmission, and three hypotheses have been proposed: the blockade of the glutamate release in the synaptic compartment, the inactivation of the glutamate sodium channels of the receiving neuron, or the interference with the cellular response to glutamate signal. Despite its modest impact on the disease, riluzole have remained the only approved drug in the treatment of ALS for almost 30 years after its approval by the United-states Food and Drug Administration (FDA) in 1995.

2. Edaravone

Evidence of high oxidative stress has been put in evidence in ALS patients (T. Cunha-Oliveira *et al.*, 2020) and is suspected to play an important role in the degeneration of motor neurons. Therefore, it was hypothesized that reducing the level of ROS could show beneficial impact on patient health and, to this extend, several antioxidant compounds have been used. Edaravone is a molecule that has been shown to protect nerve cells in the brain by reducing the level of ROS and inhibiting the oxidative stress induced apoptosis (M-S. Jami *et al.*, 2015). It is the sole tested antioxidant molecule that has been shown to have a beneficial effect on the course of the disease (H. Cho and S. Shukla, 2021). However, similarly to riluzole, it only has a modest impact on the course of the disease, prolonging the survival by a few months. Edaravone was approved by the FDA in 2017 following a clinical trial on Japanese patients (K. Abe *et al.*, 2017). However, the pool of patients selected for this trial was criticized for not being representative of the ALS population (O. Hardiman and L.H. van den Berg, 2017). Based on these doubts, the European Medicines Agency (EMA) has not approved yet the usage of this drug on European territory and its availability in France is

segregated to a temporary authorisation of usage created by the *Agence National de Sécurité du Médicament*.

3. Relyvrio

Relyvrio (formerly known as AMX0035) is a treatment based on two compounds: taurursodiol and phenylbutyrate. Both of these molecules act respectively on two cellular components and mechanisms altered in the context of ALS: mitochondria and elevated oxidative stress (C.M.P. Rodrigues *et al.*, 2003) and unfolded protein aggregates (C.T. Mai *et al.*, 2018). A recent clinical trial using the combination of these two molecules has shown some beneficial effect, prolonging survival and reducing hospitalisation risk of ALS patients (S. Paganoni *et al.*, 2020; S. Paganoni *et al.*, 2021). Based on this trial, the FDA has approved the usage of relvyrio this year (2022), however, the EMA has still not approved its utilization on European territory.

4. Non-pharmacological treatments:

Besides these disease-modifying pharmacological treatments, the patients can benefit from symptomatic treatments, which do not impact the course of the disease but are based on the improvement of the quality of life, including tracheostomy. Tracheostomy consists of the incision of the trachea to open a direct airway for long-term mechanical ventilation in a context of respiratory failure. However, this procedure is rarely used due to the heavy consequences on the patient's life as it provokes a total loss of oral communication.

Overall the treatments are limited to symptomatic management and palliative care by the lack of early diagnosis of the disease. Indeed, the diagnosis based on El Escorial criteria is based on the identification of neurodegeneration, therefore, when a patient is diagnosed with ALS, the course of the disease is already irreversible. The solution to switch from a palliative to a curative treatment would be to identify early onset biomarkers prior to neurodegeneration in order to update and revise the actual criteria paradigm and allow early diagnosis where the disease course could still be reversed.

As a future perspective, we can mention increased importance of genetic therapies illustrated by a major clinical trial ongoing that is testing whether intrathecal injections directly in the cerebrospinal fluid of antisense oligonucleotide targeting the expression of SOD1 could prolong ALS disease duration (T. Miller *et al.*, 2022).

III. Physiopathology of ALS-FTD – cellular dysregulations:

ALS pathophysiology complexity is proportional to the number of genes associated with its development. Many alterations of cellular processes are involved and the identification of the primary dysregulations from their consequences can be challenging. In fact, each of the pathways identified as impacted are considered in a hypothesis as the putative cause of the disease. In the following section we will review the cellular pathways identified in the development of the pathology.

A. Impaired protein homeostasis:

One universal hallmark of the ALS pathology is the presence of cellular inclusions in the cytoplasm of neurons and skeletal muscles (A.M. Blokhuis *et al.*, 2013; M.D. Cykowski *et al.*, 2019). However, how these inclusions are formed remains obscure and the mechanisms responsible for their apparition are still unknown. From their presence we can suspect an imbalance in the protein's synthesis and their degradation.

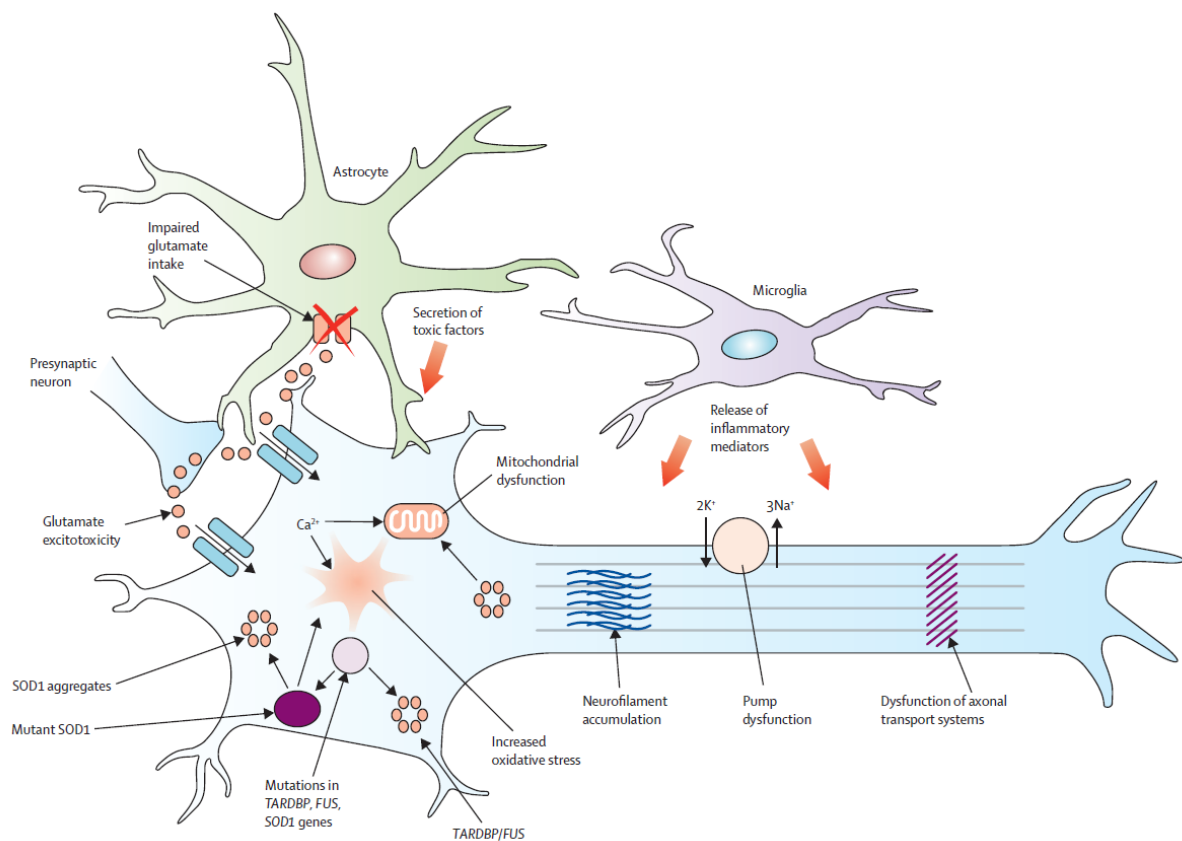


Figure 4: Illustration of the different cellular dysfunction observed in the context of ALS-FTD (derived from M.C. Kiernan et al., 2011)

The majority of the aggregates in both sporadic and familial forms are found to be positive for TDP-43 (M. Neumann *et al.*, 2006). However, the presence of aggregates in ALS patients of other pathological markers has been observed, including FUS (C. Vance *et al.*, 2009), SOD1 (Y. Hayashi *et al.*, 2016), DPRs (K. Mori *et al.*, 2013) or NF (A. Hirano *et al.*, 1984) according to the form of ALS.

1. Autophagic dysregulation:

Autophagy is a highly conserved process that is involved in protein homeostasis maintenance through their degradation. Autophagy is a term englobing 3 distinct mechanisms: macroautophagy, microautophagy and chaperone mediate autophagy (reviewed in the following section).

Macroautophagy is the major autophagic process, it relies on the targeting and engulfment of cellular components into a double membrane vacuole called autophagosome which later fused with the high acidic vacuoles lysosomes to induce the degradation and the reuse of the target molecules. Microphagy, as opposed to macroautophagy, is not selective, it only relies on the digestion of small volumes of cytosolic substrates by the lysosomes.

Among the 30 genes associated with the development of ALS, 6 are directly involved in the autophagic processes: *C9ORF72*, *TBK1* and the less ALS represented *VCP*, *OPTN*, *SQSTM1* and *UBQLN2* (S. Mathis *et al.*, 2019). At the clinical level, the most obvious sign of the autophagic involvement in the development of the ALS disease is the accumulation of autophagosomes in the cytoplasm of patients' MN (S. Sasaki *et al.*, 2011). This would suggest an increased autophagic activity in a physiological context. However, this activity is not reflected in the degradation of the typical aggregates during the course of the disease questioning about the efficiency of this system. Moreover, autophagy cargo proteins p62 are found to colocalize with the cytoplasmic inclusions of ALS patients suggesting proteostasis defects (J. Gal *et al.*, 2007, Y. Mizuno *et al.*, 2006). Stimulation of the autophagy system has beneficial effect in ALS *in vivo* and *in vitro* models (L. Marrone *et al.*, 2019; S. Lattante *et al.*, 2015). Moreover, macroautophagy efficiency have been shown to be inversely proportional to the age, and a decreased in its activity is observed in ageing models (C. Ott *et al.*, 2016; Y. Aman *et al.*,

2021). These observations link the reduced autophagic activity with the late onset development of the disease.

TBK1, the gene studied in the present study has been extensively implicated in autophagic process. Therefore, we will review more in depth its function in the context of autophagy in the following section (Part II – II.B. *TBK1* as a regulator of the autophagic process).

2. Proteasomal impairment:

In response to misfolded proteins as observed in ALS, the cell can initiate a process called the unfolded-protein response (UPR) (R. Bravo *et al.*, 2013). It results in the transport of the defective proteins toward the endoplasmic reticulum (ER) where chaperones correct the folding of the proteins. However, when the level of misfolded proteins exceeds the UPR capacity or when the chaperones are unable to restore the native conformation, a new emergency mechanism activates: the endoplasmic reticulum-associated protein degradation (ERAD) (A. Stolz and D.H. Wolf, 2010). The misfolded proteins are then released in the cytosol where they undergo ubiquitination and proteasomal degradation. Mutated *SOD1* proteins have been shown to interfere with ERAD proteins, preventing the correct ER stress response pathway (H. Nishitoh *et al.*, 2008). Moreover, DPR derived from C9ORF72 G₄C₂ repeats can reduce proteasomal activity resulting in increased ER stress and accumulation of misfolded proteins (Y-J. Zhang *et al.*, 2014). Importantly, dysregulation of the proteasomal degradation of misfolded proteins induced the formation of aggregates, which in turn activate a cascade of events that ultimately results in cell death (R. Sano and J.C. Reed, 2013). Finally, the hypothesis of an altered proteasomal pathway is underlined by the presence of ER stress marker in the motor neurons of ALS patients suggesting that this dysregulation could be responsible for their death in patients (K. Kanekura *et al.*, 2009; S. Sasaki, 2010). In addition, there are evidences of an age-dependent reduction in the level of the ER stress chaperones (A. Apostolopoulos *et al.*, 2018; D.M. Hall *et al.*, 2000), suggesting a reduced UPR and connecting the proteasomal impairment theory to the late-onset of the disease. Studies assessed the impact of chaperones supplementation on ALS *SOD1* mutant murine model with limited success, suggesting that the sole reduction in UPR is not responsible for all the subsequent neuronal cell death (Y.J.K Patel *et al.*, 2005; J. Krishnan *et al.*, 2008; P.S. Sharp *et al.*, 2008).

B. Extracellular trafficking:

Physiologically, exosomes are key players of the cell-to-cell communication and rapid interaction between the different cell types of the central nervous system is crucial for a correct activity (L.R Lizarraga-Valderrama et al. and G.K. Sheridan 2021). In the ALS context, it is suggested that exosomes carry neurotoxic elements such as aggregates or proteins with a prion-like behaviour (such as described in the previous section) subsequently inducing motor neuron death (J.M Silverman *et al.*, 2019; T. Westergard *et al.*, 2016).

C. Intracellular trafficking

MNs are highly specialized neurons, bearing very long axonal processes that allow muscle innervation. They are the longest cells of the body, their axons reaching up to 1 meter long. Due to their unique anatomical characteristic, motor neurons highly depend on intracellular trafficking to convey organelles or vesicles (containing neuronal receptors or RNA), to their synapses compartment. Axonal transport dysregulation is one of the earliest events in the development of ALS (T.L. Williamson and D.W. Cleveland, 1999; L.G. Bilisland *et al.*, 2010). For this reason, it has been considered by a part of the scientific community as causative for the development of the disease. Indeed, several mutations associated with the pathology, impacting genes not necessarily involved in the axonal transport, have been shown to impair transport dynamics early in the development of the pathology. Amongst these genes we can cite the well-studied *C9orf72*, *FUS*, *SOD1*, *TARDBP* but also genes that are more rarely mutated in the context of ALS such as *DCTN-1*, *KIF5A*, *ALS2*, *TUBA4A*, *SPG11*, *CHMP2B* or *VAPB* (D.M. Baron *et al.*, 2020). The most obvious consequence of these alterations is a defective transport of mitochondria. Indeed, lower mitochondrial axonal concentration was reported in several *in vitro* and *in vivo* experimental models (C. Vande Velde *et al.*, 2011; G.M. Mórotz *et al.*, 2012; J. Magrané *et al.*, 2014; K.J. De Vos *et al.*, 2017). These defects are thought to be mainly due to weakening of the cytoskeleton structures or impaired vesicles formation. Alterations of the neurofilament, actin or microtubules integrity have been shown to be associated with some form of ALS (D. Fil *et al.*, 2017; D.A. Figlewicz *et al.*, 1994, M.H. LaMonte *et al.*, 2002). As mentioned before, intracellular vesicle formation is altered in the context of ALS with traffic between the ER and the Golgi was reported to be inhibited (J. Atkin *et al.*, 2014; K. Soo *et al.*, 2015). Altogether, these results suggest that alteration of the

intracellular trafficking, which is a central pathway in the neuronal physiology, could be an early event of the disease leading to the dysregulation of other cellular pathways.

Moreover, it seems that altered vesicles production is not restricted to the nervous system, as muscles cells of ALS patients present an accumulation of cytoplasmic vacuoles (A. Scaramozza *et al.*, 2014).

D. Mitochondrial dysfunction and oxidative stress:

Mitochondria are commonly described as the engine of the cell due to their involvement in adenosine triphosphate (ATP) generation, but their function is much more diverse, for instance they are major regulator of cell apoptosis (S.W.G. Tait and D.R. Green, 2013) or of calcium homeostasis and therefore involved in many neuronal functions (C. Giorgi *et al.*, 2018).

Mitochondrial damaged was one of the first cellular hallmarks of ALS reported (T. Astumi, 1981). Since then, many studied have confirmed these observations and evidences of mitochondrial damages during the course of the disease accumulated in patients (K. Otamoto *et al.*, 1990; S. Sasaki *et al.*, 2007) and in *in vivo* models (C.M.J. Higgins *et al.*, 2003; M. Mattiazzi *et al.*, 2002; J. Magrané *et al.*, 2014).

Interestingly, these mitochondrial alterations have been reported to occur early in the development of the disease (J. Magrané *et al.*, 2014; C. Vande Velde *et al.*, 2011, N. Damiano *et al.*, 2006), suggesting that mitochondria loss of integrity could be a preventive therapeutic target.

It is suspected that ALS related mitochondrial damages is due to structural alteration through either formation of vacuoles within both the inner- and the outer- mitochondrial membranes (P. Pasinelli *et al.*, 2004; C.M.J. Higgins *et al.*, 2003) or sequestering of mitochondrial protein in cytoplasmic aggregates (J. Deng *et al.*, 2015; J. Deng *et al.*, 2018; P. Pasinelli *et al.*, 2004; S.Y. Choi *et al.*, 2019). The association between ALS and mutation in *CHCHD10* (S. Bannwarth *et al.*, 2014), a gene coding for a protein located between the inner- and outer- mitochondrial membrane, is a further evidence of the link between structural alterations of the mitochondria and the development of ALS. The most obvious consequences of these mitochondrial damages in the context of ALS is the increased level of toxic cytoplasmic ROS which can lead to neurodegeneration (J. Deng *et al.*, 2015; T. Cunha-

Oliveira *et al.*, 2020). However, mitochondria could also lead to neuronal death through pro-apoptotic signalling (S.W.G. Tait and D.R. Green, 2013). Finally, a disturbed mitochondrial activity is suspected to weaken the cells through an impaired calcium homeostasis subsequently increasing the sensitivity to glutamate toxicity (N. Damiano *et al.*, 2006), another ALS features that we'll discuss in the next section.

E. Glutamate excitotoxicity:

Glutamate is the most abundant excitatory neurotransmitter in the human CNS. It is released in the synaptic compartment by the pre-synaptic neuron, when in the synapse, glutamate binds to NMDA and AMPA glutamate receptor of the post-synaptic cell in order to induce a Ca^{2+} and Na^{+} influx and the subsequent membrane depolarization. An excessive level of glutamate in the extracellular medium is deleterious for the neurons, leading to extreme firing rate. Evidences of dysregulated glutamate excitatory response have been reported on two levels in the context of ALS.

First, it is suspected that an increased permeability of the glutamate receptors induces an excessive Ca^{2+} influx leading to mitochondrial dysfunction and subsequent ROS production. Indeed, ADAR2, a protein regulating the permeability of AMPA have been reported to be dysregulated in the context of ALS (S. Moore *et al.*, 2019). Moreover, evidence of a physiological higher sensibility of motor neurons would link the selective loss of motor neurons in the context of ALS to the dysregulated glutamate excitability. Indeed, specific overactivation of AMPA in a murine model lead to motor neuron degeneration and the subsequent paralysis similarly to what was observed in ALS patients (J.C. Corona and R. Tapia, 2004). The hypothesis of a higher calcium sensitivity of the motor neurons is supported by evidence of physiologically lower expression of the *GRIA2* gene coding for the AMPA subunit responsible of the permeability compared to their neighbouring neuronal cells (P.R. Heath *et al.*, 2002).

Secondly, it is suggested that glutamate accumulates in the synapse compartment because of an impaired astrocytes clearance leading to continuous excitatory signal. Indeed, astrocytes express on their synaptic process, GLT1, which maintains a low extracellular glutamate concentration and therefore prevents an excessive neuronal intake of positive ions (K. Kim *et al.*, 2011). Supporting this hypothesis, GLT1 levels have been reported to be

severely depleted, leading to a decreased activity and extracellular glutamate accumulation (L.A. Bristol *et al.*, 1996; S. Sasaki *et al.*, 2000, C. Vanoni *et al.*, 2004; A.R. Peterson and D.K. Binder, 2019). This hypothesis is supported by two other studies respectively inhibiting and boosting the expression and the activity of GLT1. Inhibiting the expression of GLT1 has been shown to increase glutamate levels extracellularly and causing neuron degeneration and a progressive motor deficit (J.D. Rothstein *et al.*, 2005) whereas enhancing the activity of GLT1 was reported to be beneficial in the SOD1^{G93A} murine model (J.D. Rothstein *et al.*, 2005).

F. Inflammation:

Upon tissue lesion or pathogen invasion, damaged cells components or motifs shared by pathogens are recognized by surrounding cells. The response to these signals, resulting in tissue repairing is called the inflammation. Once these signals are integrated, a cascade of events lead to the activation of two main pathways: the nuclear factor-kappa B (NFκB) complex and the interferon regulatory factors (IRFs) responsible for the expression of pro-inflammatory genes and the respective synthesis of cytokines/chemokines and interferons. The release of these endogenous molecules alerts the host of a danger and allow recruitment of cells of the immune system. As this process is so important, the synthesis and response to inflammatory signals is precisely tuned. When a dysregulation occurs along the synthesis of these endogenous molecules, it leads to hypo- or hyper-stimulation of the inflammatory system and provokes different types of pathologies. In ALS patients and animal model, inflammation was found to be up-regulated (K.A. Staats *et al.*, 2022; R. Tortelli *et al.*, 2020; P.A. McCombe and R.D. Henderson, 2011). However, it is still obscure whether it is a consequence of the death of MN or if the hyperstimulation of the inflammatory response leads to MN loss.

In the CNS, inflammation response is triggered by the glial cells (the myeloid precursor derived cells in the brain) composed of microglia and the astrocytes. These cells remain in a silent state physiologically but activates when exposed to immune challenge or injury. Evidence of activated myeloid cells have been reported in patients (M.R. Turner *et al.*, 2004; B.E. Clarke and R. Patani, 2020) and animal models (E.D. Hall *et al.*, 1998; B.E. Clarke and R. Patani, 2020). The microglia population can be divided into two subtypes: the M1 subgroup is activated by pro-inflammatory signals and is responsible for the increased inflammation whereas the M2 subgroup is stimulated by anti-inflammatory signals and suppresses the

inflammation and therefore neuron survival (Y. Tang and W. Le, 2016). It has been reported in the SOD1^{G93A} murine model that the ratio M1/M2 is proportional to the age (J.M. Crain *et al.*, 2013; B. Liao *et al.*, 2012), this difference could be linked to the late onset of the disease, an older brain being more sensitive to pro-inflammatory signals.

Astrocytes involvement in the development of the disease is complicated to apprehend. It is suspected that they have a minor impact on the course of the disease and deletion of the NFκB pathway doesn't improve the phenotype in the SOD1^{G93A} murine model (C. Crosio *et al.*, 2011) suggesting that they are not involved in the inflammatory state. However, despite the suggested minimal involvement in the inflammatory state of ALS models, astrocytes' impaired clearance of synaptic glutamate molecules have been suggested as an important factor in the ALS pathogenicity (K.A. Staats and L. Van Den Bosch, 2009; K. Yamanaka *et al.*, 2008).

G. Aberrant RNA metabolism:

As mentioned previously, the main cellular hallmarks of the disease is the presence of TDP43 and at a lesser extend FUS positive cytoplasmic inclusions (T. Arai *et al.*, 2006; M. Neumann *et al.*, 2006). These proteins are RNA binding proteins that are involved throughout all the RNA maturation. Therefore, the sequestration of these two proteins in the cytoplasm impact the RNA metabolism transcription defects, alternate splicing changes, miRNA biogenesis, stress granule formation, and RNA nucleocytoplasmic transport (L. Le Gall *et al.*, 2020).

First, the most obvious consequence is the loss of function of these two proteins. It is unclear what are their precise roles as they are interacting with a large number of proteins (A. Ratti and E. Buratti, 2016; M. Kamelgarn *et al.*, 2016). However, when they are inhibited as well as in the context of ALS, there is evidence of several RNA metabolism alterations: RNA mis-localization, unvalid splicing, defect in transcription (S. Reber *et al.*, 2016; C. Lagier-Tourenne *et al.*, 2012; E.S. Arnold *et al.*, 2013; A. Ratti and E. Buratti, 2016).

Besides TDP43 and FUS loss of function's impacts on RNA metabolism, their cytoplasmic aggregation has severe consequences. Indeed, highly dense RNA foci are observed in ALS patients colocalizing with TDP43, which suggests an RNAs mis-localization also being responsible for the observed altered RNA metabolism (O. Aladesuyi Arogundade *et al.*, 2019). Moreover, it was reported that when forming aggregates FUS proteins still bind and

sequester important actors of RNA splicing in the cytoplasm (Y. Yu et al., 2015; S. Reber et al., 2016; M. Kamelgarn et al., 2016), further impacting the physiological RNA metabolism.

H. Programmed cell death:

Cell death is part of the normal cellular development. It can occur in a physiologically and genetically controlled manner: we therefore talk of a programmed cell death (PCD), or in an alternative uncontrolled cell death induced by environmental causes, the necrosis. Necrosis is induced by an external injury, for instance hypoxia or inflammation and is usually considered as an accidental death when the cell cannot maintain its homeostasis with its environment. At the cellular level, necrosis results in the swelling and subsequent rupture of the plasma membrane (D. Moujalled et al., 2021). In the context of ALS, the characteristic MN degeneration has been suspected to be induced through a PCD-related mechanism (C. Guégan and S. Przedborski, 2003). There are several types of PCD: the well-known apoptosis but also necroptosis, pyroptosis, or ferroptosis (D. Moujalled et al., 2021). Regarding to ALS, two of these pathways have been suggested to be involved in neurodegeneration (J.R. Morris et al., 2017): apoptosis (L.J. Martin, 1999; K.Y. Kim et al., 2020) and necroptosis (D.B. Re et al., 2014; Y. Ito et al., 2016). As *TBK1* has been reported to be involved in the induction of these two cell processes, we will review their mechanistic insights and the functional role of *TBK1* in these cellular pathways later in the introduction (Part II – section II functional roles of *TBK1*).

IV. Modelling the ALS-FTD – state of the art

A. In vitro models

Although they suffer from the lack of tissue connectivity or functional connectivity, cellular models are powerful tools to study the role of genes involved in ALS through the expression of WT and mutant ALS associated proteins or with their respective gene deletion. They also allow genetic and compound screening which are great tools to find drug that could lead to therapeutic avenues.

The first use of unicellular model in the ALS research was the expression of a SOD1 human wild type or mutant plasmid in NSC34 cells (a hybrid mouse cell line produced to mimic motor neurons) (M.R. Cookson et al., 2002). These experiments have reported the production of intracellular ROS upon expression of mutant form of SOD1. However, this

mouse cultured cell type has been put aside with the apparition of primary culture of mouse MN in which various factors have been tested to investigate toxicity: for instance, glutamate (P. Van Damme *et al.*, 2007), astrocytes released factors (M. Nagai *et al.*, 2007) or DPR synthesis (X; Wen *et al.*, 2014).

Since the use of NSC34 cells, various types of cell lines have been used, and many ALS genes have been investigated. One model that has been extensively used to perform genetic screen in the context of ALS is the *Saccharomyces cerevisiae* budding yeast. In the context of TDP-43 overexpression, genetic screening in yeast have allowed the identification of several genes modulating the induced toxicity: *ATAXIN2* (*ATXN2*) (A.C. Elden *et al.*, 2010), *CNC1*, *DNM1*, *YBH3* (V. Bharathi *et al.*, 2021), and *DBR1* (M. Armakola *et al.*, 2010).

Furthermore, a real advance in modelling of ALS disease was made with the introduction of induced pluripotent stem cells (iPSCs) directly derived from differentiated cells of ALS patients (K. Takahashi *et al.*, 2007). This model represents enormous advantages. First, they remove the need of artificially expressing mutant transgenes to study their impact. The development of tools allowing their differentiation into differentiated cells allow to study the impact of ALS associated mutants in specific cell types such as spinal MN (Y. Maury *et al.*, 2015; J. Lenzi *et al.*, 2016)). Finally, this is the only experimental model that can allow the study of sporadic ALS through the induction of iPSCs derived from sporadic ALS patients. However, these models still have limits and they suffer from the lack of tissue connectivity. The development of iPSCs co-cultures could bypass these issues as cultured of mature MN innervating muscle cells have been developed recently (N.R.W. Martin *et al.*, 2015; N. Santhanam *et al.*, 2018; M.A. Bakooshi *et al.*, 2019). Nevertheless, these cellular models have allowed the recapitulation of characteristic hallmark of the disease such as the TDP-43/FUS/SOD1/DPRs (I.V. Chestkov *et al.*, 2014; J. Lenzi *et al.*, 2015; B. Bilican *et al.*, 2012; S. Almeida *et al.*, 2019) aggregations, NF disorganization (H. Chen *et al.*, 2014), defects in the intracellular transport (K. Zhang *et al.*, 2015), and changes in excitability (B.J. Wainger *et al.*, 2014).

Overall, although many aspects of the disease cannot be studied in these unicellular models, they can offer mechanistical insights of the cytotoxicity and the proteinopathy. Moreover, these cellular models are optimal to identify possible therapeutic target to be further tested in more complex *in vivo* model.

B. In vivo models

1. Mouse models

Murine models are the most widely studied *in vivo* models for ALS. It's a popular model as it is a mammalian species with a high genetic and physiology homology compared to human. Since the identification of the causative genes of "familial" ALS, a large number of murine models have been developed. For instance, the sole study of the toxicity of mutated SOD1 has led to the design of more than 10 models each expressing a different mutant protein (P.C. Wong *et al.*, 1995; L.I. Bruijn *et al.*, 1997; M.E. Ripps *et al.*, 1995). The first and still most used model developed is the transgenic SOD1^{G93A} overexpression line (M.E. Gurney *et al.*, 1994). Murine models for the major genetic causes of ALS have been key to better establish the pathogenicity of these major genetic factors and the consequences of these mutations upon MN degeneration (Y.F. Xu *et al.*, 2010; I. Wegorzewska, *et al.*, 2009; A. Sharma, *et al.*, 2016; J. Scekcic-Zahirovic, *et al.*, 2016; J. Jiang, *et al.*, 2016; J.G. O'Rourke, *et al.*, 2016). Although these models have led to a great advance in the comprehension of the disease, most of them are based on either the deletion of a target gene or an overexpression of a mutant. The arrival of new genetic engineering technologies is shifting the standard models with knock-in murine mutants already developed for FUS and TDP-43 mutations (S-H. Huang *et al.*, 2020; J. Scekcic-Zahirovis *et al.*, 2016). Furthermore, to define therapeutic strategies for ALS patients, an array of simpler animal models has been emerging over the last two decades as reviewed below.

2. Drosophila

Besides the mouse standard *in vivo* model, a wide number of small animal models have been developed. Due to their fast development and cheap maintenance, they are widely used. The most widely used of these small *in-vivo* models for ALS study is the *Drosophila melanogaster*. Due to its quite developed nervous system (U.B. Pandey *et al.*, 2011), the large repertoire of genetics tools design for its study (B. Ugur *et al.*, 2016) and the 40% of homology between its functional gene and their respective human orthologs (L. McGurk *et al.*, 2015), the *Drosophila* is an excellent model to study the complex genetics of ALS. Many *Drosophila* lines expressing human ALS associated mutant transgenes have been developed for instance for *SOD1* (M.R. Watson *et al.*, 2008), *TARDBP* (P.S. Estes *et al.*, 2011), *FUS* (Y. Chen *et al.*, 2011), *C9orf72* induced DPRs (S. Mizielinska *et al.*, 2014). For instance, mutant

SOD1 has been extensively studied in the *Drosophila* and several antioxidant compounds have been identified to ameliorate the phenotypes of their respective mutant SOD1 models (C. Zhang *et al.*, 2019 (1); C. Zhang *et al.*, 2019 (2); T. Wang *et al.*, 2018). Through the use of these models, many advances have been made in the field of ALS, for instance, the identification of Pur- α protein as a regulator of the toxicity of C9orf72-DPRs expression (Z. Xu *et al.*, 2013) and of the mutant FUS expression (J.G. Daigle *et al.*, 2016). Moreover, *Drosophila* is also a great model to confirm observation made in unicellular models: for instance, ATXN2 after being identified in the yeast through genetic screens, was confirmed as a modifier of TDP-43 toxicity in the *drosophila* (A.C. Elden *et al.*, 2010). Recent genetic screening in FUS and TARDBP *Drosophila* models identified an array of enhancers and suppressors, which were able to impact the pathogenic induced phenotype (M.K. Kankel *et al.*, 2020).

3. *C.elegans*

Another invertebrate *in vivo* model used in the context of ALS research is the nematode *Caenorhabditis elegans* (*C. elegans*). This model presents a simplified but highly characterized nervous system and although it has a lower genomic homology with humans than other models, cellular stress pathways are still well conserved. Transgenic expression of the four main ALS-gene has been developed in *C. elegans* (T. Oeda *et al.*, 2001; P.E.A. Ash *et al.*, 2010; T. Murakami *et al.*, 2012; M. Therrien *et al.*, 2013). The use of these models has contributed to the identification of several cellular alterations linked to the expression of mutant ALS proteins such as ER stress (A. Jablonski *et al.*, 2015) or Ca²⁺ homeostasis disruption (D. Aggad *et al.*, 2014). These models have also led to the identification of calcineurin as a regulator of TDP-43 toxicity (N.F. Liachko *et al.*, 2016). More recently, this model was used to report the differential impact of SOD1 mutant knock in expression in cholinergic vs glutamatergic neurons (N.S Baskoylu *et al.*, 2018).

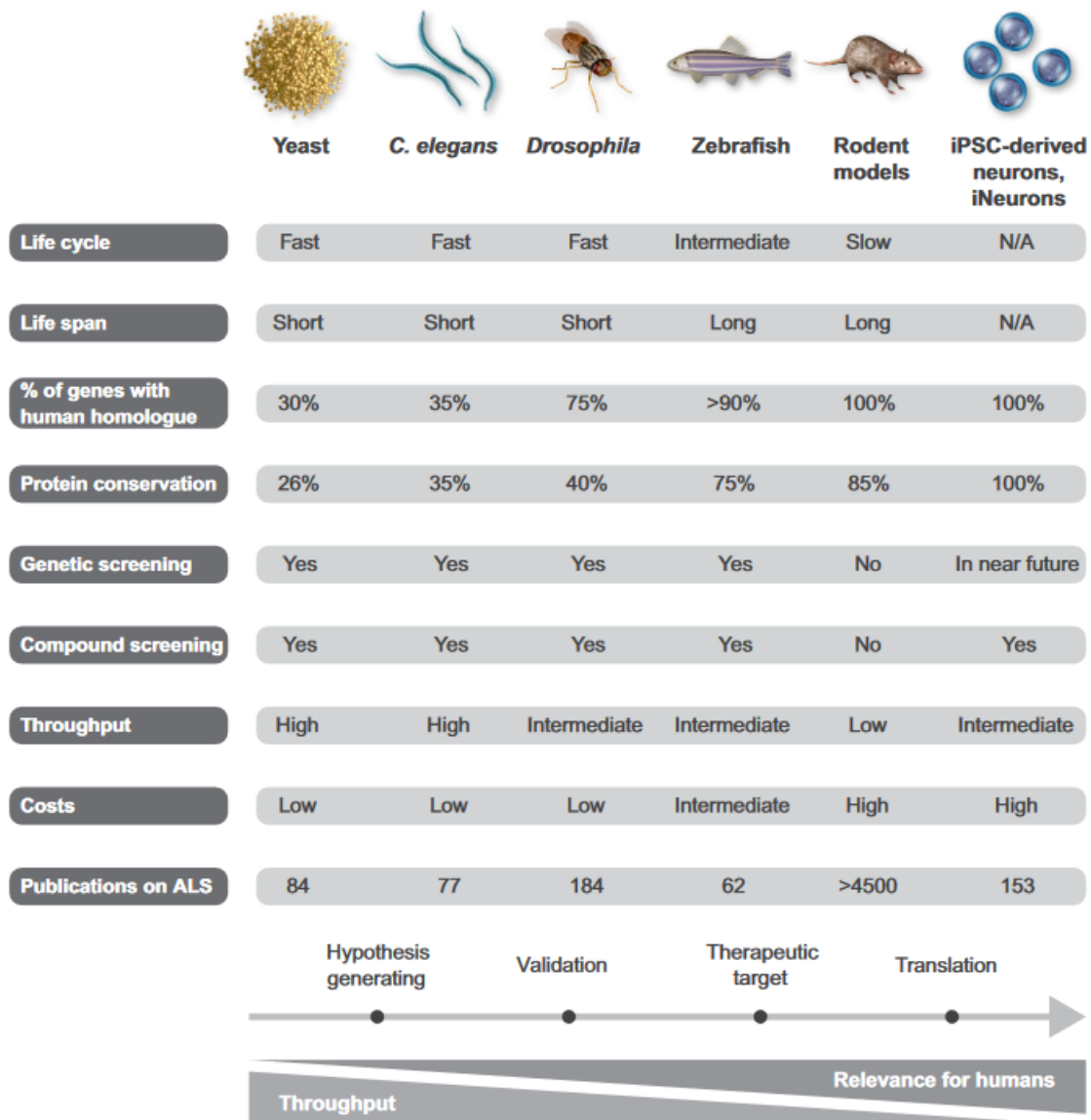


Figure 5: Advantages and limitations of the *in vitro* and *in vivo* models in the context of ALS (derived from Van Damme *et al.*, 2017)

4. Modelling ALS in the zebrafish:

The *Danio Rerio* model, or zebrafish, is in many aspects an optimal model for modelling the ALS physiopathology.

Zebrafish is a vertebrate that is part of the *Cyprinidae* fish family. The use of this model was at first limited to the understanding of the genetic basis of vertebrate development (G. Streisinger *et al.*, 1981). However, due to the high degree of conservation between its cellular mechanism and the human's, this model has grown in popularity over the years.

Technological advances led to the sequencing of its entire genome (K. Howe *et al.*, 2013), unveiling a high conservation of the genes between the zebrafish and the human (up to 80% of the human genes have a known orthologue in the zebrafish) (S.A. Patten *et al.*, 2014). Combined with this high homology with the human, the zebrafish also present some practical advantages due to their very fast development: 2 days post fertilization (dpf) larvae already present organs formation and function (C.B. Kimmel *et al.*, 1995; P. Drapeau *et al.*, 2002), with a sexually maturation up to 3 to 5 months after birth (C. Singleman and N.G. Holtzman, 2014). Moreover, zebrafish also have the advantages to give large clutches of *extra-utero* fertilized and transparent eggs which facilitates the observation and the *in vivo* or *ex vivo* fluorescence imaging (E. Kabashi *et al.*, 2010; E. Kabashi *et al.*, 2011). The *extra-utero* fertilization also facilitates genetic manipulation by antisense morpholino oligonucleotide (AMO) mediating transitory knockdown (KD) of a target transcript (B.R. Bill *et al.*, 2009) or by genome editing techniques such as CRISPR cas9 system to develop knockouts (KO) (B. Schmid and C. Haass, 2013; W.Y. Hwang *et al.*, 2013). Finally, one of the biggest advantages of the zebrafish is the experimental facility to perform drug screening due to their permeability to small molecules at the different stages of development. Moreover, due to its small size, a larva can fit in a single well of an up to 384 wells plate. Therefore, high scale drug screening can be easily performed in this model (L.I. Zon and R.T. Peterson, 2005).

In the context of ALS, numerous studies have used the zebrafish as a model to study genetic dysregulation associated with the development of the disease. Overexpression of mutant SOD1 for instance recapitulates several hallmarks of the pathology such as locomotion defects, loss of MNs, or loss of neuromuscular connectivity and subsequent degeneration (T. Ramesh *et al.*, 2010; S.A. Sakowski *et al.*, 2012). A zebrafish model of C9orf72 reduced expression has also been designed, displaying axonopathy and locomotion impairment (S. Ciura *et al.*, 2013). These defects were subsequently rescued via expression of the human C9orf72 orthologue. Finally, both decreased expressions of *tardbp* and *fus* have been investigated in the zebrafish resulting in severe motor deficits and alteration of the MNs integrity (E. Kabashi *et al.*, 2009; A-R. Bourefis *et al.*, 2020). Interestingly, both the *tardbp* and *fus* downregulation have been shown to be rescued via the expression of human TDP-43 protein (E. Kabashi *et al.*, 2011). More recently, a *tardbp* KD zebrafish model has led to

important understanding in the field by confirming the “distal axonopathy” theory, a hypothesis that claimed that pathological alterations appear during the presymptomatic phases of the disease at the neuromuscular junctions (M.L. Campanari *et al.*, 2021).

Part II: TBK1 as a novel genetic factor in ALS

I. Introduction to TBK1

A. Discovery and initial characterization

Tumor necrosis factor (TNF) receptor associated factor NF κ B activator (or TANK) is a protein thought to be involved in both the stimulation and the inhibition of NF κ B (G. Cheng and D. Baltimore, 1996; M. Rothe *et al.*, 1996). In an article of 1999, J.L Pomerantz and D. Baltimore were investigating the mechanisms involved in the stimulation of NF κ B activity by TANK (J.L. Pomerantz and D. Baltimore, 1999). The lack of homology in the signatory region of TANK led these researchers to seek for other proteins interacting with TANK: one of the identified proteins was called TANK binding kinase 1 (TBK1 also called NAK or T2K). Due to the fact that *Tbk1* mutant mice lacked the TANK-mediated stimulation of NF κ B (J.L. Pomerantz and D. Baltimore, 1999, Y. Tojima *et al.*, 2000) and died from a massive liver degeneration at E14.5 (M. Bonnard *et al.*, 2000), similarly to NF κ B mutant mice (A. Beg *et al.*, 1995; M. Tanaka *et al.*, 1999; D. Rudolph *et al.*, 2000; Q. Li *et al.*, 1999), TBK1 was first considered as a NF κ B activating kinase.

NF κ B is activated through the inactivation via phosphorylation of I κ B α , its repressor (A. Oeckinghaus and S. Ghosh, 2009). This post-transcriptional modification is performed by a family of homologous proteins called the I κ B kinases (IKK) composed of IKK α , IKK β , and IKK γ (or NEMO). Due to its presumed role in the NF κ B activation and its structural resemblance to this family of gene, TBK1 along with IKK ϵ (another highly structurally identical kinase protein) were first linked to IKK (J.L. Prochians and D. Baltimore, 1999, Y. Tojima *et al.*, 2000). However, despite their homology with IKKs, the TBK1 and IKK ϵ kinases were found to play distinct role in cell physiology (K.A. Fitzgerald *et al.*, 2003; H. Hemmi *et al.*, 2004; S.M. McWhirter *et al.*, 2004) and therefore a new classification was made separating the canonical IKK (α , β , γ) from the non-canonical IKK related TBK1 and IKK ϵ .

TBK1 is constitutively expressed throughout all the organism. Despite its low tissue specificity, its expression level is higher in some specific tissues as the nervous system, the endocrinal system, the digestive and respiratory tracts, the skin and the female tissue (M. Ulhen *et al.*, 2015 – *The human protein atlas*). Importantly, TBK1 expression is enriched in the nervous system within the cerebral cortex, the hippocampus and the basal ganglia.

B. Structure and properties

Tbk1 is a 50kb long gene encoding for a 729 amino acids (AA) long serine/threonine kinase protein. Below, I will review the 4 functional domains of TBK1 (C. Shu *et al.*, 2013; A. Larabi *et al.*, 2013; D. Tu *et al.*, 2013):

- Kinase domain (KD):

Located at the N-term sequence of the protein, it is 300 AA long. The kinase domain is the key functional domain of the TBK1 protein. It allows its activation through autophosphorylation and bear the catalytic ability of the protein: the phosphorylation. The kinase domain of TBK1 exhibited the conserved architecture of a kinase protein composed of two lobes: the C and N terminal loops with the active cleft at the intersection (D. Tu *et al.*, 2013).

- Ubiquitin like domain (ULD):

It is a 80 AA long domain shared within the IKK family. Ubiquitin is a small molecule of 76 AA that can be considered as a post-transcriptional modification: one or several ubiquitin molecules can be attached to a substrate protein targeting it for proteasomal degradation (C. M. Pickart, 2001) or altering their activity or localization (S. E. Dougherty *et al.*, 2020). Ubiquitin is highly conserved throughout the evolution as is its structure: a unique fold formed by a β -sheet with five antiparallel β -strands and a single helical segment (S. Martin-Villanueva *et al.*, 2021). Interestingly, some proteins, as TBK1 and the IKKs, bear this motif as part of their larger structures, called a ubiquitin like domain. These domains are not processed to produce a ubiquitin molecule, but in the vast majority of these proteins, they only function as protein-protein interaction region and whether they have an additional role linked to their resemblance to ubiquitin remains obscure (M. Hochstrasser, 2009). In TBK1, this motif does not bind to any known ubiquitin-binding domains but only to its own kinase domain and to the IRF3 protein (F. Iketa *et al.*, 2007). However, it has been shown that this

domain is crucial for TBK1 activation and its phosphorylation capacity (F. Iketa et al., 2007) suggesting a more important role in the dimerization or the autophosphorylation of the protein.

- Scaffold dimerization domain (SDD):

The SDD has a structural function and it allows TBK1 activation by interacting with the other subunit of the homodimer. It is a 250 AA long domain characterized by four helices that form three helical antiparallel coiled coils, which runs along the entire length of the molecule. This dimerization is allowed by 4 dimerization interfaces, each involving interactions in between the SSD domain and respectively the KD and ULD domains of the other sub-unit (A. Larabi *et al.*, 2013). The importance of the SDD domain is underlined as the sole mutation of 3 residues in the first SSD helix leads to accumulation of monomeric TBK1 (A. Larabi *et al.*, 2013). Moreover, mutant protein lacking the interaction between ULD-SDD domains results in the inhibition of TBK1's function without preventing its activation (A. Larabi *et al.*, 2013). These results suggest that the SDD is also important in the functional role of the protein besides its importance in its activation.

- C terminal domain (CTD) or TANK binding domain:

It is a 100 AA long domain involved in the interaction with adaptor proteins such as TANK, NAP1, SINTBAD (A. Goncalves et al., 2011). The truncation of the CTD does not alter the kinase activity (A. Goncalves et al., 2011) and the similar abolishment of the dimerization does not prevent binding of TANK or NAP1 to the CTD (A. Larabi et al., 2013). These adaptor proteins reside at different sub-cellular location (A. Goncalves et al., 2011) while they are competing for a TBK1 interaction and as the CTD function seems to be restricted to the interaction with these binding partners. This domain could be then used for the protein as a localization signal.

TBK1's activation requires two features: the homodimerization of the protein and its polyubiquitylation at two crucial residues (K30 and K401) (D. Tu *et al.*, 2013). Mutant proteins lacking one of those polyubiquitylated marks exhibit an impaired activation and signalling (D. Tu *et al.*, 2013). When in dimer, TBK1 undergo transphosphorylation on the Ser172 which leads to its activation via a change in its conformation: the C-loop of the KD rotates until formation of a salt-bridge between glutamate residue of the KD with a lysine

residue of the SSD. Serine to alanine substitution at the location abolishes TBK1's function (C. Shu *et al.*, 2013) and phosphomimetic mutation S279E partially restores the activity of the wildtype TBK1.

II. Functional roles of TBK1

TBK1 functional relevance has been largely investigated, and its activity has been reported to be involved in inflammatory responses (T. Yu *et al.*, 2012; Y-Y. Wang *et al.*, 2019), autophagy (B. Ritcher *et al.*, 2016; J.A. Oakes *et al.*, 2017), the insulin signalling pathway (M.A. Muñoz *et al.*, 2009) and innate immunity against bacterial and viral infections (R. Zhou *et al.*, 2020; K. Mosallanejad and J.C. Kagan, 2022).

A. TBK1 in innate immune response and inflammatory response:

1. Brief introduction to innate immunity – what are IFN and cytokines?

Upon invasion of the host by microbial pathogens such as viruses, bacteria and parasites, the first line of defence of the organism is the innate immunity. As mentioned previously, the response to these stimuli is triggered by the pattern recognition receptors (PRRs). Interestingly, some of these PRRs have been associated with ALS. For instance, toll-like receptors (TLRs) are PRRs that have been reported to be upregulated in glial cells of ALS patients (M. Casula *et al.*, 2011). Moreover the inhibition of some members of this family have been shown to be beneficial in some murine model (J.Y. Lee *et al.*, 2015; A. Fellner *et al.*, 2017). Other PRRs subclasses have also been linked to the ALS pathology such as Nod-like receptors (NLRs) (S. Johann *et al.*, 2015; S. Lehmann *et al.*, 2018) or AIM2-like receptors (ALRs) (S. Lehmann *et al.*, 2018).

These receptors, upon recognition of PAMPs/DAMPs, are responsible for the cascade of event that leads to the activation of NFκB and IRF3 and the subsequent synthesis of inflammatory cytokines and interferons (IFN) (O. Takeushi and S. Akira, 2010).

a. Inflammatory cytokines

Cytokines is a term that describes small proteins released by cells in order to interact and communicate with other cells (J-M. Zhang and J. An, 2009). There are different types of cytokines according to the types of cells where these factors originated, for instance, lymphokines are synthesized by lymphocytes, monokines by monocytes, interleukins by

leukocytes. Pro-inflammatory cytokines are mainly produced by activated macrophages, however, during cell injury, infection, invasion, and subsequent inflammation they can be synthesized by non-immune cells (J-M. Zhang and J. An, 2009).

Pro-inflammatory cytokines, upon binding, are stimulating several cellular processes mostly resulting in cellular death (ER stress, apoptosis, oxidative stress) (J.A. Smith *et al.*, 2012). Their aim is to slow down a possible infection or lesion. Amongst the most studied pro-inflammatory cytokines, we can mention IL-1 β , IL-6, or TNF- α . The anti-inflammatory cytokines are secreted to reduce inflammation and promote healing via regulation of the pro-inflammatory cytokine production. Similarly, pro-inflammatory cytokines can be both produced by immune and non-immune cells, and for instance in the CNS they can be synthesized by neurons and glial cells (L. Vitkovic *et al.*, 2001). Amongst the most studied anti-inflammatory cytokines, we can mention several interleukins such as IL- 4,10,11, or 13. One last set of specific cytokines, the chemokines are known to induce chemotaxis, a process in which chemokines are able to attract immune cells at a lesion site (C.E. Hughes *et al.*, 2018).

b. Interferons

Interferons, are a family of anti-viral cytokines named after their capacity to interfere with viral replication. Although more than 20 different IFNs have been described, only 3 IFN-receptors have been reported: IFNAR, IFNGR, IFNLR (E.V. Mesev *et al.*, 2019). These receptors are composed by 2 unique heterodimers units, which are responsible for the specificity of the binding. Upon IFN-receptor binding, two receptors associated proteins transmit the signal and further induce STAT1 or STAT2 transcription factors' nuclear translocation and the subsequent expression of interferons stimulated genes (ISGs) (S.A. Qureshi *et al.*, 1995). According to the recognized receptors, IFN are sorted into 3 sub-groups (E.V. Mesev *et al.*, 2019):

- the IFN-I: the largest group of IFN is composed by the IFN β , IFN ϵ , IFN ω and 13 types of IFN α . These IFN are specifically binding to the IFNAR.
- The IFN-II: this class is only composed by the IFN γ . These IFN are specifically binding to the IFNGR and therefore follow the classical one ligant - one receptor schem.

- The IFN-III: this group is composed by 4 types of IFN λ . These IFN are specifically binding to the IFNLR.

Importantly, in opposition to IFN-I and -III, the IFN γ are only inducing the phosphorylation of STAT1 and therefore, are not able to stimulate the expression of every ISGs. Their field of action is limited to ISGs bearing a so-called gamma-activated sequence (GAS) (L.C. Plataniias *et al.*, 2005). Furthermore, even though IFN-I and IFN-II are supposedly able to activate every ISGs, the reality is different and the pool of expressed genes is directly depending on the type of IFN and the cell type (W.M. Schneider *et al.*, 2014). As a consequence of these various pools of activated genes, the binding of an IFN results in a different cellular response such as antiviral immunity, proliferation, autophagy or apoptosis. This allows the cell to eliminate the threat by targeting multiple stages of the infection, for instance every step of the viral replication can be targeted (W.M. Schneider *et al.*, 2014; E. Yang and M.M.H. Li, 2020). Moreover, interferons are often produced in a cascade, meaning that one interferon stimulates its target cells to make additional interferons.

TBK1 is a crucial regulator of the type I IFN as described in the following section.

2. TBK1 activates IRF3

TBK1 is involved in the cascade of signalisation induced by five of the PRRs: the toll-like receptor 3 (TLR3) and 4 (TLR4), two RIG-I like receptors (RLR): RIG-I and MDA5 and the DNA sensing receptor cGAS (S. Liu *et al.*, 2015). Even if they are involved in the responses to different factors such as nucleic acid detection (X. Li *et al.*, 2013; C. Shu *et al.*, 2014) or bacterial and viral signatures (T. Kawai and S. Akira, 2006; J. Rehwinkel *et al.*, 2020), these 5 receptors all lead to the recruitment of TBK1/IKK ϵ , however, the intermediate factors involved in these signalling mechanisms are different. Indeed, within these pathways TBK1 is known to be recruited and to interact respectively with TRIF, MAVS-TRAF3 and STING (S. Liu *et al.*, 2015; T. Kawai and S. Akira, 2006; S. Paz *et al.*, 2011; C. Shu *et al.*, 2014). When activated these two closely related kinases phosphorylate interferon regulatory factor 3 (IRF3). P-IRF3 forms homodimers leading to their nuclear translocation, which further induce the expression of type I IFN and the subsequent immune response.

Interestingly, upon activation, these receptors also lead to the activation of the NF- κ B complex and the subsequent production of inflammatory cytokines by alternate pathways (Y. Liu *et al.*, 2017; T. Kawai and S. Akira, 2006).

These receptors are therefore crucial for the immune response to pathogen invasion and their dysregulation can have severe impact on the organism defence. For this reason, several viruses directly target members of these pathways to bypass the host defence (A. Zotta *et al.*, 2021; L. Qu *et al.*, 2011; M.H. Christensen *et al.*, 2016).

3. TBK1 activates NF κ B:

Although it was reported that its main function in the innate immune response was linked to the production of type I IFN, it is important not to forget its involvement in the production of inflammatory cytokines through the activation of NF κ B. Indeed, TBK1 was first discovered as a TANK binding partner involved in the regulation of NF κ B and repression of I κ B (J.L. Prochiantz and D. Baltimore, 1999). However, TBK1 dependent regulation was reported to be limited compared to its crucial contribution in the IFN synthesis (K.A. Fitzgerald *et al.*, 2003).

Nevertheless TBK1's contribution to NF κ B, although secondary, exists and the report of a specific NF κ B activation via activation of the cGAS-STING pathway supports this idea. Indeed, a recent study (S. Yum *et al.*, 2021) has designed a STING mutant line deficient in the STING-IRF3 interaction. However, this mutant line was still able to induce an anti-viral response to HSV-1, through the activation of the NF κ B response despite the impaired TBK1-phosphorylation of IRF3. This alternate innate immune response was prevented in another mutant STING line inhibiting the STING-TBK1 pathway suggesting that TBK1 was responsible for the cGAS-STING- NF κ B pathway.

It is unclear in what context the TBK1 dependent stimulation of NF κ B occurs but it was reported that this function was partially shared with IKK ϵ and that TBK1 loss could be compensated by IKK ϵ activity in opposition to IRF3 stimulation in which TBK1 activity is indispensable (K.R. Balka *et al.*, 2020). This further supports the theory of the unequilibrated dual role of TBK1 in IRF3 and NF κ B regulation. In addition, TBK1 has been reported to be involved in the regulation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome complex. NLRP3 is a NOD-like intracellular sensor that detects a

broad range of DAMPs or PAMPs, resulting in the formation and activation of the NLRP3 inflammasome (K.V. Swanson *et al.*, 2019). Upon activation, NLRP3 leads to the caspase 1-dependent release of the pro-inflammatory cytokines IL-1 β and IL-18 via activation of the NF κ B complex. NLRP3's activation is induced by the protein phosphatase 2A (PP2A) and is prevented by an inhibiting phosphorylation mark via TBK1 and IKK ϵ activity (F.A. Fischer *et al.*, 2021). Consequently, pharmacological and genetic inhibition of these two kinases has led to uncontrolled NLRP3 inflammasome response. However, the sole losses of either TBK1 or IKK ϵ were not sufficient to induce increased NLRP3 activation, suggesting redundant roles of the two kinases in its regulation (F.A. Fisher *et al.*, 2021). Besides the implication of TBK1 in this inflammation process, this suggests a more complex role of TBK1 in the regulation of NF κ B as it is in some context also preventing its activation.

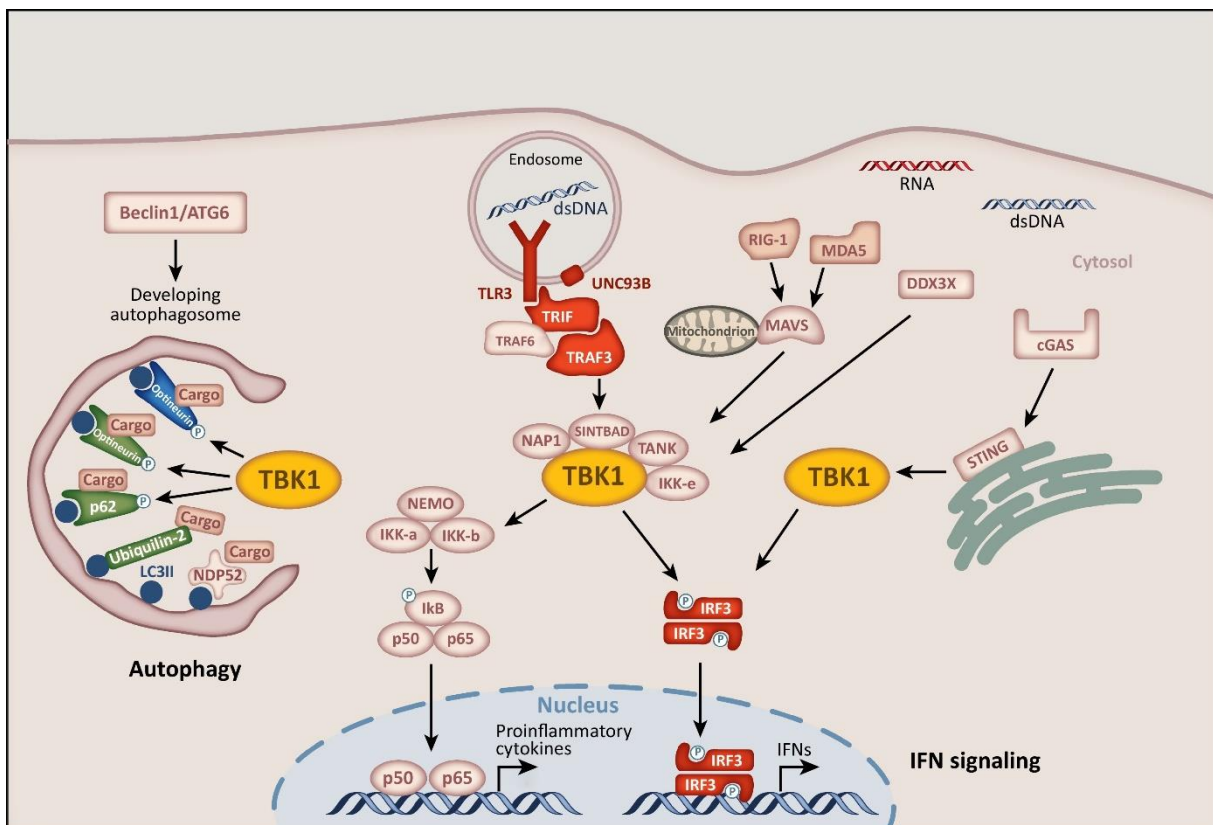


Figure 6: Schematic representation of TBK1 involvement in inflammation induction and phagophore maturation. (Derived from L. Ahmad *et al.*, 2016).

B. TBK1 as a regulator of the autophagic process:

Because TBK1 is mainly involved in the selective macroautophagy process, I will use the term “autophagy” to specifically describe this process. Autophagy initiation is regulated by the

activity of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a kinase complex involved in several cellular pathways. In the autophagy regulation, mTORC1 targets two kinases called autophagy activating kinase 1 and 2 (ULK1/2) for inhibitory phosphorylation. Upon autophagy activation, AMP-activated protein kinase (AMPK) in turn inhibits via phosphorylation mTORC1 (Y. Kiriya and H. Nohi, 2015), subsequently, ULK1/2 without their regulatory marks are free to interact with ATG13 and FIP200. FIP200 is phosphorylated by the ULK proteins within the newly formed complex which induces the formation of an immature membrane structure, the phagophore (C.H. Jung *et al.*, 2009). Formation of the immature phagophore triggers the recruitment of a second complex the phosphatidylinositol 3-kinase class III (PI3K-cIII) complex, which mediates the elongation of the phagophore and the subsequent envelopment of the proteins or organelles marked for degradation (J.H. Hurley and L.N. Young, 2017). An additional complex contributing to the phagophore's maturation is the ATG12-ATG5-ATG16 complex, which allows production of LC3-II (N. Mizushima *et al.*, 1998; M. Walczak and S. Martens, 2013). The newly formed LC3-II molecules bind to the surface of the phagophore and act as anchor for the autophagy cargo proteins in addition to their role in the elongation of the phagophore membrane (Y. Kiriya and H. Nohi, 2015). Once this process of maturation is done, the newly formed vacuole is called an autophagosome. Subsequently, the autophagosome is transported along microtubules to fuse with a low pH organelle called lysosome. The fusion of these structures is called an autophagolysosome, and the content of these structures digested by low-pH dependent enzymes (J.A. Oakes *et al.*, 2017).

Protein or organelles that are targeted to autophagy dependent degradation undergo a ubiquitin posttranscriptional modification, which consists in the addition of a ubiquitin chain on a target lysine residue (M. Rape, 2017). These ubiquitin chains are later recognized by autophagy cargo proteins such as p62, OPTN, NDP52, (D. Gatica *et al.*, 2018; J.M. Heo *et al.*, 2015) which are phosphorylated by TBK1. Autophagy cargo proteins are binding to the anchored phagophore LC3-II proteins to sequester the ubiquitinated components for subsequent autophagosome engulfment (T. Johansen and T. Lamark, 2020).

TBK1 regulates the autophagic process at several levels. As described above, TBK1 is a known regulator of autophagy cargo proteins. Indeed, it has been reported that TBK1 dependent phosphorylation on the 3 cargo proteins p62, OPTN, and NDP52 (J.M. Heo *et al.*,

2015) regulates their activity and enhance their binding capacity to LC3-II and to ubiquitinated compounds. The importance of TBK1 in this crucial process is underlined by decreased p62 mRNA levels in ALS patients bearing a *TBK1* mutation (C. Pottier *et al.*, 2015) which could result in the impairment of the autophagy process and the accumulation of the cytosolic inclusions. TBK1 is also known to interact and phosphorylate SMCR8, a member of the C9orf72 complex along with WDR41. A direct interaction between the C9orf72-SMCR8-WDR41 complex and the FIP200/ULK1/ATG13 complex was reported and it was suggesting that the C9orf72 complex was a substrate downstream of ULK1 (P.M. Sullivan *et al.*, 2016). Altogether the C9orf72 complex was suggested to regulate autophagy at 3 different levels: on the autophagy initiation, on the autophagic flux (M. Yang *et al.*, 2016), and on the lysosomal initiation (J.G. O'Rourke *et al.*, 2016). TBK1 also regulates autophagy by interacting with mTORCs. Indeed, TBK1 has been reported to stimulate via direct phosphorylation both of the mTOR complexes mTORC1/2 (C. Bodur *et al.*, 2017; A.S. Tooley *et al.*, 2021). This observation exhibits a dual role of TBK1 in both activating autophagy (via interaction with adaptors proteins and SMCR8 phosphorylation) and inhibiting autophagy (via stimulation of mTORC1 a known inhibitor of the autophagy initiation). Moreover, TBK1 has also been reported to activate RAPTOR, a known inhibitor of mTORC1 (R.J. Antonia *et al.*, 2019). Altogether, this suggests that TBK1 is an important regulator of mTORC1 activity and subsequent autophagy, however, more investigations would be required in order to understand in which context each of the regulation is occurring.

Finally, TBK1 is also regulating autophagy process indirectly through IRF3 activation and subsequent type-I interferons production. Indeed, amongst the interferon-inducible genes, ISG15 have been linked to the activation of autophagy (D. Xu *et al.*, 2015; H. Nakashima *et al.*, 2015).

C. Cell death in ALS:

As previously introduced (cf: part I section B.9), programmed cell death (PCD) mechanisms have been suspected to be responsible for the motor neuron degeneration in ALS. I will discuss the evidence reported of the involvement of these pathways in ALS pathophysiology and provide evidence for the functional roles of TBK1 within these pathways.

1. Programmed cell death:

a. Apoptosis

The discovery and definition of the apoptosis process can be attributed to John Kerr in 1972 (J.F.R Kerr *et al.*, 1972). It was considered for a long time as the only cause of PCD.

Apoptosis can be triggered by 2 distinct pathways:

- The mitochondrial (or intrinsic) induction

In response to cellular stress, a protein family called the BH3-only proteins are being upregulated (H. Puthalakath and A. Strasser, 2002). These crucial initiators of apoptosis subsequently bind and sequester the anti-apoptotic BCL-2 protein family (L. Chen *et al.*, 2005; G.L. Kelly and A. Strasser, 2019). These last proteins, in a non-apoptotic context, are preventing cell death by sequestering the two essential effectors of apoptosis: BAX and BAK (G.L. Kelly and A. Strasser, 2019). When inhibited by the BH3-only proteins, BCL-2 proteins no longer sequester BAX and BAK, which are therefore free to form oligomers. The formation of these secondary structures causes a mitochondrial outer membrane permeabilization which induces the release of both cytochrome c and DIABLO resulting in the subsequent activation of the caspases cascade. Caspases (Casp) are a family of protease that induces cleavages of hundreds of proteins upon apoptosis activation. The degradations of these proteins eventually lead to cell death.

- The death receptor (or extrinsic) induction:

Although it results in a similar cell death mechanism than the mitochondrial induction, the death receptor pathway is triggered by external stimuli. As mentioned before, cells bear PRRs at the surface of their plasma membrane to facilitate cell-cell communication. The TNF family of receptors are involved in the inflammatory process. Upon activation, TNF receptors induce the formation of an intracellular death-signalling complex (D. Moujalled *et al.*, 2021). The formation of this complex results in the activation of CASP8 and its downstream effector CASP 3/7 (H.R. Stennicke *et al.*, 1998; M. Lamkanfi and T-D. Kanneganti, 2010), which results in the subsequent trigger of apoptosis. Interestingly, these two receptors have been shown to connect as CASP8 activation can lead to the stabilisation of a BH3-only protein, BID which leads to mitochondrial outer membrane permeabilization (H. Li *et al.*, 1998; X. Luo *et al.*, 1998).

Upon apoptotic death, cells appear smaller with tightly packed organelles and chromatin condensation. At the final stage of the process, an extensive plasma membrane blebbing and a nuclear fragmentation are reported, resulting in the formation of apoptotic bodies and their release in the extracellular environment, which are eliminated by neighbouring cells via phagocytosis (S. Elmore, 2007; R. Kakarla et al., 2020).

b. Necroptosis

Historically, most researchers believed that cell death exists as a dichotomy between apoptosis and necrosis (L.J. Martin *et al.*, 1998) until it became clear that some specific cases of cellular death were not fitting in either of these boxes (C. Portera-Cailliau *et al.*, 1997). Necroptosis was only described in 2000 (N. Holler *et al.*, 2000; T. Vanden Berghe *et al.*, 2014) and is usually described as an active form of necrosis.

Importantly, necroptosis initiation is dependent of receptor-interacting serine/threonine protein kinase 1 (RIPK1) activity. RIPK1 itself is regulated by several post-transcriptional marks: phosphorylation by MAK2, TBK1, the IKK complex, TAK1 or by autophosphorylation and via polyubiquitination by cIAP2/5 and the LUPAC complex (T. Delanghe *et al.*, 2020). Necroptosis initiation is directly dependent on the deubiquitination process of RIPK1 by Lys63-deubiquitylating enzyme cylindromatosis (CYLD) (P. Vandenabeele *et al.*, 2010). However, CASP8 has been reported to cleave CYLD and subsequently prevent the activation of RIPK1 (M.A. O'Donnell *et al.*, 2011). Therefore, necroptosis initiation relies on the blockade of CASP8's activity by degradation via viral or pharmacological agent or by sequestration by cFLIPs proteins (T. Kataoka, 2005; B. Tummers and D.R. Green, 2017). Indeed, upon TNF receptor activation in a CASP8 deprived context, RIPK1, via deubiquitination by CYLD, is able to perform an activating autophosphorylation (L. Laurien *et al.*, 2020). This post-transcriptional mark allows the formation of a complex called necrosome which leads to activation of RIPK3 and subsequent activating of the mixed lineage kinase domain like pseudokinases (MLKL). This is the terminal effector of the necroptosis pathway and after oligomerization, MLKL chains translocate to the plasma membrane where they dysregulate its integrity (L. Sun *et al.*, 2012; J.M. Murphy *et al.*, 2013; A.L. Samson *et al.*, 2020). This results in membrane disruption and rapid influx of Ca²⁺ ions and water leading to the dissolution of cell.

Because this type of cell death is characterized by the disruption of the membrane, numerous cell components are released in the extracellular medium. The cellular debris resulting from necroptosis activation is recognized as DAMPs or PAMPs (in case of infected cells) further leading to the initiation of the inflammation response (M. Pasparakis and P. Vandenabeele, 2015).

2. Evidence of programmed cell death in ALS:

a. *Apoptosis in ALS:*

Multiple reports have described apoptosis-mediated cell death involved in the massive motor neuron degeneration observed in ALS. The first suspicion of apoptosis involvement came from preliminary observations reporting altered expression of BCL-2 and increased levels of BAX in the spinal cord of ALS patients (X. Mu *et al.*, 1996) and in a SOD1 mutant model (J.L. Gonzalez de Aguilar *et al.*, 2000). Consecutively, sequestration of BCL-2 by SOD1 mutant proteins was reported in the murine SOD1^{G93A} line suggesting an important role of SOD1 mutant in regulating BCL-2 protein and therefore possible trigger of the apoptosis process without active intrinsic signalling (P. Pasinelli *et al.*, 2004). The involvement of BCL-2 was further exposed as an overexpression of this anti-apoptotic protein was reported to extend the survival of the mSOD1 (S. Vukosavic *et al.*, 2000). Dysregulation of the anti-apoptotic machinery with neuronal apoptosis inhibitory protein (NAIP), a member of the anti-apoptosis IAP family (N. Roy *et al.*, 1995) shown to selectively suppress oxidative stress-induced cell death (P. Liston *et al.*, 1996), was reported to be decreased in ALS patients (O. Kano *et al.*, 2018). These results were further confirmed as the use of bromocriptine, a positive regulator of NAIP and the antioxidant response, was reported to extend survival in a murine mSOD1 model (K. Tanaka *et al.*, 2011). Similarly, evidence of increased levels of pro-apoptotic compounds have been reported with both of the BAX and BIM proteins upregulated respectively in patients and mSOD1 murine model (T. Ekegren *et al.*, 1999; C. Hetz *et al.*, 2007). Moreover, deficiencies in both of these proteins were reported to ameliorate mSOD1 mouse phenotype and extend their survival (N.A. Reyes *et al.*, 2010; C. Hetz *et al.*, 2007). Finally, caspase activation has also been reported to be involved in the development of the pathology (P. Pasinelli *et al.*, 2000; H. Inoue *et al.*, 2003), which has been further confirmed by the use of a broad anti-caspase drug ameliorating survival still in the mSOD1 mouse model (M. Li *et al.*, 2000).

Overall, these results suggest an activation of the apoptotic pathway as the major cause of neurodegeneration in the context of ALS. However, it is important to underline that these studies mostly focused on SOD1 associated ALS models.

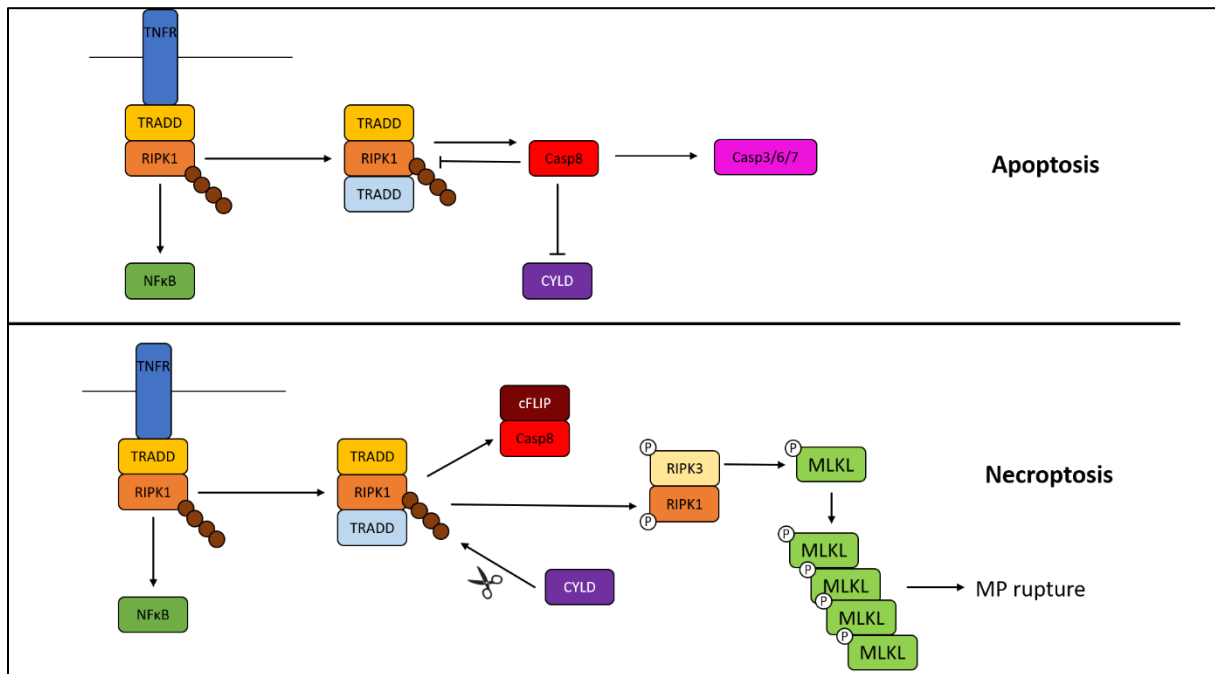


Figure 7: Schematic comparative illustration of Casp8 dependent apoptosis and necroptosis induction

b. Necroptosis in ALS

Consistent results of necroptotic involvement in the development of ALS have been reported these last years with reports of RIPK1 playing a crucial role in the course of the disease. Indeed, first evidences came from *in vitro* modelling from mSOD1 mouse and ALS patients, exhibiting a caspase independent form of PCD with a morphology that closely resembles to necrosis (D.B. Re *et al.*, 2014). Incubation with specific necrostatin-1, a RIPK1 specific inhibitor, and necrosulfonamide, a MLKL specific inhibitor, led to the rescue of the cellular death, highly suggesting the involvement of necroptosis in this model (D.B. Re *et al.*, 2014). Still in this direction, several studies have identified robust activation of the different members of the necrosome complex in an *in vitro* OPTN KD mouse model (Y. Ito *et al.*, 2016), in mSOD1 mouse (G. Dermentzaki *et al.*, 2019) or in ALS patients (M. Chevin and G. Sébire, 2020). Moreover, a double mutant TBK1-TAK1 KD murine model was reported to exhibit alleviated phenotype upon pharmacological deletion of RIPK1 (D. Xu *et al.*, 2018).

However, a recent study described a minimal implication of necroptosis in ALS, via a lack of impact from the deficiency of MLKL in the mSOD1 murine model (T. Wang *et al.*, 2019).

Results concerning RIPK1 upregulation have to be taken with caution as this kinase has also been reported to be involved in a specific type of apoptotic cell death pathway via the formation of a RIPK1-FADD-CASP8 complex (D. Ofengeim and J. Yuan, 2013). Therefore, an increase in RIPK1 expression could lead to activation of either apoptosis or necroptosis.

3. TBK1 involvement in programmed cell death

TBK1 is a known endogenous RIPK1 regulator via an inhibitory phosphorylation mark on the T189 RIPK1 residue (D. Xu *et al.*, 2018; T. Delanghe *et al.*, 2020). This phosphorylation has been reported to be an important regulator of cell death in several reports (E. Lafont *et al.*, 2018; Y. Yang *et al.*, 2022; D. Xu *et al.*, 2018). Interestingly, it was shown that TAK1, another kinase regulator of RIPK1, expression was decreased in aging human brain (D. Xu *et al.*, 2018), therefore exhibiting the aged increased sensitivity to TBK1 dysregulation in the inhibition of RIPK1 activity and further giving a possible link with the late-onset ALS disease. As a regulator of RIPK1 activity, a critical protein in the regulation of two critical PCD pathways, TBK1 appears as an important pro-survival protein.

In addition, TBK1 has been reported to directly regulate via phosphorylation the activity of the pro-survival AKT protein (Y-H Ou *et al.*, 2011). AKT is an important member of the mTOR pathway, stimulating the formation of the mTORC1 complex and further cell proliferation. These observations made a clear link between TBK1 activity and pro-survival signal.

III. TBK1 as an ALS-FTD associated gene:

A. Epidemiology and genetic features of TBK1 related ALS-FTD:

In 2015, two independent studies have exhibited a link between *TBK1* mutations and ALS through a whole exome sequencing on a European background population (E.T. Cirulli *et al.*, 2015) and a Germano-Swedish population (A. Freischmidt *et al.*, 2015). Several studies have further confirmed the involvement of TBK1 in ALS-FTD (I. Gijssels *et al.*, 2015; L. Kelly *et al.*, 2015). Approximately 0.5% of ALS cases are linked to a mutation in *TBK1* (I. Le Ber *et al.*, 2015), even though this percentage is rather low, *TBK1* remains one of the most common low-frequency mutations associated with ALS. Moreover, this percentage greatly increases

when considering ALS patients presenting an FTD comorbidity with up to 10% of cases associated with *TBK1* mutations (K.L Williams *et al.*, 2015; I. Le Ber *et al.*, 2015). *TBK1* variants have also been associated with the sole development of FTD without any motor involvement (C.A.M. Koriath *et al.*, 2017; C. Pottier *et al.*, 2015). It is considered that variations in the sequence of *TBK1* are responsible for approximately 3% of the total FTD cases (C. Pottier *et al.*, 2015) making it the fourth genetic cause of development of the FTD (S. Van Mossevelde *et al.*, 2016). Studying the prognosis of *TBK1* mutation carriers can be challenging due to the reduced numbers of patients compared to the other main genes associated with the disease. A study following 16 *TBK1* mutation carriers reported a median age of onset of 62.1 with a mean disease duration of 4.7 years (S. Van Mossevelde *et al.*, 2016).

Since the initial identification of *TBK1* mutations in ALS, more than 50 non-sense or missense variants throughout the entire sequence of the *TBK1* gene have been described (M. de Majo *et al.*, 2018). This suggests that the ALS-FTD pathology is more probably linked to a loss-of-function mechanism. So far, no gain-of-function mechanism induced by a *TBK1* variant has been reported. Therefore, the actual paradigm concerning *TBK1* associated ALS-FTD is an haploinsufficiency mechanism (A. Freischmidt *et al.*, 2015; M. de Majo *et al.*, 2018).

B. *TBK1* related ALS-FTD pathogenicity:

Overall, ALS patients bearing a *TBK1* mutation show a median age of onset compared to other types of ALS (M. Weinreich *et al.*, 2019; P.A. McCombe *et al.*, 2019; S. Van Mossevelde *et al.*, 2016; I.J. Swift *et al.* 2021). Only one study brings together data from different clinical studies concerning focusing on ALS patients bearing *TBK1* variants (I.J. Swift *et al.* 2021). From this study, analysing 110 cases, *TBK1* associated ALS patients seem to present a propensity of limb-onset with 63% of the cases concerned, in opposition to 32% with bulbar-onset, and 5% with respiratory-onset (I.J. Swift *et al.* 2021). In opposition to what could be expected due to *TBK1* involvement in the development of FTD, only 19% of the total cases had a cognitive presentation (I.J. Swift *et al.* 2021). Moreover, out of these cases presenting cognitive impairment, 29% were presenting an FTD-ALS comorbidity and 29% an unspecified dementia (I.J. Swift *et al.* 2021). Additionally, out of the 110 patients of the study, 4% were characterized by an atypical parkinsonian presentation (I.J. Swift *et al.* 2021) suggesting that *TBK1* could be implemented in the gene panel associated with development of atypical

parkinsonism. Interestingly, another study has reported alteration of the metabolism characterized by a marked hypermetabolism, weight loss, reduced fat mass and reduced appetite in *TBK1* mutation carriers patients (P.A. McCombe *et al.*, 2019). Several clinical studies have focused on cases of FTD only pathology associated with a *TBK1* mutation (C. Wilke *et al.*, 2017; Koriath *et al.*, 2016; C. Pottier *et al.*, 2015). Interestingly, these cases exhibit specific clinical features with mesencephalon and cerebellum injuries suggesting that *TBK1* associated phenotypic spectrum could be extended beyond ALS and FTD and also include progressive supranuclear palsy-like and cerebellar syndromes (C. Wilke *et al.*, 2017).

C. Modelling *TBK1* related ALS-FTD:

In the context of ALS, 2 main *in vitro* models have been developed. First the generation of hiPSC derived *TBK1*-mutant motor neurons exhibiting a reduced viability and accumulation of p62 positive cytosolic inclusions due to the failure of the phagophore's elongation phase (A. Catanese *et al.*, 2019). The second *in vitro* model developed is a human embryonic stem cells (ESCs) derived motor neurons line characterized by the expression of a *TBK1* loss-of-function mutant. This deficiency in *TBK1* activity has been linked to an impaired autophagic flux with deficient endosomal maturation and lysosomal function associated with an increased cytoplasmic and insoluble TDP-43 accumulation (J. Hao *et al.*, 2021). Interestingly, this cellular model also revealed that the loss of activity of *TBK1* in hMN was linked to an hyperexcitability state (J. Hao *et al.*, 2021).

Murine models have been difficult to establish as *Tbk1* deletion induces an early embryonic death at E14.5 due to massive liver degeneration and apoptosis (M. Bonnard *et al.*, 2000). This suggests an important role of *TBK1* in the development. To palliate this lethality, conditional deletion of *Tbk1* in neurons have been developed. In 2014, a study performing a T-cell specific *Tbk1* deletion in mice exhibited an over-activation of T-cell leading to a neuroinflammatory autoimmunity model due to the activation of the mTORC1 pathway (J. Yu *et al.*, 2014). This suggests a role of *TBK1* in the regulation of T-cell activation in the murine model. More recently, conditional neuronal *Tbk1* deletion in mice has been shown to induce several neuropathological changes as reduced dendritic spine density, cortical spinal loss, abnormal dendritic projections and p62/ubiquitin positive cytoplasmic inclusions in the Purkinje cell of the cerebellum (W. Duan *et al.*, 2019).

However, loss of *Tbk1* specifically in motor neurons did not lead to a neurodegenerative disease phenotype (V. Gerbino *et al.*, 2020). Moreover, a sole hemizygous loss of function is insufficient to provoke any phenotype (D. Brenner *et al.*, 2019; V. Gerbino *et al.*, 2020). Surprisingly *Tbk1* hemizygous loss in mice expressing ALS-linked SOD1^{G93A} mutant inversely modulates early and late already characterized phenotype: at the early stage, the heterozygous deletion impairs autophagy and accelerates the muscle denervation, whereas at later stages it alleviates microglial neuroinflammation and consequently decelerates the disease progression and extends the survival (D. Brenner *et al.*, 2019; V. Gerbino *et al.*, 2020). However, the conditional deletion of *Tbk1* in SOD1^{G93A} motor neurons increases the SOD1 aggregation and accelerates early disease onset without affecting lifespan (V. Gerbino *et al.*, 2020). These results unveil the importance of TBK1 in several pathways according to the cell types. More recently, a double mutant murine model bearing a heterozygous deletion of *Tbk1* and expressing the ALS associated human mutant TDP-43^{G298S} has been developed. In this model, the monoallelic loss of *Tbk1* aggravated the characterized muscle denervation but didn't impact the motor neuron loss or the reactive gliosis in the spinal cord (K. Sieverding *et al.*, 2020). This further suggests a role of TBK1 in the neuromuscular synapse integrity.

In addition, two recent publications have developed two independent *Tbk1* murine models which gave great insights on the understanding of TBK1 associated ALS pathology. In 2018, a study has implemented the already characterized and lethal *Tbk1* KO murine model with a new kinase dead knock-in mutation in the *Ripk1* gene (D. Xu *et al.*, 2018). These genetic manipulations resulted in the prevention of the lethality previously observed. This study therefore revealed an important role for TBK1 in the regulation of RIPK1 a crucial actor of the CASP8 apoptosis and necroptosis. Furthermore, D. Xu also reveals during his work that combined inhibition of both TBK1 and TAK1 (another kinase involved in TNF receptor response and NFκB's activation) were sufficient to induce RIPK1 activation and further neuroinflammation. Interestingly, this study has also reported that levels of TAK1 protein were decreased in an age-related manner. Therefore, these results describe a model for TBK1 associated development of ALS-FTD where a heterozygous loss of TBK1 is supported by the age-dependent decreased expression of TAK1 leading to the development of a late-onset disease such as ALS-FTD. The hypothesis of a double-hit model resulting in the induction of

TBK1 associated ALS pathology was further supported by a second murine model published in 2022. In this recent landmark study, W. Shao has reported the interplay between two disease associated genes *C9orf72* and *Tbk1*. Indeed, in a murine model bearing a GGGGCC repeat expansion within the *C9orf72* gene, TBK1 was found to be sequestered in cytoplasmic inclusions induced by the expression of these repeats. Interestingly, the double knock-out of both *C9orf72* and TBK1 worsens the condition of the mouse via endosomal disruption. Therefore, these two studies lead to the hypothesis that the sole loss of *TBK1* might not be sufficient to induce the pathology. Therefore, there is abundant emerging evidences that there is a combination of *TBK1* dysregulation and other age-related factors or additional genetic alterations that could result in the development of motor neuron degeneration upon *TBK1* mutations or reduced activity of this key factor.

Part III: metabolites dysregulation in ALS

Metabolites are an emerging topic in the ALS research as they could help the diagnosis of the patients and therefore apprehend early-dysregulations responsible for the development of the disease (H. Blasco *et al.*, 2020).

In the following section, I will review some of the main observations that have been reported in the literature describing metabolomics alterations in ALS.

I. Hypermetabolism as a cardinal feature in ALS:

Alteration of the nutritional status and its subsequent loss of weight is a common symptom of ALS patients (S.T. Ngo *et al.*, 2019). The combination of two factors is suspected to be responsible for this feature: the loss of appetite and an increased resting energy expenditure (REE) (M. Kurihara *et al.*, 2021; F.J. Steyn *et al.*, 2018). The REE represents the amount of energy required in a 24-hour period by the body in a resting state without activity. When an organism exhibits an increase in this metabolism indicator, we talk of the hypermetabolism state, which means that the body is in a higher demand of energy (L. Dupuis *et al.*, 2011).

At the cellular level, hypermetabolic features are translated through dysregulated glucose levels, which are reported as reduced in both patients and animal models (T. Lerskiatiphanich *et al.*, 2022; T.S. McDonald *et al.*, 2020). Physiologically, glucose is transferred to the cells via the activity of the GLUT family receptors. Following glucose

uptake, it is either stocked under the form of glycogen which constitute stocks for a later use or it undergoes a series of enzymatic reactions resulting in the production of ATP. Two hormones tightly regulate the glucose levels: insulin and glucagon. Insulin is secreted in the pancreas in response to high levels of glucose in the blood, promoting the glucose uptake by the peripheral tissues. In opposition, glucagon is secreted when plasma glucose levels are too low to induce the production of glucose via gluconeogenesis and glycogenolysis. Both the uptake of glucose and its regulation are suspected to induce the hypermetabolic state observed in ALS patients (T. Lerskiatiphanich *et al.*, 2022).

Glucose uptake is reduced in CNS of both ALS patients and animal model (T.W Tefera *et al.*, 2021). Supporting this observation, overexpression of the GLUT3 receptor in a *drosophila* TDP-43 ALS model, was reported to be beneficial and alleviates features of the TDP-43 proteinopathy (E. Manzo *et al.*, 2019). In contrast, glucose uptake in peripheral tissue such as skeletal muscles, liver or adipose tissue was reported to be upregulated in the context of ALS (T.S. McDonald *et al.*, 2021). Interestingly, muscle that relies mostly on anaerobic ATP production through glycolysis (glycolytic muscle fibres) have been reported to be decreased in murine SOD1 ALS model (L. Palamiuc *et al.*, 2015; S. Scaricamazza *et al.*, 2020). This reduction in muscle anaerobic ATP production was linked to the impaired activity of phosphofruktokinase, a key enzyme in the glucose metabolism (L. Palamiuc *et al.*, 2015). With a combination of deficient glucose metabolism and mitochondrial damage, muscle could then switch to a lipidic aerobic ATP production (L. Palamiuc *et al.*, 2015; S. Scaricamazza *et al.*, 2020).

Moreover, glucose can be stored for later use under the form of glycogen by glycogenesis. In ALS patients and animal models, glycogen accumulation has been also reported (L. Palamiuc *et al.*, 2015; T.W Tefera *et al.*, 2021; T.S. McDonald *et al.*, 2021). This accumulation has been associated with a reduced expression of glycogen phosphorylase and an enhanced activity of glycogen synthase (L. Palamiuc *et al.*, 2015; T.W Tefera *et al.*, 2021) further suggesting impairment in the glucose metabolism. Supporting this hypothesis, some enzymes of the TCA cycle were found to be downregulated, suggesting that a decreased utilization of the glycogen stocks could be linked to an impaired capacity of producing ATP via glucose.

Altogether, these data suggest an impaired ATP production via glucose degradation, leading to a metabolic switch towards ATP production via enhanced lipid degradation. This

hypothesis is supported by the several dysregulations in lipid levels observed in the ALS context.

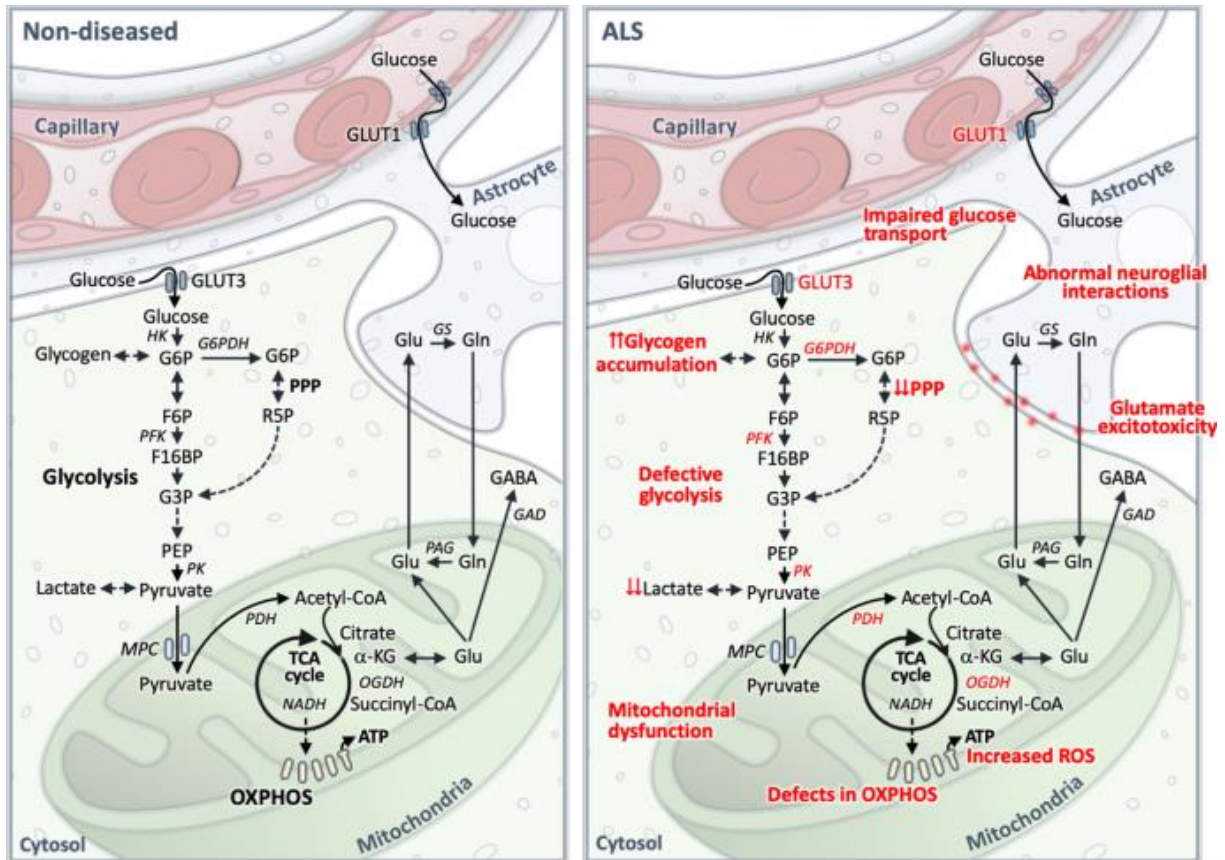


Figure 8: Schematic illustration of CNS glucose metabolism involvement in a healthy nervous system and the commonly observed defects observed in ALS. (Derived from T.W Tefera *et al.*, 2021)

II. Lipidic dysregulation in ALS:

Lipids are involved in several cellular processes: they constitute a source of energy storage, they are involved in signalling, they constitute the structural components of every cell membranes, they can be used as protein recruitment platforms, and are substrates for post-translational protein–lipid modifications (T. Harayama and H. Riezman, 2018). Due to their high concentration in the CNS, lipids dysregulation has been associated with several neurodegenerative diseases and an alteration in their levels can be induced by multiple causes.

There are eight main families of lipids, however only four of those are associated with ALS, namely the sterols, the glycerolipids, the sphingolipids and the glycerophospholipids (J.M. Godoy-Corchuelo *et al.*, 2022). Although dysregulations are diverse amongst these pathways some of their members have been considered as potential biomarkers of ALS.

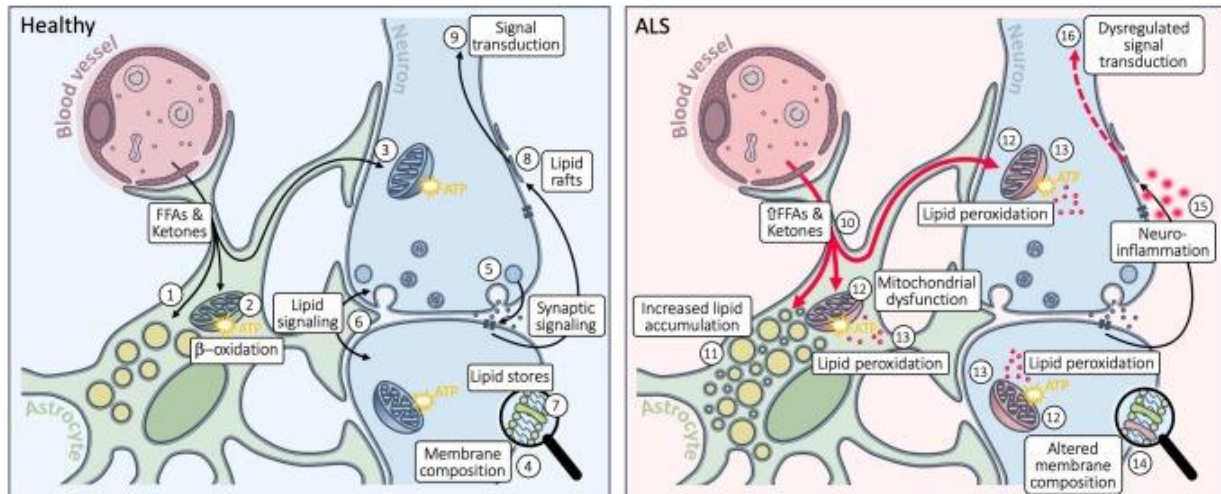


Figure 9: Schematic illustration of lipids involvement in a healthy nervous system and the observed consequences induced by lipidic dysregulation in ALS patients. (Derived from T.J. Tracey *et al.*, 2020)

The main representative of the sterol family is cholesterol. Cholesterol and its associated lipids have been extensively measured and reported to be increased in ALS patients (J. Abdel-Khalik *et al.*, 2017; R.G. Cutler *et al.*, 2002). These observations were confirmed in ALS murine *FUS* and *SOD1* mutant lines (T. Burg *et al.*, 2021; A.B. Chaves-Filho *et al.*, 2019). Moreover, the reported dysregulation of enzymes from this metabolic pathway in patients (J.C. Dodge *et al.*, 2020) and mice models (L.C. Fernández-Beltrán *et al.*, 2021) have further supported these observations. Interestingly, due to its altered levels, cholesterol has been suggested to be a potential prognosis marker in patients (L. Dupuis *et al.*, 2008; B. Chełstowska *et al.*, 2020; R. Nakamura *et al.*, 2022).

Glycerolipids, due to their specific ALS-associated signature, have also been considered as potential biomarkers of the disease course in patients (R. Huang *et al.*, 2015; J. Sol *et al.*, 2021). Indeed, according to the number of glycerols group bore by the compound, they are reported to be either down- or upregulated in patient's blood samples (E. Area-Gomez *et al.*, 2021; G. Fernández-Eulate *et al.*, 2020; S.A. Goutman *et al.*, 2022) but downregulated in the

brain or spinal cord (J.C. Dodge *et al.*, 2020; J. Hanrieder and A.G. Ewing, 2014). Interestingly, the increased level of one specific glycerolipid: arachidonic acid, seems to be shared in all types of ALS (H. Lee *et al.*, 2021; N. Shibata *et al.*, 2010).

Sphingolipid metabolism has also been reported to be dysregulated in ALS patients' blood samples (J. Sol *et al.*, 2021; E. Area-Gomez *et al.*, 2021). Interestingly, these decreased levels are accentuated along the course of the disease, and therefore could constitute an optimal biomarker to supervise the disease progression. In opposition, brain and spinal cord tissues from ALS patients present increased levels of sphingolipids (R.G. Cutler *et al.*, 2002; J.C. Dodge *et al.*, 2015; A. Henriques *et al.*, 2017), this observation was further confirmed in murine model (A. Henriques *et al.*, 2018; L.C. Fernández-Beltrán *et al.*, 2021).

Finally, the last lipidic family identified as dysregulated in ALS patients are the glycerophospholipids (E. Area-Gomez *et al.*, 2021; S.A. Goutman *et al.*, 2022). Similarly to what observed in the other lipid metabolisms, their levels are decreased in blood samples of ALS patients and increased in brain and spinal cord tissues. One specific glycerophospholipid in particular, phosphatidylcholine, has been described as a relevant biomarker of the disease as its dysregulation seems to be shared in all ALS subtypes (H. Blasco *et al.*, 2017; J. Sol *et al.*, 2021).

III. Kynurenine pathway and associate metabolisms:

A. The kynurenine pathway:

The kynurenine pathway is the main metabolism of tryptophan degradation. Products of the kynurenine pathway are further catabolized in several subsequent metabolites routes: the serotonin pathway, the immune related tetrahydrobiopterin (BH4) pathway, glycolysis, and the *de novo* nicotinamide adenine dinucleotide (NAD⁺) synthesis (V.X. Tan and G.J. Guillemin, 2019). The kynurenine pathway is active in every cell but predominantly in liver and activated in monocytic cells during inflammation (M.P. Heyes *et al.*, 1997; S.P. Jones *et al.*, 2015). Due to their capacity to cross the blood-brain barriers, some of the metabolites of this pathway such as kynurenine, 3-hydroxykynurenine, or tryptophane raised the interest as they could be used as a biomarker to supervise the progression of the disease (S. Fukui *et al.*, 1991).

Indeed, this metabolism pathway leads to the synthesis of several neuroactive toxic compounds such as quinolinic acid and 3-hydroxykynurenine (only in monocytes activated cells upon IFN- γ stimulation) (J.R. Moffett *et al.*, 2020). *In vitro*, these neurotoxic compounds have been shown to induce neurodegeneration and neuronal cell death through excitotoxicity, N-methyl-D-aspartate receptor antagonism, increased glutamate release, and the production of reactive oxygen species (L. Khaspekov *et al.*, 1989; J.P. Kim and D.W. Choi, 1987; S. Okuda *et al.*, 2002; G. Leinritz *et al.*, 2007; P. Pierozan *et al.*, 2014; J.-M. Lee *et al.*, 2017). Interestingly, all these processes overlap with mechanisms of neurodegeneration in ALS. Moreover, this pathway also leads to the production of neuroprotective compounds such as respectively kynurenic acid and picolinic acid by astrocytes and neurons respectively (R.S. Grant *et al.*, 2009; C. Klein *et al.*, 2013).

In the context of ALS, increased levels of quinolinic acid, tryptophan, kynurenin and decreased levels of picolinic acid were reported in both CSF and patients' plasma (Y. Chen *et al.*, 2010; J.-M. Lee *et al.*, 2017). These increased levels of kynurenine associated metabolites correlate with the increased neuroinflammation observed along the progression of the disease and are suspected to drive neurodegeneration. Kynurenine dysregulation has already been identified to regulate inflammation (G.J. Guillemin *et al.*, 2003), therefore the modulation of this metabolism is considered as an interesting therapeutic target (K.R. Jacobs and D.B. Lovejoy, 2018). In the nervous system, dysregulation in kynurenine pathway and increased levels of quinolinic acid have been associated with the development of several pathologies such as schizophrenia (M.E. Kegel *et al.*, 2014; F. Cathomas *et al.*, 2021), Parkinson disease (P.A. Lewitt *et al.*, 2013; D. Venkatesan *et al.*, 2020), multiple sclerosis (C.K. Lim *et al.*, 2017), Alzheimer disease (Z. Hartai *et al.*, 2007; G.J. Guillemin *et al.*, 2013) and Huntington (N. Stoy *et al.*, 2005; P.R. Sanberg *et al.*, 1989). Therefore, its link to a neurodegenerative disease such as ALS is not surprising. Although this confirmed the use of biomarkers derived from the kynurenine metabolism to accurately quantify the progression of the disease, it also prevents their use as diagnosis biomarkers as they are not specific to ALS pathophysiology.

Besides the kynurenine pathway, it is interesting to look at the impact of such dysregulation on metabolism deriving from the tryptophan catabolism, including serotonin and NAD⁺ metabolism.

B. Serotonin metabolism:

Due to its association with the regulation of motor neuron excitability and the subsequent global control of motor behaviour and its involvement in neuronal maturation and aging (A. Barreiro-Iglesias *et al.*, 2015; R. Sandyk, 2006), serotonin have been considered as a therapeutic target in the context of ALS for a long time. Moreover, it was observed that the levels of serotonin were reported to be reduced in ALS patients (L. Dupuis *et al.*, 2010). Interestingly, MN that are the most affected in ALS pathology are also the most densely innervated by serotonin neurons. In opposition, the MN that are the most resistant to ALS-related neurodegeneration receive smaller inputs from the serotonin neurons (R. Sandyk, 2006). Physiologically, serotonin interacts at the synapse with 5-HT1 receptors which are involved in the inhibition of the glutamate excretion (A. Barreiro-Iglesias *et al.*, 2015). Therefore, a reduced level of serotonin could easily be linked to an increased synaptic glutamate release and its subsequent neurotoxicity. For these reasons, exogenous input of serotonin has been reported to be beneficial on *in vivo* animal ALS models (B.J Turner *et al.*, 2003; P. He *et al.*, 2022). Furthermore, melatonin, the major downstream metabolite of serotonin metabolism, due to its ability to regulate glutamate-induced neurotoxicity, has been associated with neuroprotection in ALS patients and in ALS animal model. (J.H. Weishaupt *et al.*, 2006).

C. NAD⁺ metabolism:

Another downstream metabolism of the kynurenine pathway is the nicotinamide metabolism. Nicotinamide adenine dinucleotide (NAD), its central metabolite, is a molecule involved in many cellular reactions either as a proton receiver when oxidized (NAD⁺) or as a donor when reduced (NADH). Due to its central place in the cellular metabolism, NAD is involved in the clearance of the oxidative stress, the genomic stability and expression, RNA processing, and immunity/inflammation. Depletion of the NAD levels were observed in *in vivo* models and ALS patients (E. Blacher *et al.*, 2019; B.A. Harlan *et al.*, 2020). When looking at the nicotinamide metabolism (Fig. 12), NAD is synthesized from 3 distinct pathways: 1) the kynurenine pathway catabolizing the degradation of tryptophan into NAD; 2) the Preiss-Handler pathway catabolizing the degradation of nicotinic acid into NAD and 3) the salvage pathway. NAD is used in many physiologic reactions and in many of those result the production of NAM (Fig. 12). NAM can be then retransformed into NAD through the salvage

pathway via an intermediate called nicotinamide mononucleotide (NMN). NMN can also be formed through the conversion of another molecule called nicotinamide riboside (NR) via the action of nicotinamide riboside kinase.

Several hypotheses have been emitted to explain these reduced levels. First as mentioned previously, in physiological conditions, quinolinic acid is degraded into nicotinic acid, and ultimately NAD⁺ (Fig. 10). However, in a neuroinflammatory state, quinolinic acid is degraded at a much lower rate and is instead released by activated microglia (J.A. Fifita *et al.*, 2021). Therefore, the NAD levels could be depleted in ALS context due to impaired *de novo* synthesis associated with excessive release of quinolinic acid induced by the neuroinflammation. Besides *de novo* synthesis, the salvage pathway has also been suspected to be responsible for the depleted level of NAD in ALS context. Indeed, most NAD degradative reactions lead to the formation of nicotinamide, which is subsequently retransformed into nicotinamide mononucleotide (NMN), a direct NAD precursor, via the activity of nicotinamide phosphoribosyltransferase (NAMPT) (Fig 10). NAMPT exists in an intracellular and extracellular form (X. Wang *et al.*, 2017). Interestingly, intracellular NAMPT levels were reported to be downregulated in ALS patients in opposition to the extracellular form which exhibits increased levels (X. Wang *et al.*, 2017). Moreover, neuronal *Nampt* deletion was reported to induce motor reduced capacity and death in a murine model (X. Wang *et al.*, 2017). Supporting this hypothesis, nicotinamide riboside (NR) supplementation, a direct precursor of NMN without NAMPT involvement, was shown to be beneficial SOD1^{G93A} ALS mice model (B.A. Harlan *et al.*, 2020; E. Obrador *et al.*, 2021). Moreover, a clinical trial involving NR supplementation in ALS patients is ongoing.

The final hypothesis concerns the indirect link of NAD with axonal degeneration via the sterile alpha and TIR motif—constraining 1 (SARM1) hydrolase activity. This protein is involved in the Wallerian axonal degeneration upon lesion through the degradation of NAD into nicotinamide (J. Gerdtts *et al.*, 2015). The involvement of this process in ALS pathophysiology was put in evidence in a TDP-43 mutant murine model where *Sarm1* deletion prevents MN degeneration (M.A. White *et al.*, 2019). Therefore, NAD level could be depleted due SARM1 degradation upon axonal degradation.

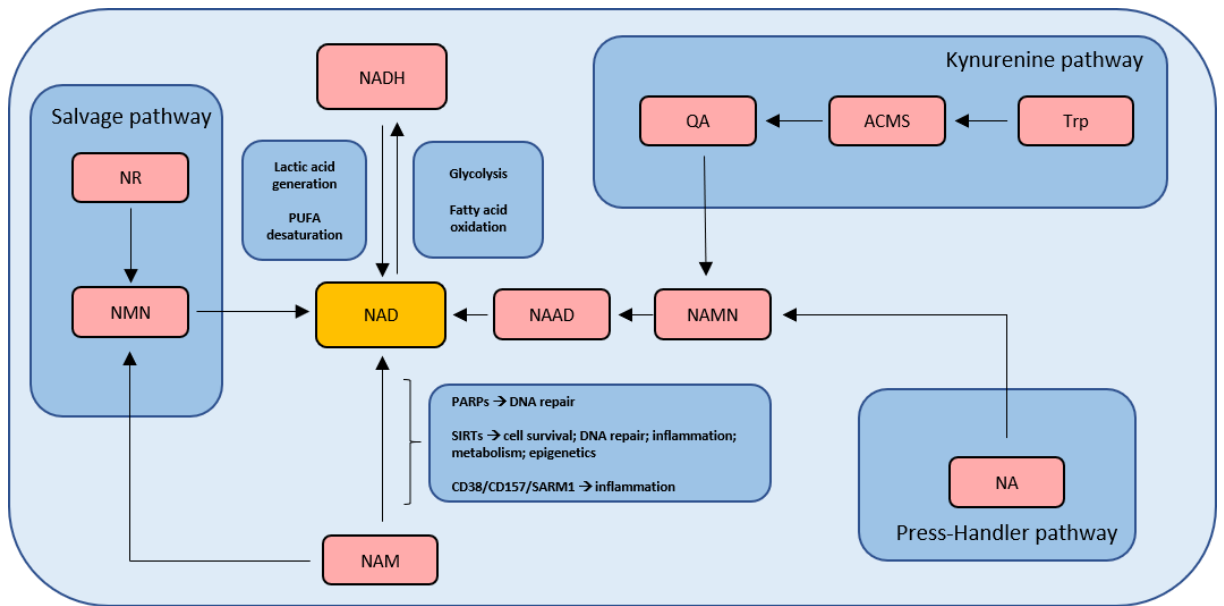


Figure 12: Graphical representation of the NAD metabolism

Abbreviations: Trp: tryptophan; ACMS: α -amino- β -carboxymuconate- ϵ -semialdehyde; QA: quinolinic acid; NA: nicotinic acid; NAMN: nicotinate mononucleotide; NAAD: nicotinic acid adenosine dinucleotide; NAD: nicotinamide adenosine dinucleotide; NR: nicotinamide riboside; NMN: nicotinamide mononucleotide; NAM: nicotinamide; PARPs: poly ADP-ribose polymerases; SIRT6: sirtuins; PUFA: polyunsaturated fatty acid

Results

The results section describes the development of two zebrafish models targeting the expression of the TBK1 orthologue, *Tbk1*.

I. Background and state of the art-TBK1 in ALS

ALS is fatal neurodegenerative disease characterized by the progressive degeneration of both the upper and the lower motor neurons. The pathogenicity is characterized by the death of MN leading to the denervation of muscles. At the molecular level, multiple pathways are involved in the development of ALS including autophagy dysregulation, mitochondrial dysfunction, inflammation, axonal transport and oxidative stress (M.C. Kiernan *et al.*, 2011). The main neuropathological hallmark of the disease is the presence and the accumulation of TDP-43 and ubiquitin positive inclusions within the motor neurons (M. Neumann *et al.*, 2006; A.M. Blokhuis *et al.*, 2013). In 2015, two independent studies have demonstrated causative mutations of TBK1 in ALS patients through a whole exome sequencing (E.T. Cirulli *et al.*, 2015) (A. Freischmidt *et al.*, 2015). Subsequent studies have confirmed *TBK1* mutations as a major cause of ALS as well as in another related neurodegenerative disease, FTD (K.L Williams *et al.*, 2015; C. Pottier *et al.*, 2015; I. Le Ber *et al.*, 2015). A study on a French population in particular have revealed that *TBK1* genetic alterations occurred more frequently in patients presenting an ALS-FTD comorbidity (10.8%) than those with ALS alone (0.5%) (I. Le Ber *et al.*, 2015). Nonsense and/or missenses mutations associated to ALS are located throughout the *TBK1* genetic sequence, giving rise to a loss-of-function hypothesis thought to be responsible for the disease. Pathological studies of *TBK1* mutation carriers show accumulation of ubiquitinated aggregates in their motor neurons and the glia in several area of the CNS (especially in the spinal cord and the motor cortex) as well as fronto-temporal lobar degeneration (S. Van Mossevelde *et al.*, 2016; C.A.M Koriath *et al.*, 2017; M. Weinreich *et al.*, 2020).

TBK1 is a serine-threonine kinase known to be an activator of the innate immunity through the induction of type I interferon (IFN-I) and the production of inflammatory cytokines via the regulation of NFκB (S. Yum *et al.*, 2021; L. Ahmad *et al.*, 2016). TBK1 has also been shown to regulate the inflammatory TNF-mediated cell death through inactivation of the RIPK1 protein (D. Xu *et al.*, 2018; E. Lafont *et al.*, 2018). In ALS patients, the loss of *TBK1* results in

lower but sufficient IFN-I induction via compensatory pathways, but high autoinflammation due to the TNF-induced cell death caused by dysregulated RIPK1 (J. Taft et al., 2021).

Moreover, in the context of ALS, TBK1 role in the protein degradation pathway, autophagy, has been thoroughly investigated. Indeed, TBK1 is shown to control the early stages of autophagy at several levels (J.A. Oakes et al., 2017) and has been shown to be crucial in the clearance of aggregates (R.M. Hedge et al., 2020; I. Raya *et al.*, 2022). To better elucidate the functional relevance of TBK1 in ALS animal models have been developed, including murine model lacking the expression of *Tbk1* (M. Bonnard *et al.*, 2000). However, the study of this model was challenging because of a massive liver degeneration leading to an early embryonic death at E14.5. TBK1 was reported to control CASP8-related apoptosis and necroptosis activations via regulation of RIPK1 function (E. Lafont *et al.*, 2018; M. Delhase *et al.*, 2011). This observation lead to hypothesis that the lethality observed in the TBK1^{-/-} murine model was associated with the activation of cellular death pathways. Indeed, in 2018, a study has implemented a novel model by combining the deletion of TBK1 with a RIPK1 kinase-dead knock-in mutation (D. Xu *et al.*, 2018). Importantly, the authors prevented lethality in these double knockouts as compared to TBK1 deletion mutant. Moreover, combined inhibition of both *Tbk1* and *Tak1* (a gene coding for another kinase involved in TNF receptor response and NFκB activation) were sufficient to induce RIPK1 activation (D. Xu *et al.*, 2018). Interestingly, this study has also reported that the levels of TAK1 protein were decreasing in an age-related manner, therefore suggesting a convincing model for TBK1 associated development of ALS-FTD where the heterozygous loss of TBK1 is supported by the age-dependent decreased expression of TAK1 leading to the development of a late-onset disease such as ALS-FTD.

Further defining the potential role of TBK1 in the development of ALS-FTD, a recent study (W. Shao *et al.*, 2022) has reported the functional connection between the disease associated *C9orf72* and *TBK1* genes. Indeed, TBK1 was found to be sequestered within the cytoplasmic inclusions containing DPRs translated via the expression of *C9orf72* hexanucleotide repeats in a mouse model. Moreover, mutation in *Tbk1* worsened the condition of the murine model via endosomal disruption (W. Shao *et al.*, 2022).

Using the powerful vertebrate model, zebrafish, I targeted the unique *TBK1* orthologue, *tbk1* in order to define the pathogenic consequences related to *tbk1* loss of expression to better

understand TBK1 in the context of ALS-FTD pathophysiology and to open therapeutic strategies for these disorders.

II. Phenotypic characterization of a new *tbk1* KD zebrafish model

2.1 Generation of the *tbk1* KD zebrafish model

In order to disturb *tbk1* expression in zebrafish, we took advantages of the high homology with its human orthologs. Indeed, genomic sequences between the *TBK1* genes of these two species are shared at 63.16%. Consequently, the two encoded proteins also share a high homology with up to 71.39% of nucleotide identity and a conserved four domains architecture.

In order to study the depletion of Tbk1 in the zebrafish model, I initially targeted the expression of *tbk1*, as a hemizygous loss of expression of *tbk1* has been reported in mutant TBK1 ALS-FTD patients (A. Freischmidt *et al.*, 2015; E.T. Cirulli *et al.*, 2015). To induce this partial decrease in *tbk1* expression, we generated an antisense morpholino oligonucleotide (AMO) complementary to the mRNA start codon sequence of *tbk1*. An AMO is a stabilized RNA complementary to a target mRNA which sterically prevent the binding of ribosome and the subsequent translation of the gene without noticeable degradation of the target mRNA. However, this technique is limited by the progressive degradation of the AMO in the developing zebrafish embryo. Indeed, it is commonly accepted that 4 days post injection, the concentration in AMO is insufficient to alter the expression of the gene anymore (D.Y. Stainier *et al.*, 2017), restricting the analysis to the first 4 days of development. For these reasons, the analyses of the AMO injected fish have been restricted in this study mainly to the 2nd day post fertilization (dpf). During this period, the development of the motor neurons and their migration has been completed as secondary motor neurons enter the myotome around 34 hours post fertilization (hpf) (P.Z. Myers *et al.*, 1986). As a control of injection, a control AMO – bearing a sequence that does not target any sequence in the zebrafish transcriptome – has been used. This control condition will be called further on the “standard control” or “mismatch condition”.

After quantifying the decrease in the expression of the *tbk1* gene by western blot (Fig. 1), I analysed the possible impact on the swimming capacity of the larvae, locomotor alterations being the main clinical hallmark of the loss of *TBK1* expression in patients (I.J Swift *et al.*, 2021).

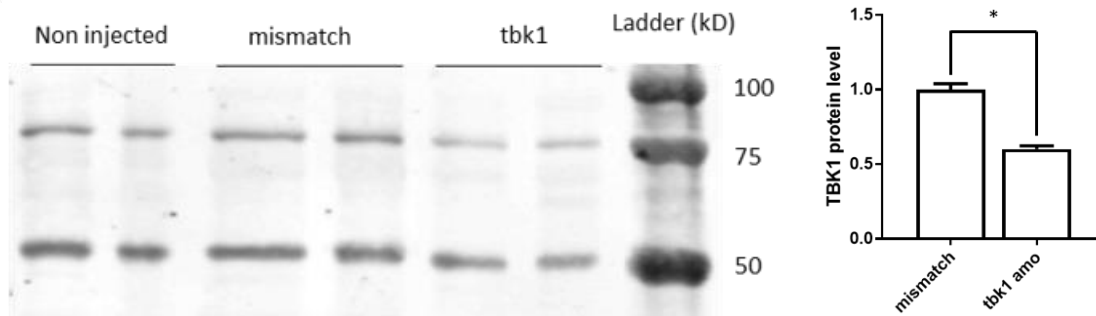


Figure 1: 2dpf *tbk1* AMO injected larvae exhibit reduced expression of *Tbk1*:

Left, immunoblot analysis of *Tbk1* protein in total extract from WT, standard control and *tbk1* AMO injected embryos at 48hpf. *Tbk1*'s band is detected around 80 kDa. Control antibody targeting α -Tubulin protein was performed to normalize samples. α -Tubulin's band is detected around 50 kDa. Right, densitometry quantification of *Tbk1* protein level normalized to α -Tubulin. (Two-way ANOVA with Sidak's multiple comparisons test. * $p < 0, 05$).

2.2 *tbk1* reduced expression leads to altered larvae swimming capacity

In order to assess the impact of *Tbk1* depletion on the locomotor activity of the zebrafish larvae, we performed a touch escape evoked response test at 2dpf. This exercise consists in inducing an escape response through non-invasive stimulation of the larva's tail (T.E. Sztal *et al.*, 2016; S. Ciura *et al.*, 2013; A-R. Bourefis *et al.*, 2020). The trajectory of the larva's response is recorded, the distance of the trail and the velocity are calculated. Theoretically, we expect that a modification in the locomotor system's responses due to either alteration of the muscle fibres or spinal cord integrity could result in a reduced velocity or distance swum (S. Li *et al.*, 2020; I. Dehnisch Ellström *et al.*, 2019).

Using this approach, we observed that *Tbk1* KD induces an alteration of the locomotor capacity of the larvae at 2dpf compared to the larvae injected with the control AMO (Fig. 2). Indeed, the *Tbk1* depleted larvae exhibit shorter and slower swimming tracts as compared to the control larvae. This alteration of the locomotor capacity was even more marked on the velocity as the *tbk1* depleted fish swam two-fold slower than the controls (Fig. 2).

To our knowledge, this is the first study demonstrating that the sole heterozygous loss of *tbk1* leads to motor defects in an animal model.

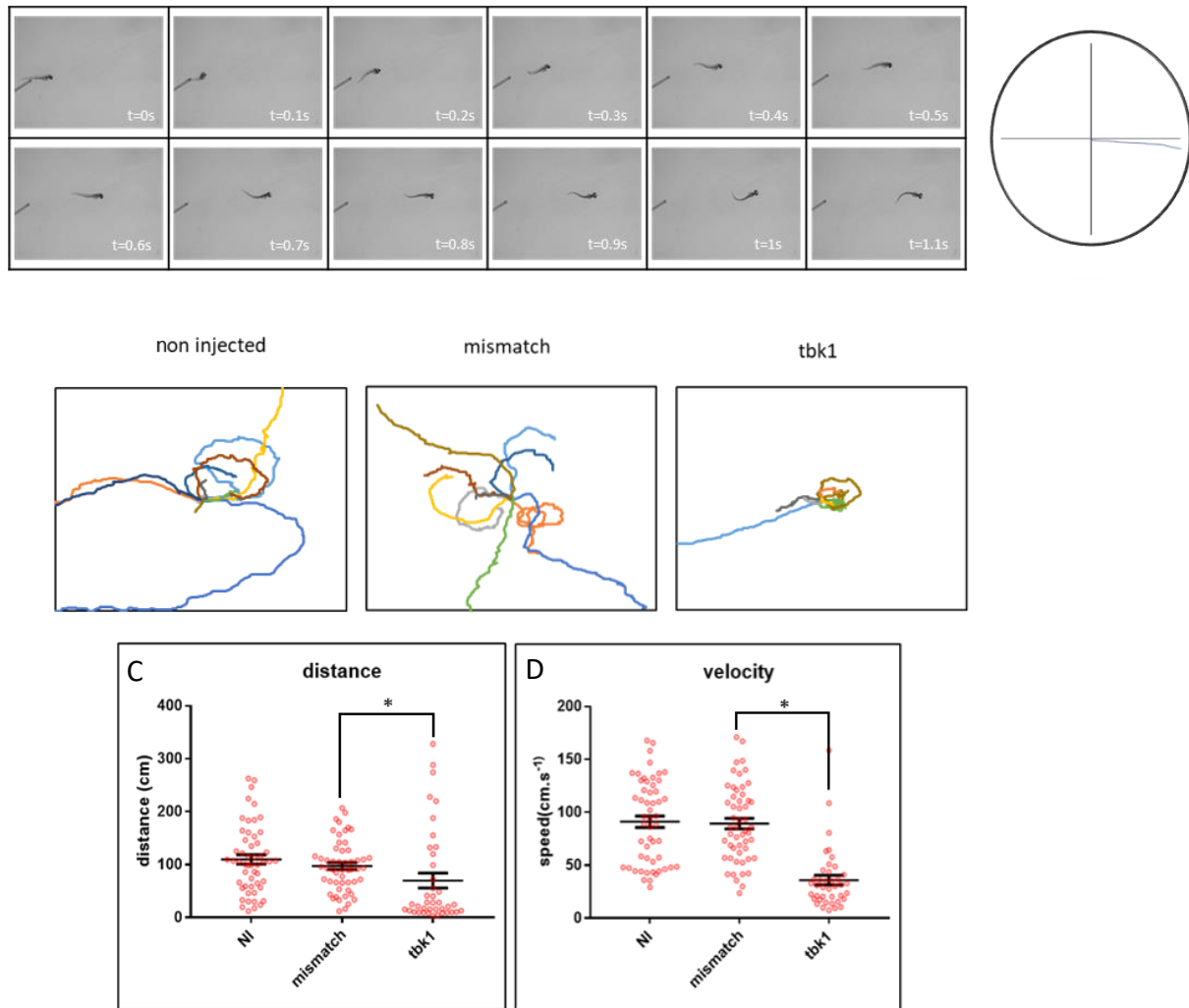


Figure 2: *tbk1* AMO injected larvae exhibit locomotor deficits at 2dpf:

(A) Graphical illustration of the touched evoked escape response test. Left, zebrafish larvae were stimulated at the tip of their tails and swim response were recorded. Right, trace representing the swim response induced by TEER of the larvae imaged on the left panel. (B) Traces representing the swim response of zebrafish larvae injected with the *tbk1* AMO compared to non-injected controls and standard control injected zebrafish larvae during the touch-evoked escape response assays (TEER). C-D. Quantitative analysis of TEER. *tbk1* AMO injected larvae showed a significant decrease in the distance travelled (C) and in the velocity (D). (Two-way ANOVA with Sidak's multiple comparisons test. * $p < 0, 05$).

After characterizing the impact of the partial loss of *tbk1* in the 2dpf larva at the behavioural scale, I determined whether *tbk1* KD leads to alterations in motor neurons as well as their innervating targets, through the neuromuscular junction (NMJ).

2.3 Defects in locomotion are associated with spinal cord alteration upon *tbk1* KD

To define whether impaired mobility could be linked to deficits at the level of the neuromuscular junction, I performed a detailed analysis in the zebrafish larvae. As the death of motor neurons being the main hallmark of the ALS pathogenicity (M.C. Kiernan *et al.*, 2011), I investigated the integrity of the spinal cord using a transgenic zebrafish line bearing an *RFP* gene under the regulation of a specific motor neuron promoter: *mnx* (S. Knafo *et al.*, 2017). Therefore, I assessed the axonal projections of the motor neurons upon depletion of *Tbk1* expression and measured the axonal length. As the size of the larvae can be variable, I rationalized the length of the axons with the thickness of the spinal cord. Interestingly, I did not observe any difference in the size of the *Tbk1* depleted larvae compared to the controls (Fig. 3) and did not associate any axonal alterations from the spinal motor neuron projections due to the partial loss of *Tbk1*. This suggests that the axonal projections of MN in this model appear to develop correctly upon depletion of *Tbk1*.

Using the double transgenic *Tg(mnx1:gal4)/Tg(UAS:RFP)* zebrafish line, I also quantified the number of MN of the *tbk1* KD larvae and compared it with the mismatch condition. Interestingly, we quantified a severe reduction in the number of MN upon depletion of *Tbk1*, with in average 15 MN per somite in the *Tbk1* depleted larvae in opposition to the average 20 MN observable in the control larvae (Fig. 3).

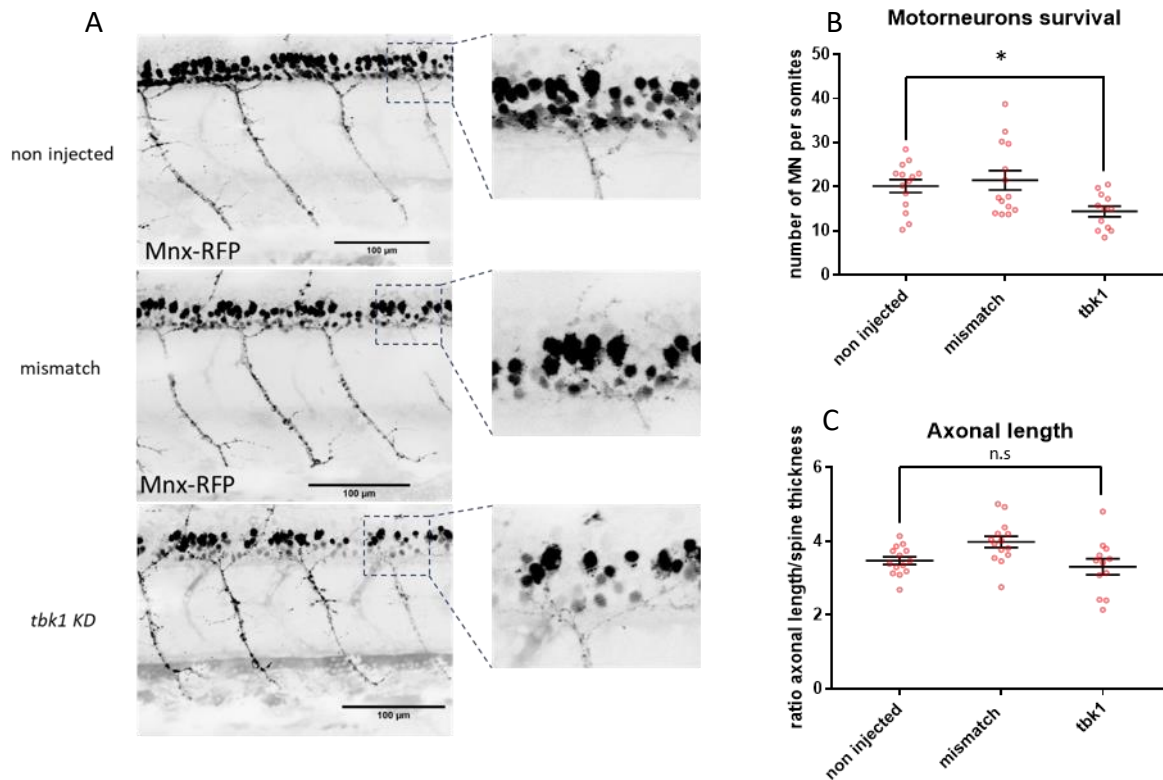


Figure 3: *tbk1* knock-down causes spinal motor neuron loss.

(A) Double transgenic *Tg(mnx1:gal4)/Tg(UAS:RFP)* embryos at 2dpf were used *in vivo* for the quantification of spinal motor neuron axonal length and number. (B) Quantification of average motor neuron numbers in the spinal cords at 2dpf. (C) Quantification of motor neuron axonal length rationalized to spinal cord thickness at 2dpf. (Two-way ANOVA with Sidak's multiple comparisons test. * $p < 0, 05$).

In order to assess the impact on the muscles, we took advantage of the transparency of zebrafish larvae. Indeed, it is possible to observe and measure the muscular fibres without any labelling under polarized light. However, after quantification, we didn't observe any differences in the length of the muscular fibres upon depletion of *Tbk1* compared to the mismatch condition (Fig. 5). This suggests that the reduced number of motor neurons observed in the *tbk1* KD condition does not impact the integrity of the muscular fibres.

To determine the *Tbk1* role upon motor neuron development, I mounted whole larvae at earlier developmental stages: 23 and 32 hpf and quantify the number of spinal MN using the double transgenic *Tg(mnx1:gal4)/Tg(UAS:RFP)* zebrafish line. I selected these specific times

point as they correspond to both the initiation and the termination of the secondary MN migration (P.Z. Myers *et al.*, 1986). Interestingly, no significant differences in the number of MN was observed at these two developmental stages (Fig. 4). Therefore, spinal MN loss observed at 2dpf was likely not due to a developmental delay, but to the activation of neurodegenerative pathways.

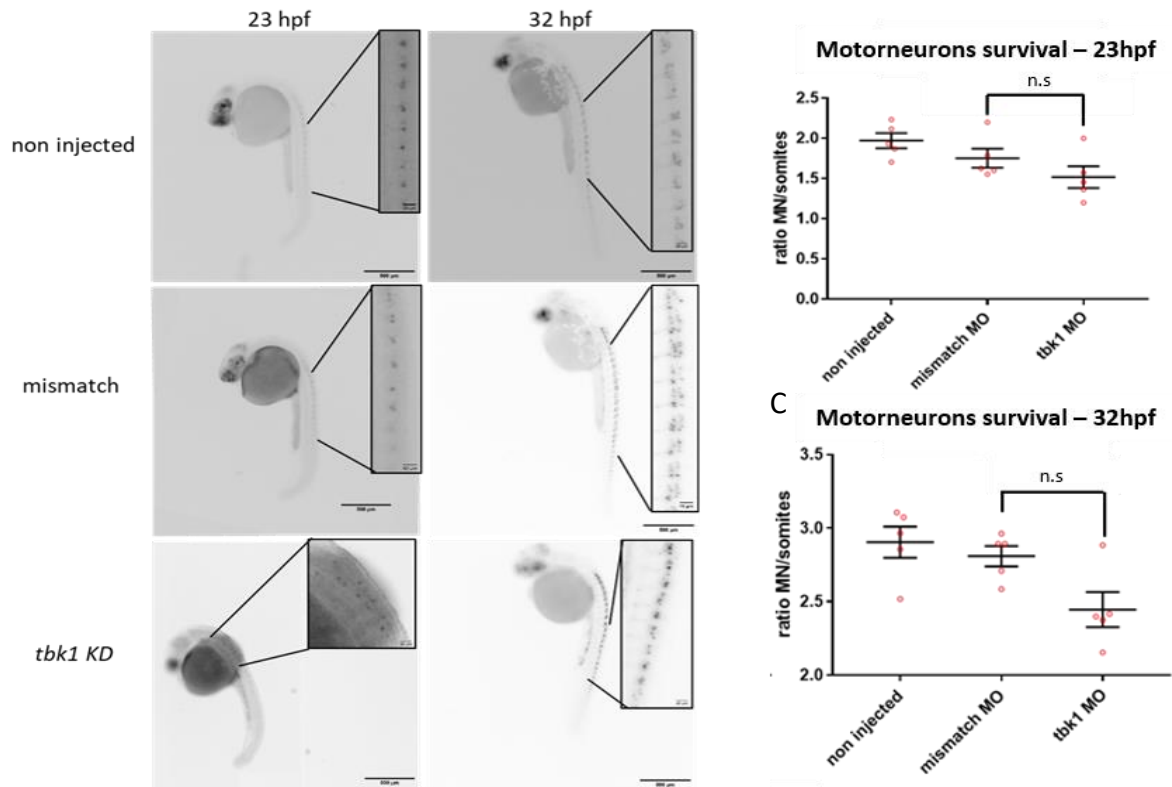


Figure 4: *tbk1* induced loss of spinal motor neurons is not linked to developmental delay:

(A) Double transgenic *Tg(mnx1:gal4)/Tg(UAS:RFP)* embryos at 23hpf and 32 hpf were used *in vivo* for the quantification of spinal motor neuron number. (B) Quantification of average motor neuron numbers in the spinal cords at 23hpf. (C) Quantification of average motor neuron numbers in the spinal cords at 32hpf. (Two-way ANOVA with Sidak's multiple comparisons test)

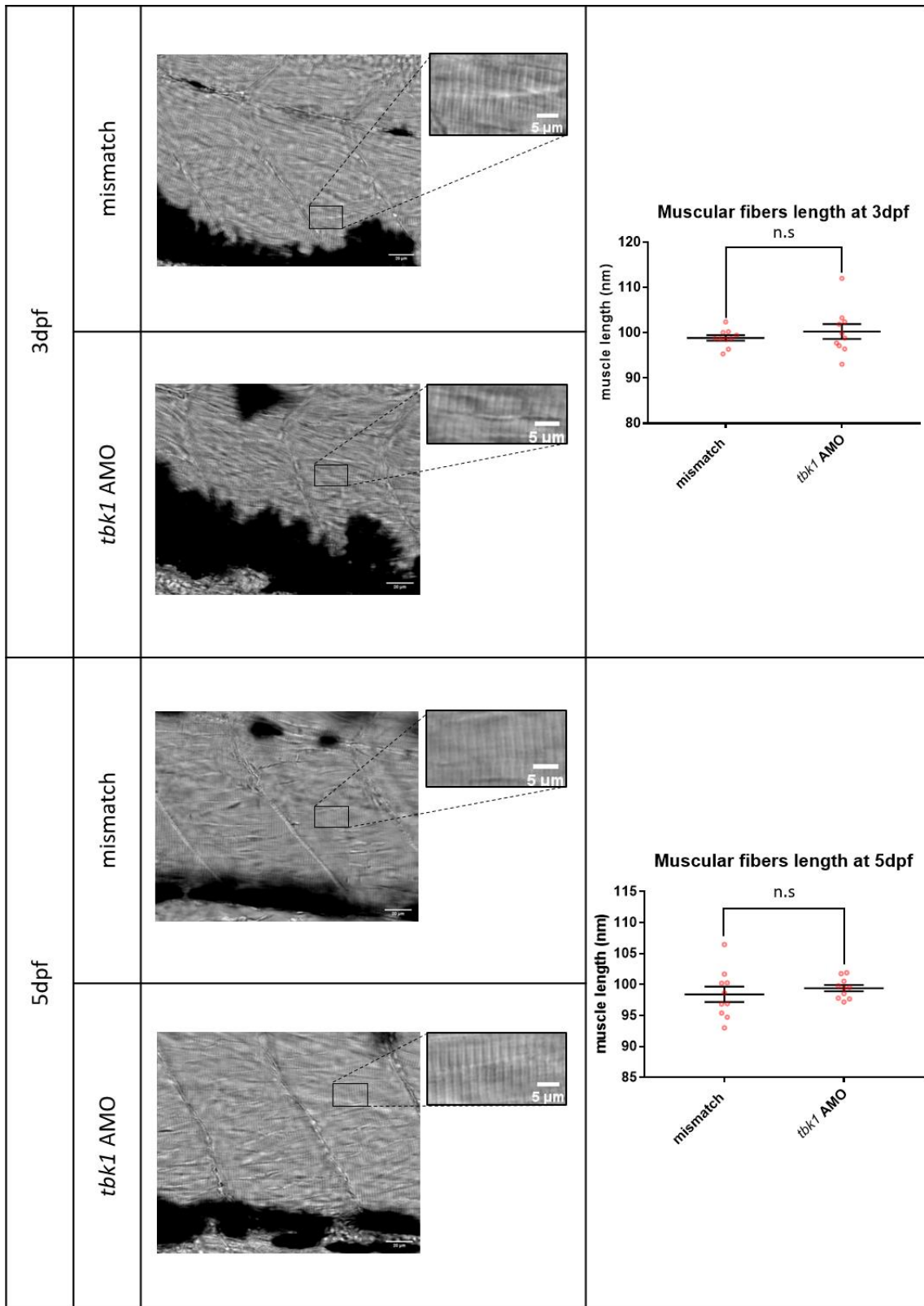


Figure 5: *tbk1* depletion does not lead to increased muscular fibre atrophy:

(A) Non-injected WT zebrafish embryos at 3dpf and 5dpf were used in vivo for the quantification of the muscular fibres' length. (B) Quantification of the muscular fibres' length at in non-injected WT

larvae at 3dpf. (C) Quantification of the muscular fibres' length at in non-injected WT larvae at 3dpf. (Two-way ANOVA with Sidak's multiple comparisons test)

In order to study Tbk1 depletion in a more extended time frame and through another genetic methodology, we developed a new *tbk1* knock out model using the CRISPR/Cas9 system allowing a longer and more complete phenotypic analysis.

III. Phenotypic characterization of a new *tbk1* KO zebrafish model

The first results obtained using AMO shedding light on the importance of studying the total loss of *tbk1* expression in the zebrafish, I designed the strategy to obtain a *tbk1* KO zebrafish line.

3.1 Generation of the *tbk1* KO zebrafish model

Generation of the *tbk1* KO zebrafish line was performed using the CRISPR technology (M. Jinek *et al.*, 2012). Our aim was to take advantage of the high percentage of identity between the human and the zebrafish genomic and protein sequences (respectively 60 and 70%) to induce a deletion within the *tbk1* kinase domain sequence. We targeted this region for two reasons: 1) the kinase domain of Tbk1 bears its catalytic activity and therefore its deletion would suppress its functional role; 2) Tbk1's kinase domain is encoded by the 5 first exons of the gene, therefore inducing a deletion within increasing the chance of generating an early stop codon or a frame shift impacting the whole transcript. To optimize the cutting efficiency of the Cas9, we adopted a strategy combining two sgRNA at the same time to induce a double strand break at two different loci and therefore generating the deletion of a long and significant segment of the kinase domain-coding region. This technique has been very efficient in inducing deletion mutants (Wu *et al.*, 2018).

We designed the sgRNA using the CHOPCHOP software (K. Labun *et al.*, 2019) and selected the top scored sequences to create 2 pairs of guides targeting respectively the exons 1 and 2 and the exons 4 and 5 of the *tbk1* gene, both creating a 700bp deletion (Fig. 6). Targeting exon 1-2 and 4-5 was assessed by genotyping individual embryos using a set of primers in this region. Importantly, *tbk1* deletion efficiency was quite high with up to 70% of *tbk1* KO

homozygous larvae when genotyped (Fig. 6). The efficiency was further confirmed by a control injection of sgRNA targeting the *tyrosinase* gene, which is responsible for the pigmentation of the zebrafish (E. Camp et al., 2001), assessing the efficacy of this procedure as early as the first day post fertilization.

However, as we are going to show later in this thesis, similarly to what is observed in the murine model, the homozygous deletion of *tbk1* leads to lethality early in the development, around 9dpf. This lethality associated with the loss of *tbk1* makes generating a KO fish line impossible. Considering the efficiency being high enough at the first generation, we decided to pursue the study with larvae directly injected with the Cas9 mix at one cell egg. This strategy doesn't allow to control the nature of the cut induced in *tbk1* but as we are looking to silence the gene, the certainty of having 70% of homozygous *tbk1* KO fish is appropriate for the following analyses performed in this study. Furthermore, this strategy allows to define the role of TBK1 early in motor neuron development.

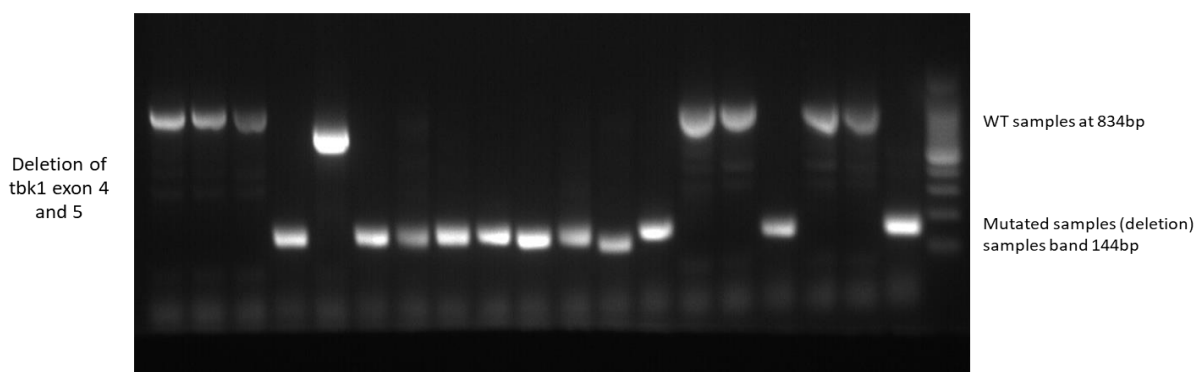
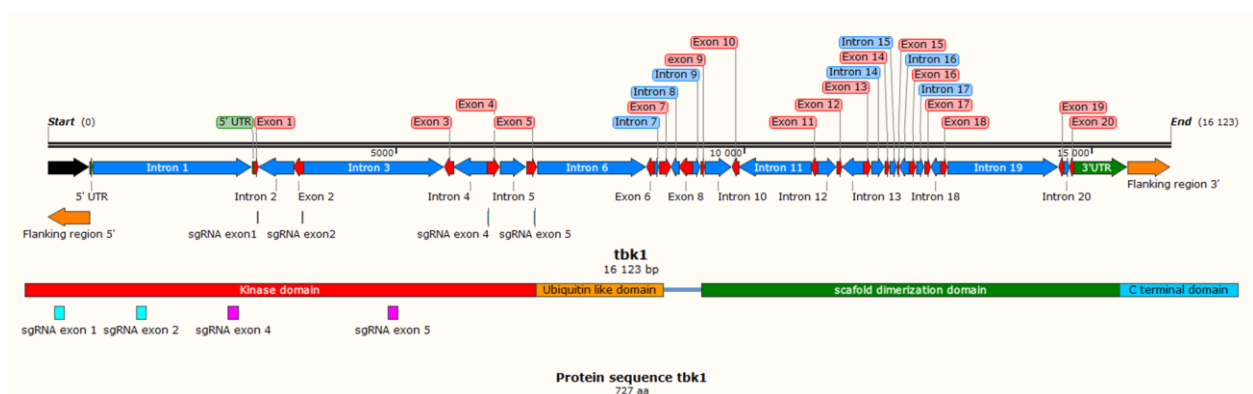


Figure 6: target *tbk1* CRISPR induction of a 700 base pairs deletion

(A) Graphical representation of the CRISPR approached design to induces a 700 base pairs deletion within *tbk1* genes at two different loci. One set of guides RNA was designed to target a 20 nucleotides

sequence within respectively the exon 1 and exon2. The other set of guides RNA was designed to target a 20 nucleotides sequence within respectively the exon 4 and exon 5. (B) Agarose migration after polymerase chain reaction amplification of DNA oligos derived from CRISPR injected larvae at 2 dpf. Each band correspond to one individual zebrafish larva.

3.2 *tbk1* KO leads to altered larvae swimming capacity

Similarly to the characterization of the *tbk1* AMO model, I initially assessed the locomotor capacity of the fish at 2dpf with a TEER test. Interestingly, the larvae carrying the *tbk1* deletion did not exhibit any significant difference in the distance swam or their velocity upon stimulation of the evoked response (Fig. 7). Following this analysis, I assessed the spontaneous swimming capacity at later developmental stages by performing an analysis using the zebabox module (viewpoint technology ©). This device automatically tracks the trajectory of several larvae in a 96 or 48 wells plate on a defined time period, and gives the opportunity to implement the analysis with a sound or a light stimulus to measure an escape response. The protocol used in this study was based on a 40 minutes period of free-swimming capacity analysis followed by 2 sound stimuli of 450Hz and 80 dB separated by a 10 minutes rest period where the escape responses were measured (Fig. 8). At 5 and 6 dpf, we quantified a significant decrease in the locomotor activity of the larvae associated with the loss of *tbk1* expression (Fig. 8). Indeed, the distance swum in the escape response induced by a sound stimulus was decreased by half in both KO larvae condition compared with the non-injected ones. This decreased was even more striking when analysing the free-swimming capacity, the larvae were almost immobilized when not exposed to any stimuli (around 25cm swum during 40 minutes whereas the non-injected larvae were travelling almost 100cm).

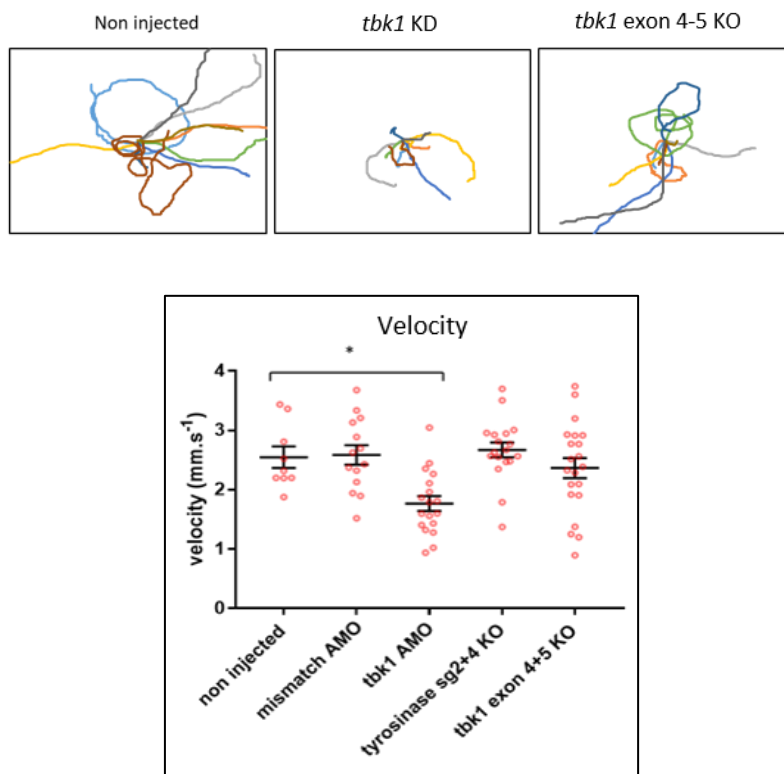


Figure 7: CRISPR *tbk1* deleted larvae do not exhibit locomotor deficits at 2dpf

(A) Traces representing the swim response of zebrafish larvae bearing a genomic deletion of the exons 4 and 5 of *tbk1* gene compared to larvae injected with the *tbk1* AMO and to non-injected controls during the touch-evoked escape response assays (TEER). (B). Quantitative analysis of TEER. *tbk1* KO larvae did not show any significant differences in their velocity nor distance swum (data not shown) compared to non-injected larvae travelled. (Two-way ANOVA with Sidak's multiple comparisons test. * $p < 0, 05$).

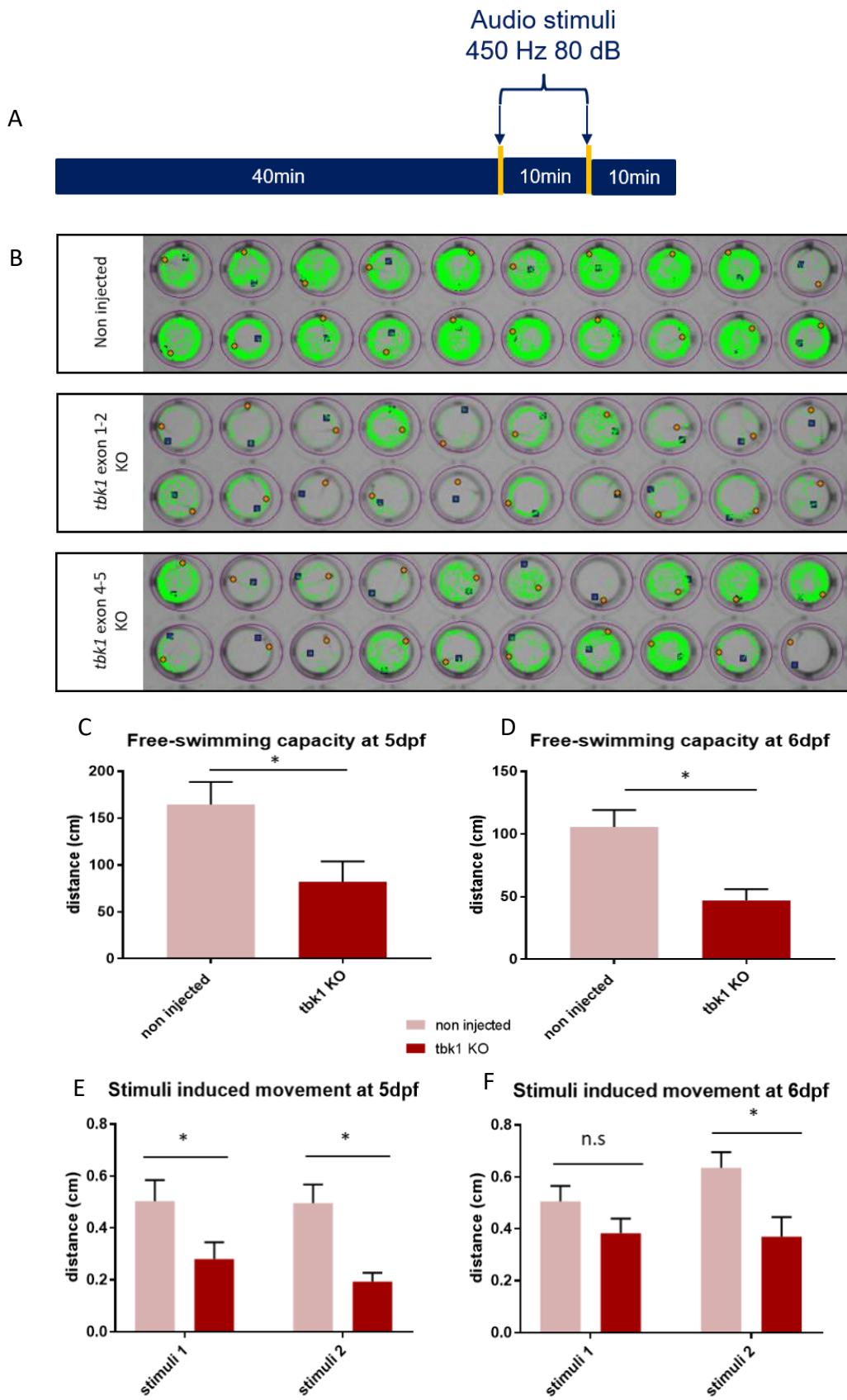


Figure 8: CRISPR induced genomic *tbk1* deletion lead to impaired swimming capacity at 5 and 6 dpf

(A) Graphical illustration of the protocol used to assess the locomotor capacity of the *tbk1* deleted zebrafish larvae. The protocol is divided in two parts: a 40min period of free swimming followed by 2 sound stimuli separated by 10 without stimulation. (B) Traces representing the swimming trajectory of *tbk1* KO zebrafish larvae and non-injected controls during the viewpoint test. (C-D) Quantification of the distance swum during the first 40 minutes of the protocol for the larvae bearing a *tbk1* exon 4 and 5 deletion compared to WT non-injected larvae at 5 dpf (C) and 6dpf (D). (E-F) Quantification of the distance swum in response to the two sound stimulations for the larvae bearing a *tbk1* exon 4 and 5 deletion compared to WT non-injected larvae at 5 dpf (E) and 6dpf (F). (Unpaired t-test comparative analysis. * $p < 0, 05$)

From the difference in the timing of apparition of the locomotor phenotype between the *tbk1* AMO and the *tbk1* KO CRISPR models, we can generate two hypotheses:

First, one difference between the two models is the expression of *tbk1* maternal mRNA in the first phases of development. Indeed, the *tbk1* AMO is targeting mRNA, this mode of action prevents as well the expression of mRNA transmitted by the mother and deposited in the egg during oocytes maturation, however the CRISPR technology targeting the larvae DNA is not impacting the expression of maternal mRNA. Therefore, this change in the phenotype apparition is suggesting a possible role for *Tbk1* during the 10 first hours of development. This will be assessed by qPCR at various stages of development. Importantly, this period is critical for the correct development of the locomotor system as the three primary motor neurons are being generated during the ninth and the tenth hours post fertilization.

Secondly, this difference in the timing of apparition could be induced by phenomenon of genetic compensation induced by the complete deletion of *tbk1*. Indeed, it is known that some TBK1 functions can be compensated by other proteins, for instance, TBK1 dependent activation of NF κ B is rescued by IKK ϵ when *TBK1* is silenced (K.R. Balka *et al.*, 2020). This compensation could be dependent of a threshold in TBK1 expression, explaining why it doesn't happen in the AMO injected larvae.

To answer these two hypotheses, unbiased analysis of the metabolic dysregulations as well as proteomic analysis to define pathophysiological alterations upon *Tbk1* depletion locomotor phenotype at 5 and 6 dpf were performed.

3.3 *tbk1* KO is lethal early in embryogenesis

After assessing the impact on the locomotor capacity of the *tbk1* KO larvae, we also assessed whether there were other metabolic features during larval development. Interestingly, gross morphological analysis of larvae at 8dpf did not reveal any reduced length in the *tbk1* KO larvae as compared to control conditions (Fig. 9). Interestingly, at 9dpf, we quantified a mortality rate of 90% in the *tbk1* KO larvae compared to the 30% rate in non-injected or larvae injected with a control sgRNA construction targeting the *tyrosinase* gene, responsible for the pigmentation (Fig. 10). Therefore, we performed an unbiased analysis to define altered pathways that lead to increased mortality in the Tbk1 depleted zebrafish larvae.

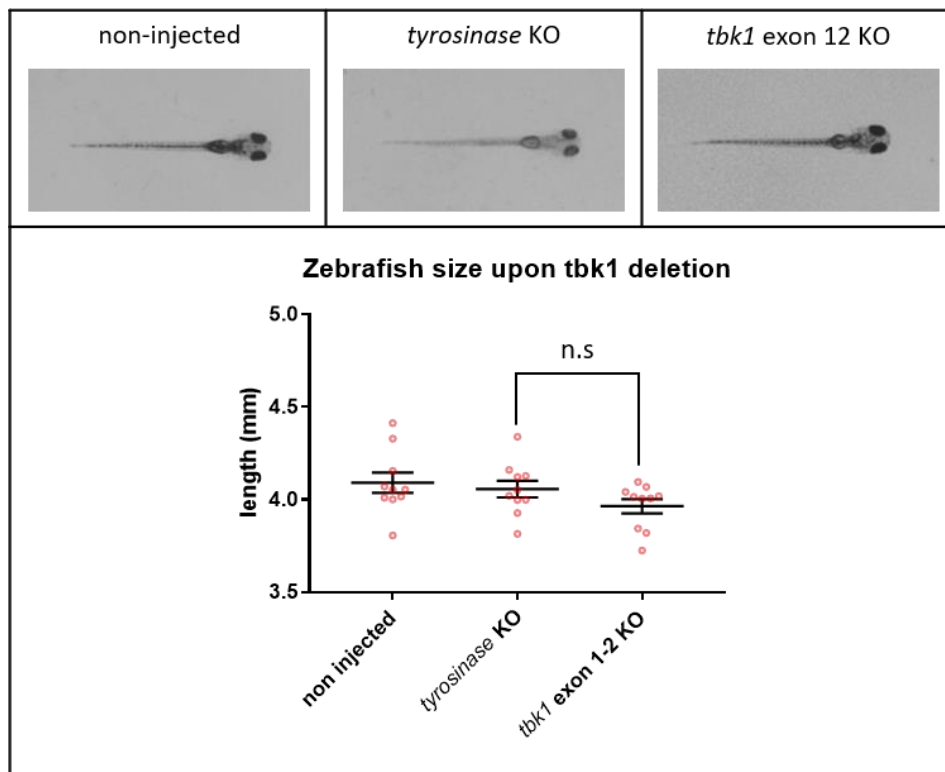


Figure 9: deletion of *tbk1* in zebrafish does not impact the size of the larvae at 8dpf.

Top, Images illustrating the size of an 8dpf *tbk1* deleted zebrafish larvae compared with a tyrosinase KO and a WT non-injected larva. Bottom, comparative quantification of the size of the larvae at 8dpf. *tbk1* deleted larvae did not exhibit a reduced size compared to control conditions. (Two-way ANOVA with Sidak's multiple comparisons test * $p < 0, 05$)

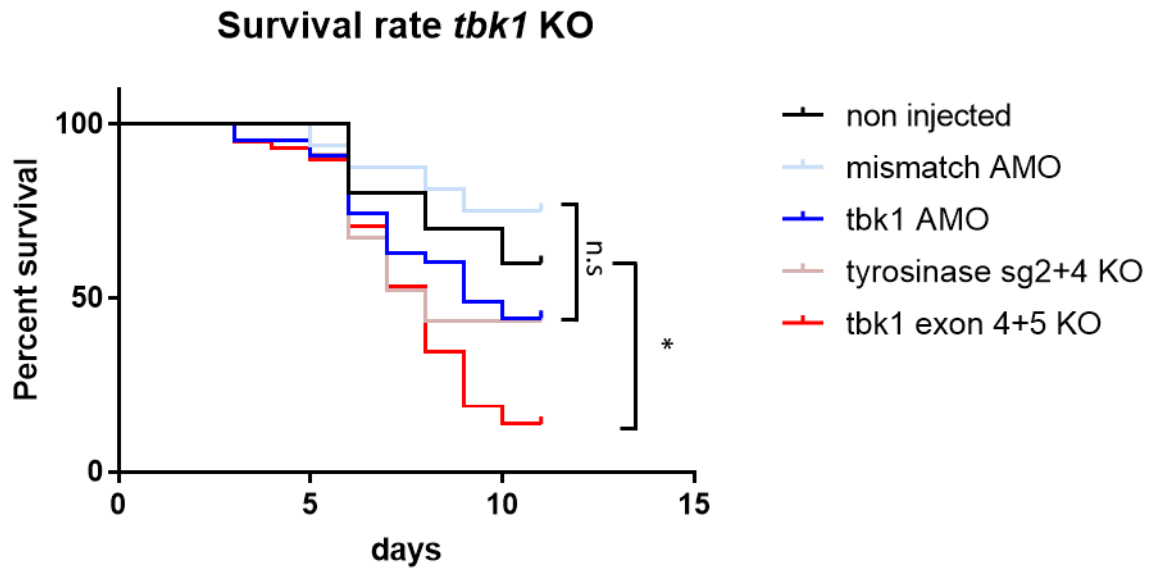


Figure 10: *tbk1* deletion is lethal early in the development at 9dpf

Curve comparing survival rate of *tbk1* KO larvae with tyrosinase KO larvae, *tbk1* KD larvae, standard-control AMO injected larvae, and WT non-injected larvae. (Log-Rank (Mantel Cox) test. * $p < 0, 05$)

IV. Metabolomic characterization of the *tbk1* KO zebrafish model

In order to investigate the molecular dysregulations responsible for the apparition of the two phenotypes observed upon deletion of *tbk1*, two metabolomic analyses were performed at 6dpf, a developmental stage characterized by the deficit in locomotion and at 8 dpf a day preceding the initiation of the larval mortality.

Metabolism is a term that encompasses every cellular reactions occurring in a living organism to sustain life. Metabolites are small molecules involved either as an intermediate or as an end product of the metabolism. Dysregulations in the level of these metabolites reflect alternations of specific cellular pathways, unveiling the mechanistic insight of a pathology. Moreover, metabolomics have been of great interest lately to develop new strategies of diagnosis based on the surveillance of metabolites levels used as biomarkers of the disease progression. Therefore, analysing the metabolite profile, or metabolomics, of our *tbk1* KO model could help explain the apparition of these two phenotypes (locomotor

deficits and lethality) and the presence of biomarkers observed in patients could also further confirm our ALS model.

4.1 Metabolomic profile of the *tbk1* KO larvae at day 6

After a first round of analysis comparing the metabolites level between *tbk1* KO, WT and *tyrosinase* KO larvae, it became clear that even though they don't present any phenotype in exception with the loss of their pigmentation, the *tyrosinase* KO larvae are characterized by a specific metabolomic profile, different from the non-injected, that could mask metabolic alterations that are aberrant in the *tbk1* KO model. Therefore, we pursued the analysis using only WT larvae as a control condition, and included the analysis on both sgRNA exons 1-2 KO and exons 4-5 KO conditions to define metabolic alterations that are present upon *tbk1* loss of function.

After measuring the metabolites through mass spectrometry, we start looking common dysregulated metabolites in the *tbk1* 1-2 exon KO and 4-5 exon KO models. We implemented a list of 131 identified metabolites and their respective concentration in the samples on the metaboanalyst © software (Z. Pang *et al.*, 2021). At 6dpf, we identified mainly two metabolites that were dysregulated with a q-value under 5% in both of the conditions: nicotinamide (NAM) and glycerol-3-phosphate (G3P) (Fig. 10).

As an intermediate of the glycolysis pathway, G3P depletion in our model could indicate an impaired glucose metabolism. However, the level of glucose or pyruvate don't seem to be impacted by the loss of *tbk1* in our analysis so it is complicated to conclude anything through the sole loss of this compound.

Depletion of the NAD levels was observed in *in vivo* model and in ALS patients (E. Blacher *et al.*, 2019; B.A. Harlan *et al.*, 2020). Increased levels of NAM, its direct precursor, suggests a similar decreased pool of NAD in our model. Moreover, when performing the analysing while pooling the two *tbk1* KO conditions versus the non-injected larvae, a new metabolite appears to be significantly increased upon deletion of *tbk1*, the quinolinic acid (QUIN); another precursor of NAD. Interestingly, in the brain, QUIN is only synthesized by microglia or astrocytes, therefore, its increased levels unveiled a possible implication of the immune

cells of the brain in the development of the phenotype observed in the context of *tbk1* deletion.

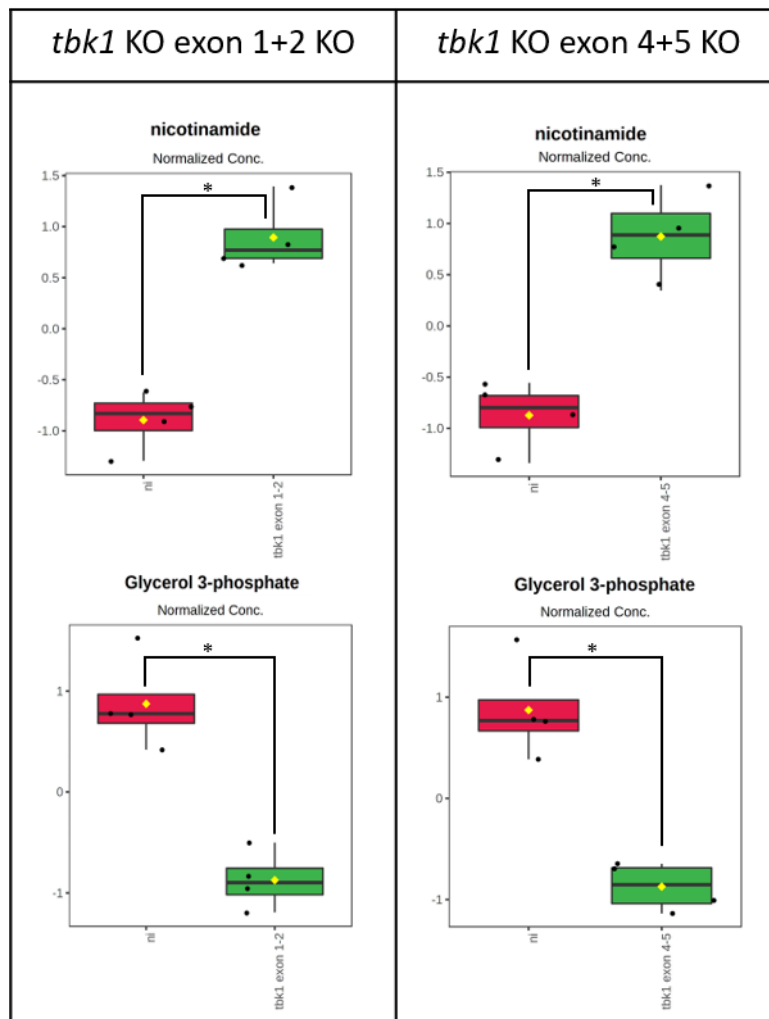


Figure 11: Nicotinamide and glycerol 3-phosphate exhibit dysregulated levels upon *tbk1* deletion at 6dpf

6dpf whole larvae level of nicotinamide and glycerol 3-phosphate metabolites are respectively up- and downregulated upon deletion of the exons 1 and 2 (left) or of the exons 4 and 5 of the *tbk1* gene in comparison with non-injected WT larvae (statistical analysis performed by two-sample t-tests implemented with a false discovery rate. * $q < 0, 05$).

An enrichment analysis on the metabolomics results was performed to identify dysregulated pathways. Out this analysis, we identified one common dysregulated metabolic pathway in both of our models: the nicotinate and nicotinamide metabolism (Fig. 12).

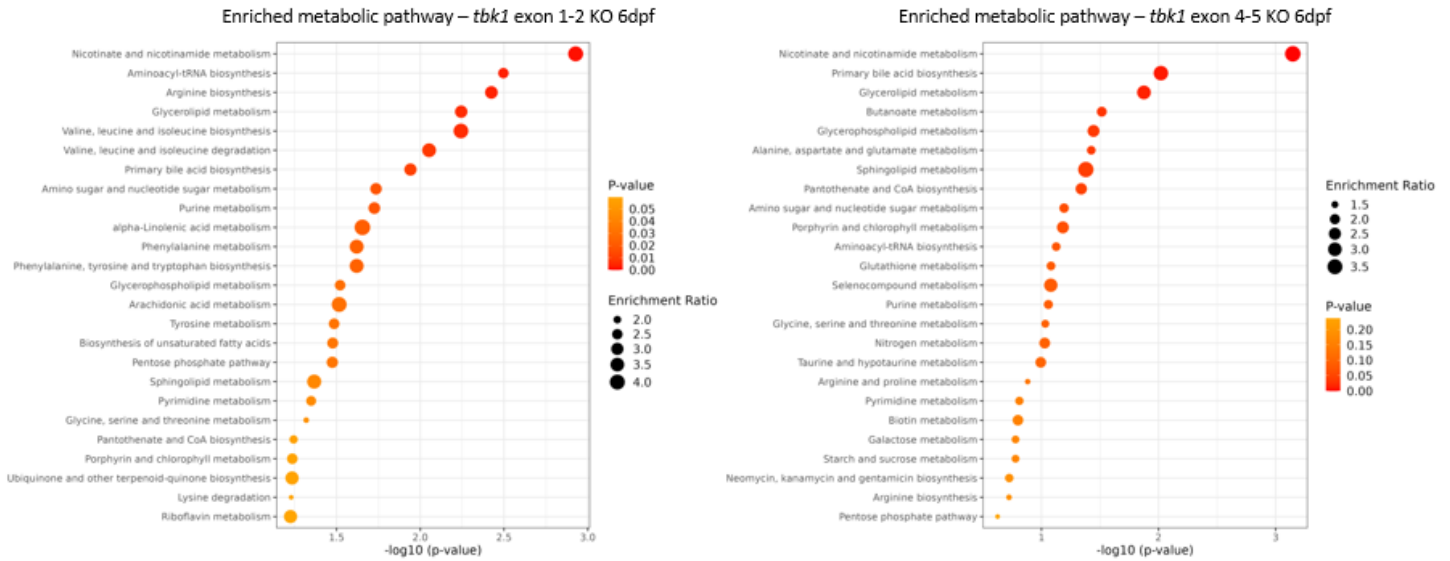


Figure 12: Nicotinate and nicotinamide metabolism are significantly dysregulated in both of the *tbk1* KO conditions at 6dpf

Dot plot of the enrichment analysis results. The size of the circles per metabolite set represents the Enrichment ratio and the colour represents the p-value. Analyses performed on 6dpf larvae bearing a deletion of the exons 1 and 2 of the *tbk1* gene (left) or a deletion of the exons 4 and 5 of the *tbk1* gene (right) compared with non-injected WT larvae. Enrichment Ratio is computed by Hits / Expected, where hits = observed hits; expected = expected hits. (Statistical analysis performed by two-sample *t*-tests).

In this study, five identified metabolites are involved in this metabolic pathway and out of these 5 compounds, 3 are characterized by a common significant change in their levels (p -value > 0.05) upon *tbk1* deletion in the two models compared with non-injected control larvae: NAM and QUIN present increased levels (as shown in the previous section) in opposition to NAD which exhibit lower levels. In addition, the *tbk1* exon 1-2 KO model exhibit a significantly decreased level of L-aspartate (a precursor of QUIN) but this decrease is not shared with the *tbk1* exon 4-5 condition. The last compound identified belonging to the NAD metabolism is NADP, the phosphorylated form of NAD, however its level is not impacted by *tbk1* loss.

4.2 Incubation with nicotinamide riboside rescue *tbk1* KO larvae's altered locomotion

As the NAD salvage pathway was significantly impacted, through increased NAM levels and the decreased NAD pool in our model, this suggests an impaired salvage of NAD. With this hypothesis, I initiated a treatment at early developmental stage through incubation of the larvae with a precursor of NAD: the nicotinamide riboside (NR). NR has been shown to reduce the degeneration of spinal MN and to reduce the characteristic neuroinflammation observed in the hSOD1 G93A mouse model (E. Obrador *et al.*, 2021a). Moreover, NR in combination with pterostilbene, an antioxidant compound, has been proposed as a therapeutic treatment for ALS (E. Obrador *et al.*, 2021a) and clinical trials of using this compound mix are ongoing.

Following a toxicity analysis of NR in non-injected larvae (data not shown) we used the concentration of 10 μ M diluted in dimethyl sulfoxide (DMSO). Our control condition was incubation with DMSO only. Because the 9 days long incubation in NR was too deleterious for the larvae (data not shown), we designed a protocol based on alternating incubation of 10 μ M NR incubation with periodic NR-free water. The larvae were kept in NR free water until 3dpf and then incubated for a day in 10 μ M NR. This first treatment was followed by a NR-free water incubation for a day and finally larvae were kept in 10 μ M NR until the end of the experiment (Fig. 14). During this period, I assessed the locomotor ability of the larvae at day 5 and 6. Importantly, I observed a striking rescue of the locomotor activity of the *tbk1* KO larvae incubated with 10 μ M NR in comparison with those incubated in DMSO only (Fig. 13). Indeed, the total distance swum during the test was no longer significantly decreased compared to non-injected WT control larvae with NR supplementation. Strikingly, at 6dpf, the distance swum by the *tbk1* KO larvae incubated in 10 μ M NR was even significantly increased when compared to the larvae incubated with DMSO only (Fig. 13). However, the incubation in 10 μ M of NR did not impact the lethality observed at 9dpf upon deletion of *tbk1* (Fig. 13). Indeed, at 9dpf, a mortality rate of 90% was observed in the *tbk1* KO larvae treated with NR or in control conditions.

These results suggest that restoring the levels of NAD is sufficient to rescue the locomotor ability of the model, highlighting the importance of NAD metabolism in the pathogenicity of *tbk1* deletion. It is important to mention that this is the first report to link *tbk1* function with the regulation of NAD pathway so far. Furthermore, these results also imply the involvement

of another mechanism, independent from the NAD depletion, responsible for the lethality induced by *tbk1* deletion.

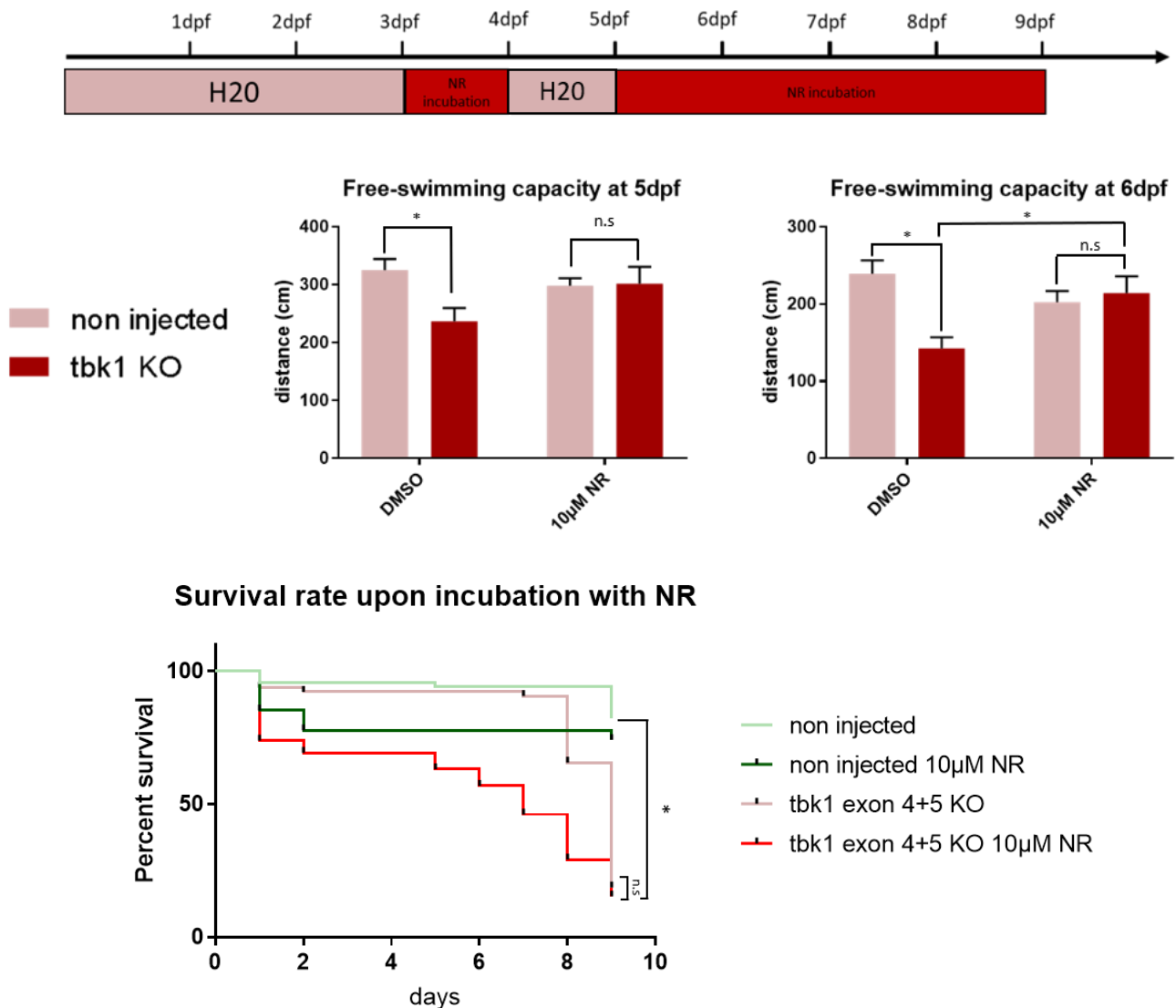


Figure 13: NR incubation is able to rescue locomotor deficits observed in 5 and 6 dpf larvae but does not impact the high lethality due to *tbk1* deletion.

(A) Schematic representation of the incubation protocol, axis represent the stage of development in days post fertilization (dpf). In dark red incubation with 10 μM NR, in light red incubation in DMSO. (B-C) Quantification of the distance swum during the activity test for the larvae bearing a *tbk1* exon 4 and 5 deletion compared to WT non-injected larvae with a 10 μM NR supplementation of DMSO incubation at 5 dpf (B) and 6 dpf (C). (Unpaired t-test comparative analysis. * $p < 0, 05$). (D) Curve comparing the survival rate of *tbk1* KO larvae supplemented with 10 μM NR with *tbk1* KO larvae incubated in DMSO. Non-injected WT larvae were used as negative control. (Log-Rank (Mantel Cox) test. * $p < 0, 05$).

After demonstrating that a dysregulation in the metabolism of nicotinamide is responsible for the apparition of the locomotor phenotype, we next investigated which molecular mechanisms could be responsible for the lethality observed upon deletion of *tbk1*. We therefore perform a similar metabolomic analysis on *Tbk1* depleted larvae at 8dpf.

4.3 Metabolomic profile of the *tbk1* KO larvae at day 8

At 8dpf, we did not identify any dysregulated metabolites in the *tbk1* exons 4 and 5 deletion (q-value inferior to 0.05). However, upon deletion of the exons 1 and 2 of *tbk1*, we identified 3 metabolites that were significantly dysregulated (Fig. 14): quinolinic acid (as observed at 6dpf), uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), and uridine monophosphate (UMP).

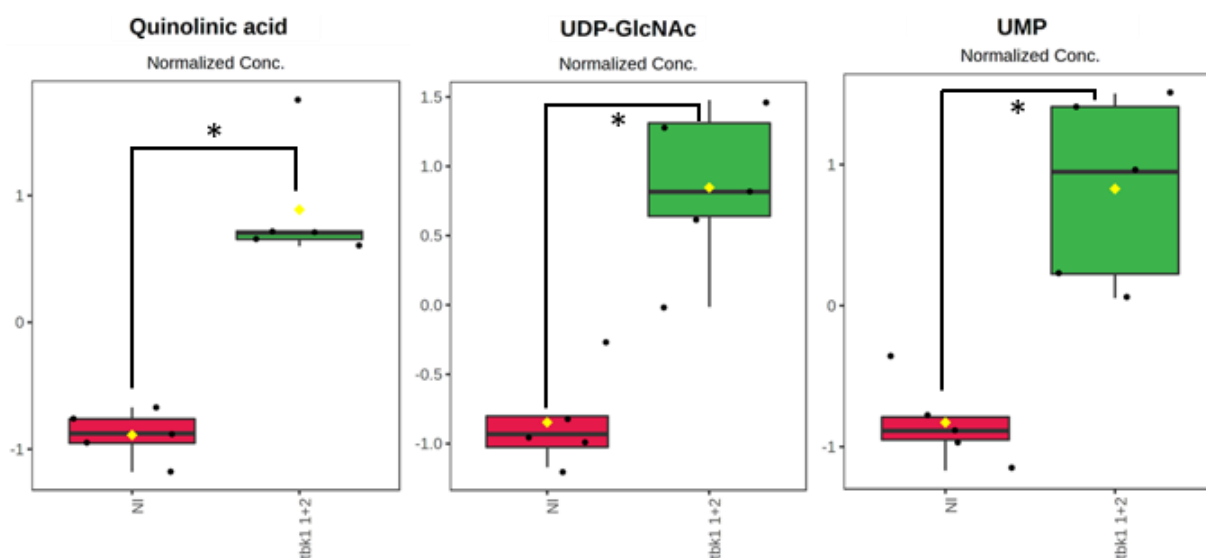


Figure 14: Quinolinic acid, UDP-GlcNAc and UMP exhibit dysregulated levels upon *tbk1* exons 1 and 2 deletion at 8dpf

8dpf whole larvae level of quinolinic acid, UDP-GlcNAc and UMP are upregulated upon deletion of the exons 1 and 2 of the *tbk1* gene in comparison with non-injected WT larvae. (statistical analysis performed by two-sample t-test implemented with a false discovery rate. * $q < 0, 05$).

An increase in quinolinic acid levels, could again suggest a dysregulation in the NAD pathway, however we saw in the previous part that an alteration of the nicotinamide metabolism does not appear to be involved in the lethality phenotype observed. QUIN has been reported to be a neurotoxic compound via several mechanisms such as protein dysfunction, oxidative stress stimulation, glutamate excitotoxicity induction, mitochondrial dysfunction, autophagy, apoptosis or neuroinflammation (J.-M. Lee *et al.*, 2017).

UDP-GlcNAc and UMP are two uridine-based compounds. Therefore, an increase in their level could suggest a dysregulation in the metabolism of uridine. Uridine is a pyrimidine nucleoside involved in several biological processes such as phospholipid synthesis, glycogen storage, or RNA synthesis (G.P Connolly and J.A. Duley, 1999). Uridine has also been suggested to present neuroprotective and neuroregenerative abilities notably due to its involvement in synapse formation (B.S. Baumel *et al.*, 2021; R.J. Wurtman *et al.*, 2010). Moreover, uridine supplementation was reported to be beneficial in the context of several neurodegenerative disease such as Huntington disease (J.A. Saydoff *et al.*, 2003; J.A. Saydoff *et al.*, 2006), Parkinson disease (M. Cansev *et al.*, 2008), and more importantly, ALS (D.J. Amante *et al.*, 2010). In a metabolic context, increased uridine levels are associated with an increased ATP production and an enhanced glycolytic energy production (D.J. Amante *et al.*, 2010). UDP-GlcNAc is a compound that is widely used in the cell metabolism as a substrate for the O-GlcNAc protein modification (J.C. Chatham *et al.*, 2021). Interestingly, O-glycosylation has been reported to be involved in neurodegenerative disease such as Alzheimer disease (A.C. Wang *et al.*, 2016; S.A. Yuzwa *et al.*, 2012) and more importantly in the context of ALS in a rat SOD1^{G93A} model where decreased levels of O-glycosylation were reported (N. Lüdemann *et al.*, 2005). Although it was not mentioned in this study, a decrease in O-glycosylation could suggest an increase in UDP-GlcNAc level similarly as what we observed in our analysis.

Similarly to the 6dpf analysis, it is impossible to identify a clear implication of a compound in the apparition of the lethality observed upon deletion of *tbk1* in our larvae when considering the metabolites as independent from each other. Therefore, we performed an enrichment analysis on our 8dpf data. Four pathways are identified as significantly dysregulated (Fig. 15): 1) the nicotinamide pathway similarly to what was observed at 6dpf; 2) the

glycosylphosphatidylinositol (GPI) anchor biosynthesis; 3) the pyrimidine metabolism; 4) the glycerophospholipids metabolism.

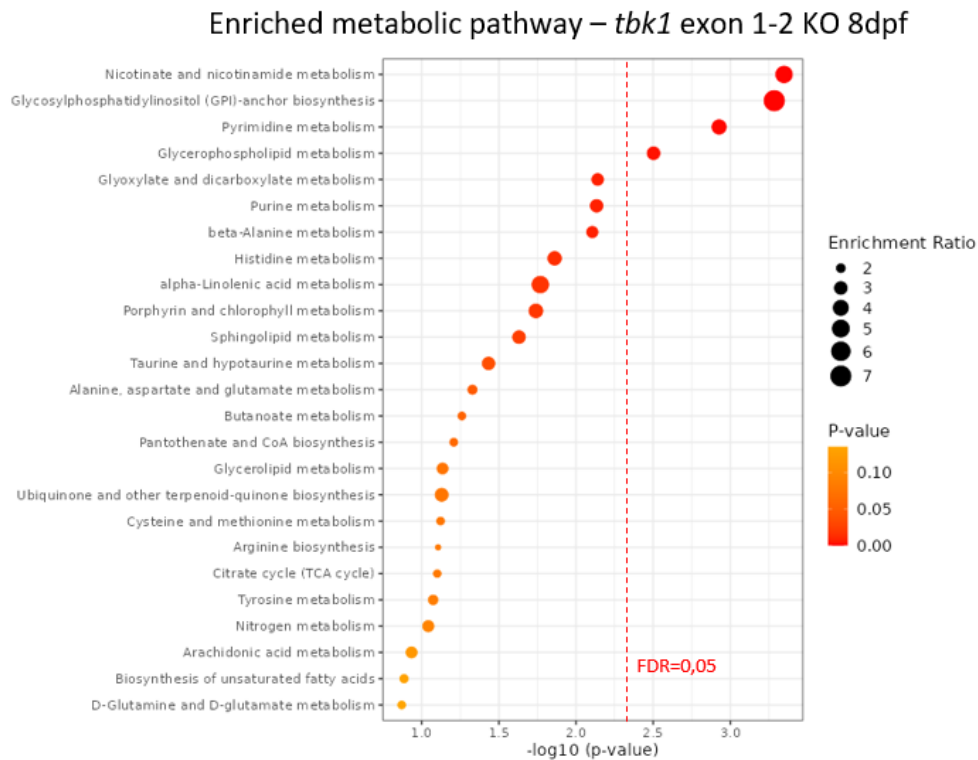


Figure 15: Nicotinate/nicotinamide, GPI anchor biosynthesis, pyrimidine and glycerophospholipid metabolisms are dysregulated in the pooled *tbk1* KO conditions at 8dpf

Dot plot of the enrichment analysis results. The size of the circles per metabolite set represents the enrichment ratio and the colour represents the p-value. Analyses performed on 8dpf larvae bearing a deletion of the *tbk1* gene compared with non-injected WT larvae. Enrichment ratio is computed by $Hits / Expected$, where hits = observed hits; expected = expected hits (Statistical analysis performed by two-sample t-tests).

Apart from the NAD metabolism dysregulation, no significant association was able to pinpoint metabolic dysregulation at 8dpf to explain the increased mortality upon deletion of *tbk1*. A metabolomic-proteomic comparative analysis was performed to unravel altered factors and pathways that could be involved in motor neuron degeneration.

V. Proteomic characterization of the *tbk1* KO zebrafish model

5.1 Proteomic profile of the *tbk1* KO larvae at day 8

Similarly to the metabolomic analysis, we select larvae at 8dpf for lysis and subsequent mass spectrometry. We used again the two *tbk1* KO conditions (exon 1-2 KO and exon 4-5 KO). Out of the 8640 identified proteins, 211 were significantly dysregulated by an ANOVA analysis adjusted with a false discovery rate. To confirm our model, we first check the expression level of *tbk1*. *Tbk1* expression appeared significantly decreased from the ANOVA (p -value=0.043), however when adjusted with a false discovery rate, its expression was no longer significantly dysregulated (q -value=0.073). When looking in details, it became clear that this was due to a lower efficiency of the *tbk1* exon 4-5 KO approach. We therefore decided to focus exclusively on the *tbk1* exon 1-2 KO condition as we could assess the correct decrease in expression of *tbk1* in this pool of samples.

The new *tbk1* exon 1-2 KO versus non-injected larvae analysis identified 115 new significantly dysregulated proteins bringing the total number to 326. We implemented the 326 compounds on the string © online software (L.J. Jensen *et al.*, 2009). Out of this analysis, the software grouped proteins according to different features as the biological process, their molecular function, or the cellular pathway (using KEGG resources) they are involved in. When looking at the biological processes identified, immune response was overrepresented. To define whether dysregulated immune response was present in the *tbk1* KO model, I tested anti-inflammatory compounds in the next section.

5.2 Inhibition of the inflammatory response via caffeic acid incubation doesn't rescue *tbk1* KO induced lethality

Caffeic acid (CA), an anti-inflammation, anti-oxidant and anti-carcinomic compound has been shown to exhibit beneficial effect in the SOD1^{G93A} model (C.V. Fontanilla *et al.*, 2012). The anti-inflammatory feature of this compound relies on the inhibition of the arachidonic acid, an inflammatory compound that was significantly increased in ALS models (H. Lee *et al.*, 2021; A. Trostchansky *et al.*, 2018). Arachidonic acid is degraded by the cyclooxygenase (COX) 1 and 2 enzymes into lipidic cytokines, (H. Tallima *et al.*, 2017) with COX1 orthologue upregulated in our proteomic analysis. 10 μ M of CA was selected as the optimal concentration and tested on the locomotor phenotype observed at 5dpf. No significant

difference in the swimming capacity was observed in the *tbk1* KO larvae treated with 10 μ M of CA (Fig. 16). This suggest that a dysregulated immune response might not be solely responsible for motor defects observed in the *tbk1* KO model. Similarly, no alteration of the lethality upon CA treatment was observed (Fig. 16). However, it is important to point out that CA has limited targets and the impact of other anti-inflammatory compounds should be assessed on the phenotypes observed upon *tbk1* deletion.

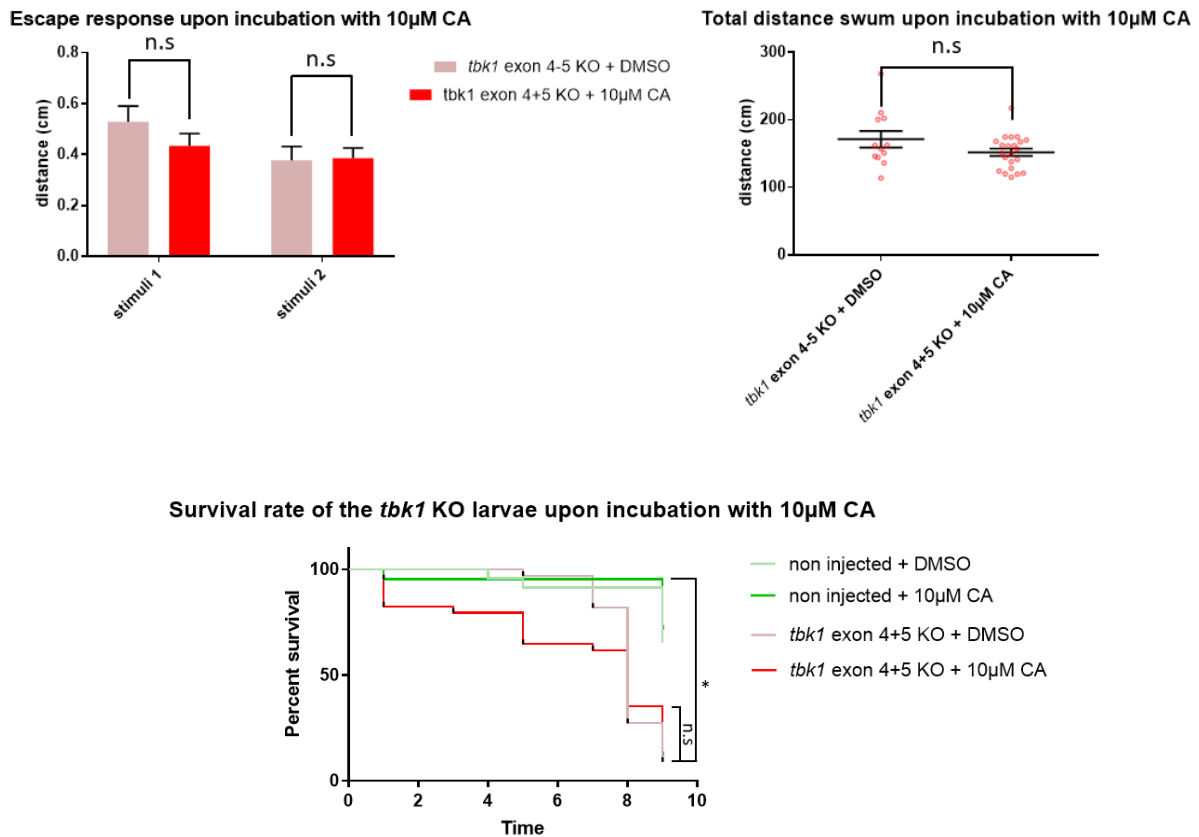


Figure 16: Supplementation with 10 μ M of caffeic acid did not rescue the development of phenotype induced by the deletion of *tbk1*.

(A) Quantification of the distance swum upon sound stimulation for the larvae bearing a *tbk1* exon 4 and 5 deletion supplemented with a 10 μ M CA supplementation or with DMSO incubation at 5 dpf. (Unpaired t-test comparative analysis). (B) Quantification of the distance swum by larvae bearing a *tbk1* exon 4 and 5 deletion supplemented with a 10 μ M CA supplementation or with DMSO incubation at 5 dpf. (A-B) Unpaired t-test comparative analysis. (C) Curve comparing the survival rate of *tbk1* KO larvae supplemented with 10 μ M CA with *tbk1* KO larvae incubated in DMSO. Non-injected WT larvae were used as negative control. (Log-Rank (Mantel Cox) test. * $p < 0, 05$).

VI. Combined metabolomics-proteomics analysis of the *tbk1* KO zebrafish

In order to perform the combined metabolomic and proteomic analysis, we selected the significantly dysregulated molecules and identified three main cellular processes: the necroptosis, the purine metabolism and the apoptosis.

Dysregulation of necroptosis and apoptosis in our model can be directly linked to motor neuron degeneration observed in the *tbk1* KD as well as the increased mortality observed at 9dpf in the *tbk1* KO model. Importantly, these two pathways can be activated upon RIPK1 aberrant activation. This kinase is physiologically sequestered via phosphorylation and ubiquitination at the plasma membrane in a TNF-receptor interacting complex. In this complex, RIPK1 plays an important role in the transmission of inflammatory signals. It is important to note that one of the regulatory phosphate group that can control RIPK1 activation is added through the activity of TBK1 (T. Delanghe *et al.*, 2020).

The mechanistic insight of these two programmed cell death pathways was reviewed in the introduction (Metabolites dysregulation in ALS – part II.C.1)

As TBK1 is an important regulator of RIPK1 activity, an induced cell death via the activation of these pathways is a convincing hypothesis. Moreover, *Tbk1* KO induced lethality in a murine model has been rescued through the additional deletion of *Ripk1* (D. Xu *et al.*, 2018).

The analysis suggests contradictory hypotheses concerning these two pathways. First, the necroptosis pathway appeared as severely enriched upon *tbk1* deletion (top hit when using the KEGG pathway data pool). However, when looking independently at the dysregulated proteins, *cas8* appears upregulated suggesting an inhibition of necroptosis, but *Cyld* level as well suggests the opposite (Fig. 17). Moreover, *Bax*, a crucial activator of the mitochondrial apoptosis induction, is downregulated in our model whereas *Cytochrome c*, which is a caspase activator complex downstream of *Bax*, is upregulated (Fig. 17). It is important to mention that apoptosis process was reported to be activated in the liver of *Tbk1* *-/-* mouse model (M. Bonnard *et al.*, 2000). Altogether, these data highly suggest the involvement of programmed cell death mechanisms in our model with a detailed analysis of these

pathways, needed at the molecular level. Importantly, necroptosis has been shown to be implicated in the development of neurodegenerative diseases (S. Zhang *et al.*, 2017) and that upregulation of necroptotic cell death occurs upon *TBK1* downregulation in the context of ALS (D. Xu *et al.*, 2018).

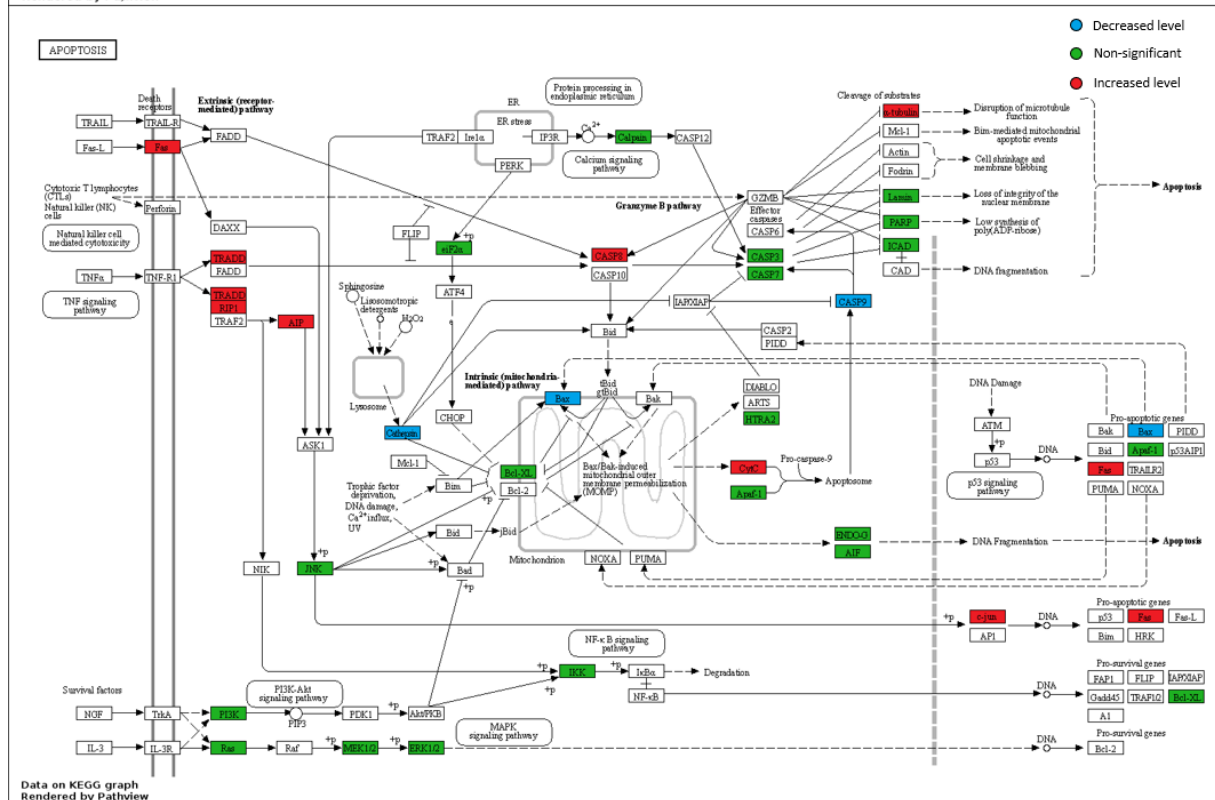
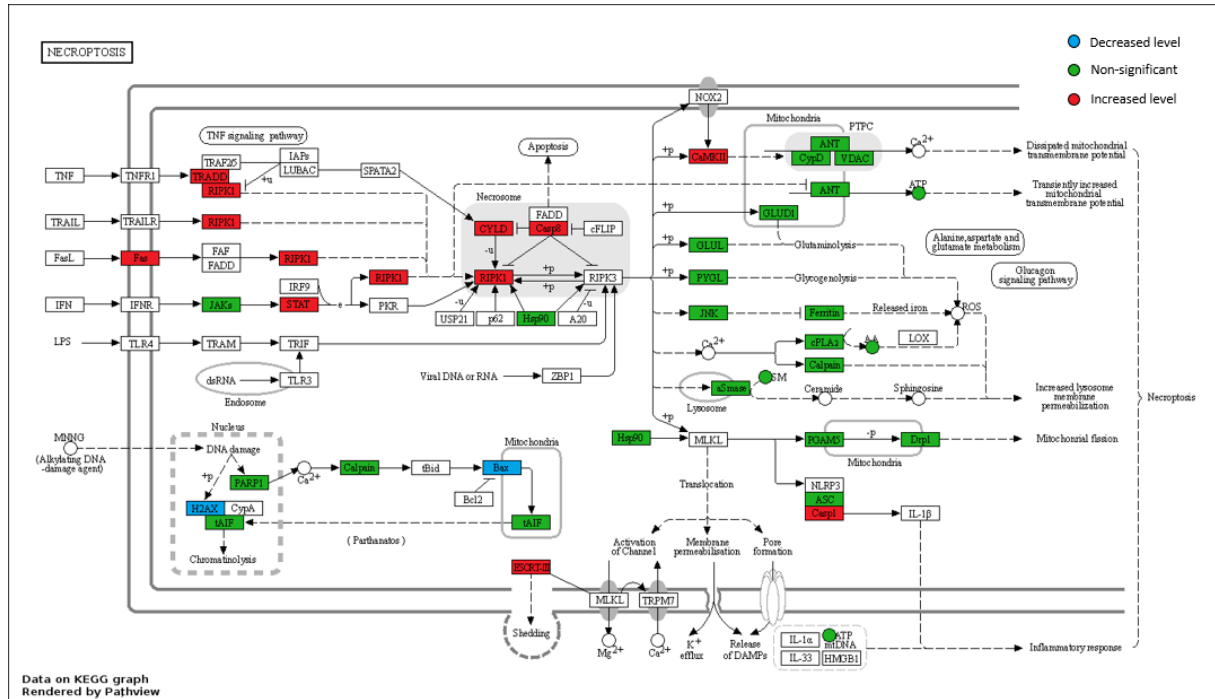


Figure 17: Schematic overview of the programmed cell death pathway:

Top, representation of the necroptosis pathway implemented with proteins and metabolites identified in our analysis. Bottom, representation of the apoptosis pathway implemented with proteins and metabolites identified in our analysis. Circle represents metabolites, rectangle represents proteins. Compounds identified in the proteomic and metabolomic analysis of 8dpf larvae are coloured. In blue compounds identified as significantly downregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In red compounds identified as significantly upregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In green compounds which are not significantly dysregulated (p -value > 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. Adapted from pathview © (W. Luo et al., 2017).

DISCUSSION

Phenotypic features of *tbk1* reduced expression

Two zebrafish models of *tbk1* loss of expression were developed during this thesis. These two models rely on different genetic approaches. The first one is a transient and ubiquitous KD based on the prevention of the *tbk1* transcript translation. The second model is based on the genomic excision of the kinase domain-coding region and the subsequent repression of *tbk1* catalytic activity.

Upon partial deletion of *tbk1* expression, we reported the reduction of the locomotor capacity of the zebrafish larvae at the developmental stage of 2 dpf. We further associate this impaired swimming ability to a decreased number of spinal MN without visible muscular alterations. Moreover, we report that the loss of these cells was not due to a developmental delay as their number is progressively decreasing when looking at different stages. Therefore, the remaining hypothesis is that this loss of spinal motor neurons is linked to cell death. However, more evidences are needed to claim the presence of neurodegeneration in our models, such as immunostaining of specific cell death markers (B.A. Smitch and B.D. Smith, 2012). Moreover, if cell death is proven to be involved in our model, it would be interesting to determine what cellular processes are responsible. Indeed, they are at least dozens of ways for neurons to die (M. Fricker *et al.*, 2018) and identifying the cellular process associated with the possible death of motor neurons in our model would give us great insight concerning the mechanisms of *tbk1* KD impact on the spinal cord integrity and the subsequent impaired locomotion characterising our model. Interestingly, MN loss was not associated with major muscle defects.

Interestingly, the phenotype observed in this model is slowly rescued over time. Although not surprising when using morpholinos as they are degraded over time, in our context it is complicated to explain how the organism can recover from neuronal cell loss. This could be due to regeneration of MN progenitors. Indeed, zebrafish possess the capacity throughout all their life of regenerating spinal MN via reactivation of quiescent progenitor cells from the spinal MN progenitor domain (S.R. Alper and R.I. Dorsky, 2022; M.J. Cardozo *et al.*, 2017). This process was observed early in development, with 3dpf damaged spinal MN being regenerated 48h later (J. Ohnmacht *et al.*, 2016). Preliminary results show that the number

of spinal MN is no longer different in *tbk1* KD larvae compared to the negative control condition (data not shown), further supporting the hypothesis of a regeneration of the lost MNs. Moreover, it is important to mention in a study implying *tbk1*, a crucial regulator of the inflammatory response, that inflammatory cytokines are been reported to play an important role in the initiation of the regenerative mechanism (T.M. Tsarouchas *et al.*, 2018; N. Kyritsis *et al.*, 2012). Although this hypothesis seems, in our opinion, plausible, we still need to confirm it. One strategy would be to combine the decreased expression of *tbk1* with the incubation of the larvae in an immunosuppressant compound such as dexamethasone, which has been shown to prevent the regeneration (N. Kyritsis *et al.*, 2012). Therefore, the characterisation of the apparition of the motor phenotype upon reduced expression of *tbk1* still remains to be investigated more in depth: identity of the lost MN, confirmation of a cellular death involvement, identification of the mechanism involved in the phenotype rescue upon *tbk1* KD.

Interestingly, the phenotype observed upon genomic deletion of sequence coding for Tbk1's kinase domain appears delayed on a developmental time-scale. Indeed, the decrease in the swimming capacity is observed at a later stage, 5dpf, in comparison with the KD model. We have emitted several hypotheses possibly explaining this delay in the apparition of a phenotype:

The first hypothesis states that maternal *tbk1* mRNA could influence the apparition of the phenotypic features in these two models. Indeed, early embryonic development occurs in the absence of transcription and therefore relies essentially on the translation of maternal origin mRNA molecules deposited within the egg during oocyte maturation (S.A. Harvey *et al.*, 2013). The CRISPR technology impacts positively or negatively genic expression through direct alteration of the genomic sequence. Therefore, *tbk1* expression through translation of maternal mRNA can still occur within the first hours of the embryonic development, even in a CRISPR induced KO context. In opposition, the *tbk1* KD model relying on injection of AMOs and the subsequent blockade of the translation, *tbk1* maternal mRNA is no longer expressed. Therefore, we designed both a model in which the first stage of development occurs without expression of *tbk1* – but with its expression being rapidly restored within the following days –; and a model in which *tbk1* is still being expressed within the first developmental stages – but no longer as soon as the embryo start relying on its own transcriptional machinery. It is

considered that the transcription machinery of the embryo is able to totally maintain the cell physiology at the gastrula stage, therefore the usage of maternal mRNAs is restricted to the first 10 hours of development (H. Chang *et al.*, 2018). *tbk1* has been shown to be expressed in the gastrula stage at 5.25 hpf (Y.W. Hu *et al.*, 2018), therefore its expression in early development is indeed permitted by the maternal mRNA stocks. However, this hypothesis implicates an impaired activity of *tbk1* within the first hours of development reverberating on the locomotor function at 2dpf. The eventuality of raising a stable *tbk1* KO zebrafish homozygous line to answer this hypothesis is made impossible by the induced lethality. Therefore, generating a model with complete repression of the *tbk1* maternal mRNA's expression and a time lasting phenotype would require the combination of the two approaches: injecting *tbk1* AMO in one cell eggs produced by a heterozygous *tbk1* KO stable line. However, even such approach has its limitations. indeed, selecting the homozygous larvae would require to perform genotyping through the cut of the tail's tip which would influence the swimming ability of the larvae and therefore might bias the phenotypic analysis.

A second hypothesis could explain the delay in the apparition of the locomotor phenotype. Three alternates splicing transcripts of *tbk1* have been identified (Y.W. Hu *et al.*, 2018; J. Zhang *et al.*, 2020) in the zebrafish. The products of these transcripts have been reported to interfere with Tbk1 physiological function by competing in the binding of Irf3 (L. Zhang *et al.*, 2016) or by inducing the degradation of both full length Tbk1 and Irf3 (J. Zhang *et al.*, 2020). *tbk1* AMO by directly preventing the ribosomes to recognize the ATG codon also inhibits the expression of Tbk1's isoforms whereas the CRISPR approach only prevent the expression of a kinase active protein, and therefore does not prevent the expression of non-functionally active Tbk1's isoforms. Due to their involvement in the regulation of the IFN production via the inhibition of Tbk1-Irf3 interaction, we cannot exclude that the difference observed in the phenotype apparition is linked to their expression.

A last hypothesis would be the existence of compensatory mechanism. Indeed, it was reported that genetic compensatory mechanisms require the degradation of mRNA to activate (M.A. El-Brolosy *et al.*, 2019). This would explain why a possible compensatory mechanism would only occur in the KO model as AMO's activity does not implicate the degradation of the target mRNA. Moreover, the genetic compensation hypothesis is

supported by the activity of *Ikke*, a kinase presenting overlapping function with *Tbk1* (K.R. Balka *et al.*, 2020). The measurement of *ikke* mRNA levels via qPCR could unshadow an increased expression traducing a compensatory effect of *Ikke* upon *Tbk1* loss.

Metabolomic and proteomic targets in the *tbk1* deletion mutants

In order to further understand the apparition of this locomotor phenotype, we performed a metabolomics analysis. From this analysis we have been able to associate the apparition of locomotor defects with a decreased level of NAD⁺ in the *tbk1* KO larvae. Indeed, incubation with the NR precursor of NAD⁺ was sufficient to rescue the locomotor activity of the *tbk1* KO larvae. This is not the first-time that decreased level of NAD⁺ are reported in the context of ALS (E. Blacher *et al.*, 2019; B.A. Harlan *et al.*, 2020). However, to our knowledge this is the first association between TBK1's impaired function and NAD metabolism dysregulation. This suggests that through an unknown mechanism, TBK1 regulates, indirectly or not, NAD⁺ stock. It is not clear at which step of the NAD⁺ metabolism TBK1 is involved but the decrease in NAD⁺ stock combined with an increase in NAM level suggest several mechanisms. Several catabolic reactions are associated with the degradation of NAD⁺ into NAM. Therefore, one could think that in response to TBK1 loss of activity, the rate of one of these enzymes is increased and therefore lead to the accumulation of NAM at the detriment of NAD⁺ level. Indeed, through their activity, the enzymes responsible of these reactions are regulating the pool of NAD⁺. Amongst these enzymes we can mention the PARPs family of protein (involved in the post-transcriptional addition of poly-ADP-ribosyl marks), the SIRT6 protein (involved in proteic deacetylation), and the SARM1 and CD38 hydrolases (generating adenosine diphosphate ribose from NAD⁺) (N. Xie *et al.*, 2020). Interestingly, some of these NAD⁺ regulatory enzymes have been associated with ALS. For instance, SARM1 is a physiologically inactive protein that is involved in the axonal degradation upon lesion (Essuman *et al.*, 2017). Mutation in *SARM1* leading to constitutive activation of the encoded SARM1 protein was reported to be associated with the development of the ALS pathology (A.J. Bloom *et al.*, 2022). Therefore, such a scenario could also be plausible in our model. To further deepen this hypothesis and investigate a potential increased rate of activity of these enzymes, their respective expressions should be quantified either at the transcriptional or protein level respectively by qPCR and WB.

A second hypothesis that could explain this situation is an impaired salvage pathway. Indeed, upon synthesis, NAM is reintroduced into the nicotinate metabolism by being reconverted into NMN, the direct precursor of NAD⁺. The NR being able to rescue the phenotype, it is unlucky that the reaction responsible for NMN conversion into NAD⁺ is impaired. Therefore, this hypothesis relies on a possible impaired activity of the enzyme responsible for the catabolic production of NMN from NAM. This enzyme is called nicotinamide phosphoribosyltransferase (NAMPT) and to confirm its possible increased activity, an expression assay should be performed either at the mRNA or the protein levels.

Moreover, glucose metabolism defects are suggested as the level of glycerol-3-phosphate (G3P) is impaired in our model. G3P is an intermediate in the glycolysis pathway. As mentioned in the introduction, glucose metabolism is altered in ALS patients because of either impaired cellular glucose uptake (T.W Tefera *et al.*, 2021), defective glucose synthesis regulation (T.S. McDonald *et al.*, 2021), or altered expression of enzymes involved in the catabolic degradation of glucose (L. Palamiuc *et al.*, 2015; T.W Tefera *et al.*, 2021). G3P being directly derived from glucose metabolism, its dysregulation suggests an hypermetabolism state as observed in patients (P. Fayemendy *et al.*, 2021). However, as the level of glucose or pyruvate do not seem to be impacted by the loss of *tbk1* in our analysis, it suggests that G3P depletion in our model could be linked to another catabolic reaction. An investigation of the different reaction involving G3P would be required to understand this decreased level.

We also report a high lethality upon complete deletion of *tbk1* in the zebrafish larvae model. M. Bonnard in 2000 has published a study based on a *Tbk1* KO mouse model where she reports the death of the embryo early in development (at E14.5) (M. Bonnard *et al.*, 2000). This induced lethality, associated with the liver's degeneration and an impaired expression of the inflammatory related complex NFκB, unveiled a crucial role for TBK1 in the liver development. A link with the lethality observed in our zebrafish model could be easily done. However, I have not assessed the liver integrity of the larvae and therefore we cannot affirm that our larvae die from a liver failure. It would be an interesting feature to analyse but as the hepatic system is spared in ALS patients, we decided to focus on understanding the apparition of ALS hallmark in our model. Moreover, I assessed the responsibility of an hyperinflammatory state in the *tbk1* KO zebrafish's developmental death by inhibiting the crucial neuroinflammation actor arachidonic acid through incubation with caffeic acid

without success. This suggests that a dysregulation in the arachidonic acid dependent inflammatory response is not involved in our model, however, we still need to investigate the responsibility of inflammation by inhibiting other aspect of this cellular process by using glucocorticoid compounds for instance (Y. Xie *et al.*, 2021) or directly inhibits quinolinic acid (which exhibit increased level in our model) via blockade of its upstream enzyme KMO (G. Constantino, 2014). Moreover, to further confirm the link between our zebrafish and the murine *tbk1* KO models, it could be interesting to assess the integrity of the zebrafish liver at 8dpf.

Since the subjacent molecular mechanisms associated with the *Tbk1* KO induced lethality have never been extensively explored, we performed a combined proteomic and metabolomic characterisation of the zebrafish just prior to their death (8dpf). The increase in quinolinic acid confirms the involvement of inflammation in our model which deepen our will of pursuing the investigation of inflammation in our model. Furthermore, the combined proteomic-metabolomic analysis also suggested the dysregulation of two programmed cell death mechanisms in our model: necroptosis and apoptosis. Interestingly this correlates with a study perform in the murine model in which a combined deletion of *Tbk1* and a kinase dead mutation in *Ripk1* was sufficient to significantly delay the lethality induced by the loss of *tbk1* (D. Xu *et al.*, 2018). However, as RIPK1 is involved in both the necroptosis mechanism and the CASP8-associated apoptosis, this study does not allow to identify the cell death mechanism involved in the model. In our case, although the identification of these two pathways as possible therapeutic targets is really exciting, we have not been able to confirm their involvement in the lethality induced by *tbk1* KO in the zebrafish larvae because of a lack of time. Therefore, in my opinion, the first experiment to perform in the continuity of this work would be the assessment of a possible rescue of the lethality via incubation with different drugs targeting one or both of these mechanisms. Amongst the drug identified to perform this series of test, we are considering the use of the following drugs: 1) in the continuity of D. Xu study in the murine model an inhibitor of RIPK1 activity: necrostatin-1 therefore inhibiting both necroptosis and apoptosis (X. Gao *et al.*, 2022), 2) an apoptotic specific inhibitor such as decylubiquinone (inhibitor of the mitochondrial induced apoptosis) (A.R. Bourefis, 2019) or Z-IETD-FMK (Casp8 inhibitor) (Z. Qi *et al.*, 2021) 3) necroptosis specific inhibitors such as necrosulfonamide (inhibitor of the MLKL activity – even though

not orthologs have been identified in the zebrafish) (D. Liao *et al.*, 2014) or GSK872 (an inhibitor of RIPK3 activity) (W. Wen *et al.*, 2022). This drug screening experiment will unveil the suggested responsibility of these death pathways in the apparition of death in our model.

Additionally, the last molecular features of our model are the dysregulations of the purine and the pyrimidine metabolisms. These were not reviewed in the results section as nothing can be concluded out of the data from our analyses. However, because of their involvement in the composition of DNA and RNA, purines and pyrimidines are ones of the most abundant metabolites in living organisms. These metabolisms rely on the production of (poly)nucleotides, which are the crucial actors of the metabolism as they are used as cofactors in most of the metabolic pathways. During these reactions, phosphate groups are cleaved from their structures which eventually lead to the formation of the purine (adenine, guanine and inosine) or pyrimidine (cytosine, uridine, and thymidine) nucleosides (A.M. Pedley *et al.*, 2017). Nucleosides are the structural components of the nucleic acid that allows coding of the genetic information.

The end reaction of the purine pathway is the degradation of the purines into uric acid via the generation of hypoxanthine (Fig. 18). In our data set, we observed a disequilibrium between the levels of nucleotides, which are either unchanged or significantly increased (GMP) and the levels of nucleosides which are all decreased upon *tbk1* deletion (Fig. 18). This is supported by increased levels of cytosolic 5'-nucleotidases-I (cN-I) in our model. This enzyme catalyzes the degradation of nucleotides to nucleosides. Accumulation of cNs proteins has been exhibited in the context of neuromuscular pathologies as they are colocalizing with characteristic inclusions of these diseases (H. Pluk *et al.*, 2013). It could be interesting to investigate the presence of inclusions in our model to further confirm this hypothesis. To our knowledge, this is the first study to suggest an association between ALS and a cN-I dysregulation.

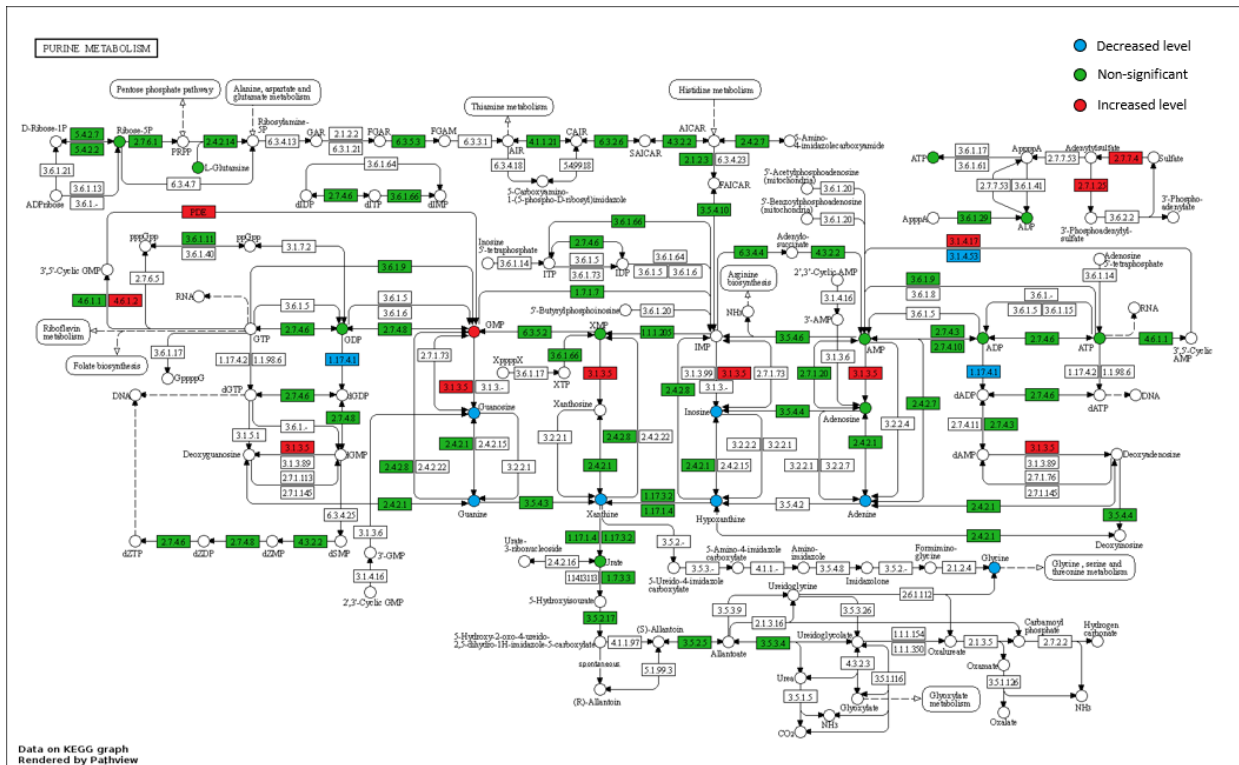


Figure 18: Schematic overview of the purine metabolism:

Circle represents metabolites, rectangle represents proteins. Compounds identified in the multi-omics analysis of *8dpf* larvae are coloured. In blue, compounds identified as significantly downregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In red, compounds identified as significantly upregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In green compounds which are not significantly dysregulated (p -value > 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. Adapted from pathview © (W. Luo et al., 2017).

Abbreviations: [PDE and 3.1.4.17]: cGMP-inhibited 3',5'-cyclic phosphodiesterase B; [4.6.1.2]: atrial natriuretic peptide receptor 1; [1.17.4.1]: ribonucleoside-diphosphate reductase large subunit; [3.1.3.5]: cytosolic 5'-nucleotidase 1A-like isoform; [3.1.4.53]: cAMP-specific phosphodiesterase 4; [2.7.1.25 and 2.7.7.4]: 3'-phosphoadenosine 5'-phosphosulfate synthase

Concerning the pyrimidine dysregulation, it can be directly linked to the alteration of the UDP-GlcNAc level as seen in the results section. In the analysis we observe a significant increase in some mono-phosphate pyrimidine (CMP and UMP) (Fig. 19). However, the levels of their respective di-phosphate forms remain unchanged upon loss of *tbk1* (CDP and UDP)

(Fig. 19). This suggests that the catabolic steps of the pathway are not altered. Similarly, increased levels of CMP and UMP suggest that the *de novo* synthesis path is functionally active (Fig. 19). However, orotate, a precursor of UMP exhibited decreased levels, questioning about a possible overactivation of the *de novo* synthesis step (Fig. 19). In the proteomics data, none of the identified enzymes involved in this pathway are significantly dysregulated upon *tbk1* loss. Therefore, the observed increased levels of CMP and UMP must be imputed to another catabolic reaction and further investigations would be needed in order to understand their involvement in our model.

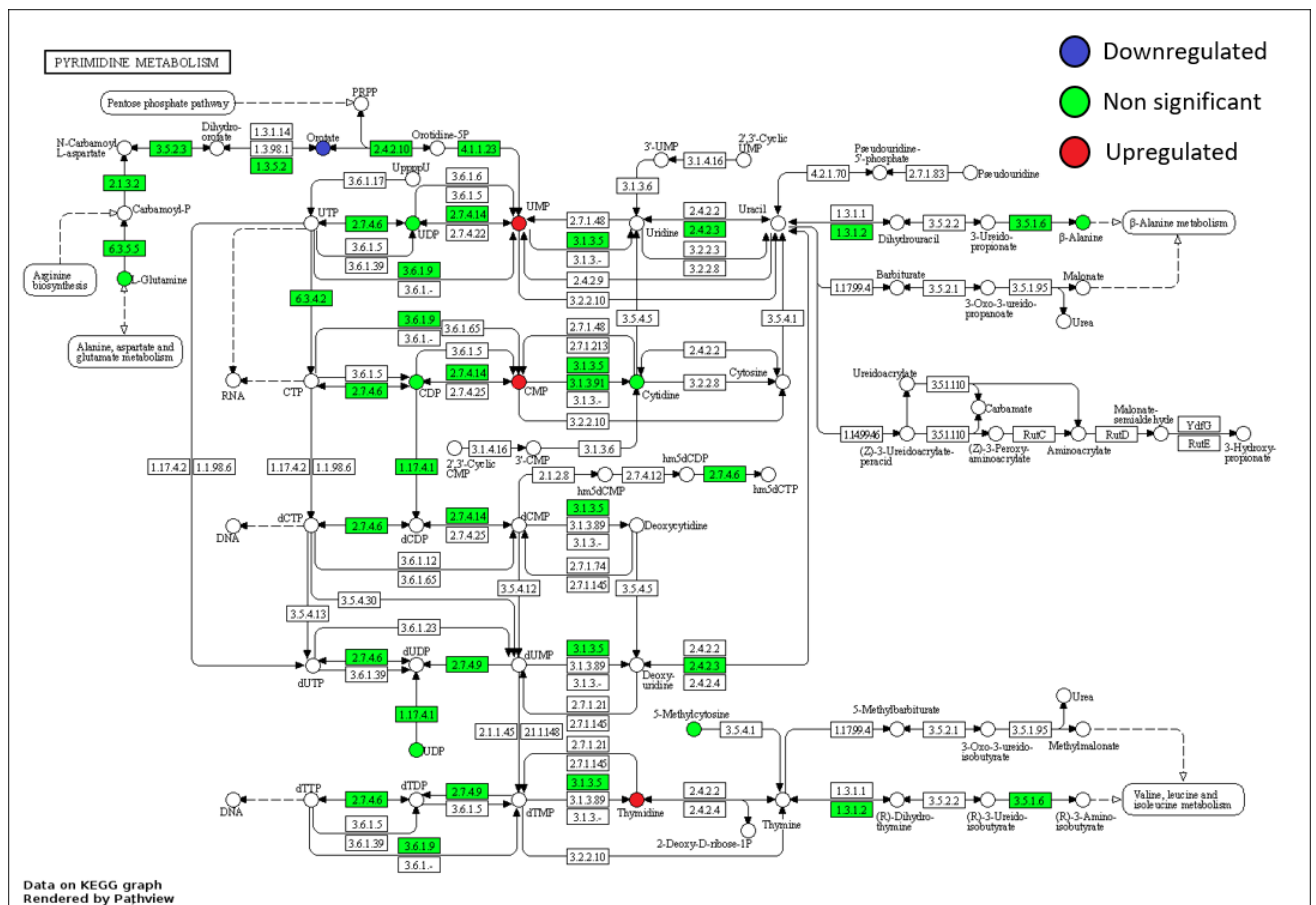


Figure 22: Schematic overview of the pyrimidine metabolism:

Circle represents metabolites, rectangle represents proteins. Compounds identified in the multi-omics analysis of *8dpf* larvae are coloured. In blue, compounds identified as significantly downregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In red, compounds identified as significantly upregulated (p -value < 0.05) upon *tbk1* deletion in comparison with non-injected WT larvae. In green compounds which are not significantly dysregulated (p -value > 0.05)

upon *tbk1* deletion in comparison with non-injected WT larvae. Adapted from pathview © (W. Luo et al., 2017)

Limitations and future directions of this study

It is important to mention that although it recapitulates some hallmarks of the disease, this model still lacks some strength. First, we have not been able to report the presence of TDP-43 aggregates, which is the main cellular hallmark of the disease. Similarly, major targets and pathways involved in neurodegeneration, including autophagy and immunity can be impacted by ageing. Therefore, studying a late onset disease in a larval and embryonic model can lack specific age-related cellular processes. Moreover, the sole heterozygous loss of *TBK1* in mouse, in opposition to our model, has not been reported to be sufficient to provoke the apparition of a phenotype in other models (D. Brenner *et al.*, 2019; V. Gerbino *et al.*, 2020). Therefore, we cannot exclude the hypothesis that zebrafish exhibit some specific *Tbk1* related features that are not shared with higher complexity organisms. For all these reasons, the model designed in this study should be more considered as a tool to understand *Tbk1* functional role. However, this model could be improved to better mimic ALS if implemented with another “hit”. Indeed, because its development only occurs in a fraction of individuals with a genetic predisposition with the majority individuals develop ALS with no known mutation or genetic risk factor, this disease is thought to be caused by multiple factors (A. Al-Chalabi *et al.*, 2014; A. Chiò *et al.*, 2018; S. Vucic *et al.*, 2018). This “multiple hit” or “multiple step” hypothesis is further supported by its late onset-feature as its development is caused by ageing (T. Niccoli *et al.*, 2017) as well and by the high number of genes involved in its development (S. Mathis *et al.*, 2019). This hypothesis was further deepened in the context of *TBK1* related ALS, D. Xu in its 2018 study shown that the sole *Tbk1* heterozygous loss was sufficient to induce an ALS-like phenotype when combined with the age-related reduced levels of MAP3K7 (or TAK1), another regulator of RIPK1’s activity (D. Xu *et al.*, 2018). Moreover, in the same study, they report that according to the age of the mouse, Ripk1 activation has different outcome. Therefore, one strategy in order to create a double hit model involving *tbk1* loss in the zebrafish would be to generate a stable *tbk1* KO heterozygous line and investigate the consequences of this impaired expression at later stages with protein levels modified by the ageing process (through spinal cord, muscle or

brain dissection and subsequent metabolomics/proteomics analyses). Moreover, another strategy would be to perform a double heterozygous knock out of the *tbk1* and *map3k7* and grow a stable line. However, to generate these lines and to perform the analysis are time consuming and could be performed in the continuity of the project. In addition, the second strategy would also require to assess map3k's expression. This example is based on the sole observations report in D. Xu's study but several other strategies could be developed, for instance additional *tbk1* heterozygous loss has also been reported to have an impact on SOD1, TDP-43 or C9orf72 mice models (W. Shao *et al.*, 2022; D. Brenner *et al.*, 2019; K. Sieverding *et al.*, 2020).

To conclude, this work was aiming to generate a zebrafish model of *tbk1* loss in the context of better understanding *TBK1* associated ALS-FTD pathology. Here we show that *Tbk1* loss of function can lead to locomotor defects and neurodegeneration such as observed in the ALS or FTD pathologies. We have been able to characterize the phenotype induced by the loss of *tbk1* expression. Interestingly, due to its fast development, zebrafish is the first *in vivo* model where the sole loss of *tbk1* is associated with deficits in locomotor functions induced at least partly by alteration of the NAD⁺ level. Moreover, we have also been able to partially confirm what was observed in the murine *tbk1* KO model with an increased lethality early in the development (9dpf). Furthermore, a metabolic analysis demonstrated that activating the NAD⁺ pathway through NR can rescue motor deficits in the *tbk1* KO model. Importantly, a combined proteomics and metabolics analysis pointed out to the necroptosis and apoptosis cell death pathways that will be investigated in detail at the molecular and pharmacological level in this novel model of ALS-FTD.

MATERIALS AND METHODS

Experimental model maintenance and handling:

Adult and larval AB wildtype zebrafish (*Danio rerio*) were maintained at the Institut Imagine, Paris, zebrafish facility and bred according to the National and European Guidelines for Animal Welfare. All procedures were approved by the Institutional Ethics Committees at the Research Centers of Imagine. Embryos were raised at 28°C and kept in egg-water (0.06g/L aquarium salt (Instant Ocean, Blacksburg, VA) in Milli-Q water +0.01 mg/L methylene blue) until 5 days post fertilization. After 5 days post fertilization embryos were transferred in water. Egg clutches were collected from a single cross and injected at the one-cell stage. In this study both male and female fishes were used. The following transgenic lines were used for imagery *Mnx:RFP*.

Morpholino and sgRNA synthesis and injection:

Microinjections of antisense morpholino oligonucleotide (AMO) were performed on wildtype and *Mnx:RFP* transgenic AB fishes at the one cell stage using a PV 820 Pneumatic PicoPump injector (World Precision Instruments, USA) and heat-pulled borosilicate glass needles. *tbk1* morpholino (synthesized by Gene Tools, Philomath, USA) targeting the initial ATG codon of the *tbk1* gene (AMO-*tbk1*: 3'-ggccgtactctgcatgatgactgta -5') were injected at the one cell stage to realize a transient knockdown of *tbk1*. Mismatch control morpholino bearing a nucleotide sequence (mismatch 5'-cctcttacctcagttacaattata-3') that does not bind any zebrafish mRNA was used to exclude any injection-related phenotype. Microinjections were performed at 0.45mM for both the *tbk1*-targetting morpholino and the mismatch control.

Target nucleotide sequence for guide RNA design were selected using the CHOPCHOP software and scoring method and obtained directly synthesized as RNA oligo nucleotides (Alt-R® CRISPR-Cas9 crRNA, IDT, USA). Sequences are listed below:

sgRNA *tbk1* exon 1: GCCACAGCUAACGUGUACCG

sgRNA *tbk1* exon 2: CGUCAAGCUGUUCGCCGUCG

sgRNA *tbk1* exon 4: AUGAAUCACCU GCGCGAGUA

sgRNA *tbk1* exon 5: CUGCCGUUCAGACCCUUCGA

sgRNA *tyrosinase* exon1_1: CAGUAUCCUCACUCAGGAGU

sgRNA *tyrosinase* exon1_2: UCCAGCUGUCCACUACCGAG

Guide RNAs were fused with a Cas9 recruitment sequence (Alt-R® CRISPR-Cas9 tracrRNA, 1072532, IDT, USA) by mixing 2µL of each type of nucleotides. Incubation was performed at 50µM for 5min at 95°C. To prevent Cas9 precipitation 0.4µL of a HEPES buffer was added (200mM HEPES-NaOH pH7.5, 1.5M KCl). To induce an 800bp deletion in the *tbk1* gene, guide RNAs were used by pair. Injection solution was prepared by mixing 1µL of each guide solution with 2µL of Cas9 enzyme (Alt-R® S.p. Cas9 Nuclease V3, 1081058, IDT, USA) to obtain a final volume of 4µL. Microinjections were performed on wildtype AB fishes at the one cell stage using a PV 820 Pneumatic PicoPump injector (Wold Precision Instruments, USA) and heat-pulled borosilicate glass needles.

PCR amplification:

To assess the correct deletion induced in the *tbk1* gene PCR amplification of the target region was performed. For each injection set DNA extraction from 20 larvae was performed through incubation in 40µL of extraction buffer (25mM NaOH, 0,2 mM EDTA) for 1h at 95°C, followed by the addition of 40µL of TrisHCl (40 mM, pH 5.5). Solution was stored at 4°C until further manipulation. PCR amplification was performed using the following *tbk1* primers (synthesized by Eurofins, USA):

tbk1 exon 1-2 deletion: F 5'-TCTGAAGCTCATGCCTGTGTG-3';

R 3'-CCTGAGAAGAGCTGCTGGATTA-5'

tbk1 exon 4-5 deletion: F 5'-ACACATCACATTCACCTCAAACTAA-3';

R 3'-GCTCGTCATGCTAACTCCACT-5'

For each sample 2µL of DNA was mixed with 10µL Hot Start Taq 2X Master Mix (NEB, M0496), 0.8µL of each primer and 6.4µL of sterile water. PCR amplification program was optimized for 30 cycles at an annealing temperature of 60°C. Migration gel was prepared at 1% using agarose (A9539, Sigma Aldrich) in 1X TAE (A4686, PanReac AppliChem).

Locomotion analysis of zebrafish embryos:

Locomotor activity was assessed at 2 days post fertilization through a touch-evoked-escape-response test. Larvae were placed at the centre of a 15cm Petri dish and their tails were lightly touched with a tip. This stimulus provokes an escape response that was recorded using a grasshopper digital camera (Point Grey) at the speed of 30 frames per second. Larvae were this way scored for the distance swam, time travel and their velocity using the fiji plugin manual tracking.

At later stage (>3dpf) locomotor activity was assessed using a spontaneous locomotion test. Larvae were maintained in a 96 wells plate and their total locomotor activity was record using a ZebraBox (Viewpoint Life Sciences, Lyon, France). The protocol used was composed of 2 distinct periods: 1) 40 minutes of free-swimming without any stimulus (dark environment), 2) 2 stimuli separated by a 10 minutes period to avoid any habituation effect. Each stimulus consists of a 1 second sound (450 Hz, 80 dB) and an exposition to bright light. Total swim activity was analyzed using ZebraLab V3 software © (Viewpoint Life Sciences, Lyon, France). The escape response induced by the stimuli was quantified by measuring the distance swam 5 seconds after stimulation.

Whole-mounts zebrafish larvae imaging and muscle-axonal measurements:

At 50hpf Mnx-RFP larvae were anesthetized with tricaine and fixed in 4% FA during 30 minutes at 4°C and agitating. Larvae were then washed 3 times in PBS 1X at 4°C. The larvae were mount by removing the cephalic part in a 70% glycerol (G9012, Sigma Alrich) solution. Image were taken with an apotome (Apotome.2, Zeiss) at 20x magnification and a laser of 561nm (HXP 120V, OSRAM) for spinal integrity investigation or bright light for muscle fibres measurements.

Immunoblotting:

30 µg of proteins from zebrafish lysates (equal amount of protein in each lane) were separated by sodium dodecylpolyacrylamide gel electrophoresis. Samples were denatured at 98°C for 7 minutes. The separated proteins were transferred to nitrocellulose membranes (0.45µm, Life Sciences) and probed with the following primary antibody: NAK1/TBK1 antibody (ab109735, Abcam), and α-tubulin antibody (T5168, Sigma-Aldrich) was used as a loading control. The blots were incubated with the corresponding fluorescent secondary

antibody and the signal was detected using the ODYSSEY® CLx. The intensity of bands in each of the lanes from the Western blots were measured by ImageJ.

Drug incubation and survival measurement:

Group of 12-10 larvae were kept in 12 wells multi-dish in 3.5mL of either egg-water (0.06g/L aquarium salt (Instant Ocean, Blacksburg, VA) in Milli-Q water +0.01 mg/L methylene blue) at 28.5 °C from 1dpf to 5dpf or water after 5dpf. Larvae were feed daily with paramecia and media was changed every two days. When needed media was implemented with 10µM of caffeic acid (C0625, Sigma Alrich) or nicotinamide riboside (SMB00907, Sigma Alrich) and dimethyl sulfoxide was used as negative control (D2438, Sigma Alrich). Number of dead larvae was scored each day for 12 days.

Statistical analysis:

All data values for the zebrafish experiments are represented as average standard error of mean (SEM) with significance determined using one-way ANOVAs. Differences between groups were identified via post hoc comparisons, specified in the legend of each figure. All analyses were performed using Prism 5.0 (Graph Pad, CA). Significance level was set at $p < 0.05$.

Metabolomics analysis:

Zebrafish larvae were collected respectively at 6 and 8dpf and stored at -80°C until analyses. After addition of an extraction solution made of 50% methanol, 30% acetonitrile, and 20% water (G.M. Mackay et al., 2015) in ratio of 1ml per 30mg of larvae, the samples were vortexed for 5 minutes at 4°C , then centrifuged at 16,000g for 15 minutes at 4°C . The supernatants were collected and separated by liquid chromatography-mass spectrometry (LC-MS) using SeQuant ZIC-pHilic column (MilliporeSigma). The aqueous mobile-phase solvent was 20 mM ammonium carbonate plus 0.1% ammonium hydroxide solution, and the organic mobile phase was acetonitrile. The metabolites were separated over a linear gradient from 80% organic to 80% aqueous for 15 minutes. The column temperature was 50°C , and the flow rate was 200 µL/min. The metabolites were detected across a mass range of 75 to 1,000 m/z using the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) at a resolution of 35,000 (at 200 m/z) with electrospray ionization and polarity switching mode.

Lock masses were used to ensure mass accuracy below 5 ppm. The peak areas of different metabolites were determined using Thermo Fisher Scientific TraceFinder software using the exact mass of the singly charged ion and known retention time on the HPLC column.

As a part of the routine analytical pipeline, the recommendations of the metabolomics Quality Assurance & Quality Control Consortium were applied. The routine quality controls included regular equipment maintenance (Thermo Fisher Scientific) and the use of standard operating procedures for sample extraction, storage, and analyses. General practices also included weekly test runs to ensure system stability and quality of runs. Regarding the quality controls (QCs) in relation to this study, we used a) pooled interstudy QC, b) process and extraction blanks, c) system stability blanks, d) solvent blanks, e) long-term reference standard interlaboratory QC mix to ensure system stability, and f) samples that were blinded and loaded in randomized order. The analyses of pooled samples' QC showed no significant difference in metabolites' levels between QCs.

Data analysis was performed in the MetaboAnalyst 5.0 software (Z. Pang et al., 2021). Samples were normalized by sum and each metabolite level was adjusted by autoscaling. PCA was performed including all the metabolites identified. Hierarchical clustering was performed using the Ward method including all the metabolites identified.

Proteomics analysis:

Sample preparation: S-TrapTM micro spin column (Protifi, Hutington, USA) digestion was performed on 20µg of whole zebrafish larvae lysate according to manufacturer's instructions. Briefly, samples were reduced with 20mM TCEP and alkylated with 50mM CAA (chloroacetamide) for 15min at room temperature. Aqueous phosphoric acid was then added to a final concentration of 1.2% following by the addition of S-Trap binding buffer (90% aqueous methanol, 100mM TEAB, pH7.1). Mixtures were then loaded on S-Trap columns. Five extra washing steps were performed for thorough SDS elimination. Samples were digested with 1.5µg of trypsin (Promega) at 47°C for 1h30. After elution, peptides were vacuum dried.

LC MS/MS: The tryptic peptides were resuspended in 20µL of 2% ACN, 0.1% Formic acid in HPLC-grade water and an amount of 400ng was injected on a nanoElute (Bruker Daltonics, Germany) HPLC (high-performance liquid chromatography) system coupled to a timsTOF Pro

(Bruker Daltonics, Germany) mass spectrometer. HPLC separation (Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile) was carried out at 250nL/min using a packed emitter column (C18, 25 cm×75µm 1.6µm) (Ion Optics, Australia) using a gradient elution (to 2 % solvent B during 1 min; 2 to 13% during 41min; 13% to 20% during 23min; 20% to 30% for 5min; 30% to 85% for 5min and finally 85% for 5min to wash the column). Mass-spectrometric data were acquired using the parallel accumulation serial fragmentation (PASEF) acquisition method. The measurements were carried out over the m/z range from 100 to 1700 Th. The range of ion mobilities values from 0.75 to 1.25 V.s/cm² (1/k0). The total cycle time was set to 1.16s and the number of PASEF MS/MS scans was set to 10.

MS Data Processing and Bioinformatics Analysis: Data analysis was performed using MaxQuant (version 2.0.1.0) and searched with Andromeda search engine against the UniProtKB/Swiss-Prot *Danio rerio* database containing also the Trembl entries (release 01-2022, 46841 entries). To search parent mass and fragment ions, we set a mass deviation of 10 ppm for the main search and 40 ppm respectively. The minimum peptide length was set to 7 amino acids and strict specificity for trypsin cleavage was required, allowing up to 2 missed cleavage sites. Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and N-term acetylation (Prot N-term) were set as variable modifications. The false discovery rates (FDRs) at the peptide and protein level were set to 1%. Scores were calculated in MaxQuant as described previously (S. Tyanova *et al.*, 2016). Proteins were quantified according to the MaxQuant label-free algorithm using LFQ intensities and protein quantification was obtained using at least 1 peptide per protein. Finally, match between runs was allowed during the analysis.

Protein groups output from MaxQuant was then processed in Perseus (S. Tyanova *et al.*, 2016) (v1.6.15.0). LFQ intensities values were log₂ transformed and the reverse and common contaminants hits were removed as well as the proteins only identified by site. For further statistical analysis, categorical annotation was then created in order to label the 12 samples as followed: 6 “non-infected” (NI) replicates were labelled as the NI group and 6 TBK12 replicates (TBK12) were labelled as the TBK12 group. This groups separation was then used to keep proteins with at least 5 valid values (over 6) in at least one group. Next, the data were imputed to fill missing data points by creating a Gaussian distribution of random numbers with a standard deviation of 33% relative to the standard deviation of the

measured values and 2.5 standard deviation downshift of the mean to simulate the distribution of low signal values. Finally, the remaining 8484 proteins were submitted to 2-groups (NI vs TBK12) Student's T-test (volcano plot, FDR=0.05 and S0=1). From this comparison, 904 proteins were found statistically significant.

Abbreviations

Abbreviation	Full name
5-HT1	<i>Serotonin 1A receptor</i>
AA	Amino acid
ADAR2	<i>Adenosine Deaminase Acting on dsRNA</i>
AKT	<i>Protein kinase B</i>
ALR	<i>AIM2-like receptors</i>
ALS	<i>Amyotrophic Lateral Sclerosis</i>
ALS2	<i>Alsin Rho Guanine Nucleotide Exchange Factor</i>
AMDA	<i>γ -α-amino-3-hydroxy-5-methylisoxazol-4-propionate</i>
AMO	<i>Antisense morpholino oligonucleotide</i>
AMPK	<i>AMP-activated protein kinase</i>
ATG13	<i>Autophagy Related 13</i>
ATP	<i>Adenosine tri-phosphate</i>
ATXN2	<i>Ataxin 2</i>
BAK	<i>BCL2 Antagonist/Killer</i>
BAX	<i>Bcl-2-associated X protein</i>
BCL-2	<i>B-cell lymphoma 2</i>
BH4	<i>Tetrahydrobiopterin</i>
BID	<i>BH3 interacting-domain death agonist</i>
BIM	<i>Bcl-2 Interacting Mediator of cell death</i>
C9orf72	<i>Chromosome 9 open reading frame 72</i>
CA	Caffeic acid
Ca ²⁺	Calcium
Cas9	<i>CRISPR-associated protein 9</i>
CASP	<i>Caspase</i>
CD38	<i>Cyclic ADP ribose hydrolase</i>
cFLIP	<i>Cellular FLICE-like inhibitory protein</i>
cGAS	<i>Cyclic GMP-AMP Synthase</i>
CHCHD10	<i>Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10</i>
CHMP2B	<i>Charged Multivesicular Body Protein 2B</i>
ciAP2/5	<i>Baculoviral IAP repeat-containing protein</i>
CMP	<i>Cytosine monophosphate</i>
cN	<i>Cytosolic 5'-nucleotidases</i>
CNC1	<i>Carney complex type 1</i>
CNS	Central nervous system
COX	<i>Cyclooxygenase</i>
CRISPR	<i>Clustered Regularly Interspaced Short Palindromic Repeats</i>
CSF	Cerebro-spinal fluid
CTD	<i>C-terminal domain</i>
CYLD	<i>CYLD Lysine 63 Deubiquitinase</i>
DAMP	<i>Damage-associated molecular pattern molecules</i>
DBR1	<i>Debranching RNA Lariats 1</i>
DCTN-1	<i>Dynactin Subunit 1</i>
DIABLO	<i>Diablo IAP-Binding Mitochondrial Protein</i>
DMSO	<i>Dimethylsulfoxide</i>
DNA	<i>Deoxyribonucleic acid</i>

DNM1	<i>Dynamin 1</i>
dpf	Days post fecundation
DPR	Dipeptide repeats
EMA	European medicines agency
ER	<i>Endoplasmic reticulum</i>
ERAD	<i>Endoplasmic reticulum associated degradation</i>
FADD	<i>Fas Associated Via Death Domain</i>
FDA	US Food and drug agency
FIP200	<i>RB1-inducible coiled-coil protein 1</i>
FTD	Fronto-temporal dementia
FUS	<i>Fused in Sarcoma</i>
G3P	<i>Glycerol-3-phosphate</i>
G4C2	GGGGCC hexanucleotide repeat
GABA	<i>γ-Aminobutyric acid</i>
GAS	<i>Gamma activated sequence</i>
GLT1	<i>Glutamate transporter 1</i>
GMP	<i>Guanine monophosphate</i>
GPI	<i>Glycosylphosphatidylinositol</i>
GPL	<i>Glycerophospholipid</i>
GRIA2	<i>Glutamate Ionotropic Receptor AMPA Type Subunit 2</i>
hpf	Hours post fecundation
IAP	<i>Inhibitor of Apoptosis</i>
IFN	<i>Interferon</i>
IFNAR	<i>Interferon-α/βreceptor</i>
IFNGR	<i>Interferon-γ receptor</i>
IFNLR	<i>Interferon-λ receptor</i>
IKBa	<i>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor</i>
IKK	<i>Nuclear factor kappa-B kinase</i>
IL	<i>Interleukine</i>
iPSC	<i>Induced Pluripotent stem cells</i>
IRF	<i>Interferon regulatory factor</i>
IRF3	<i>Interferon Regulatory Factor 3</i>
ISG	<i>Interferon-stimulated gene</i>
ISG15	<i>Interferon-stimulated gene 15</i>
KD	<i>Kinase domain</i>
KIF5A	<i>Kinesin Family Member 5A</i>
KMO	<i>Kynurenine 3-Monooxygenase</i>
LC3	<i>Microtubule-associated protein 1A/1B-light chain 3</i>
LUPAC	<i>Linear ubiquitin chain assembly complex</i>
MAK2	<i>Male Germ Cell Associated Kinase 2</i>
MAP3K7	<i>Mitogen-activated protein kinase kinase kinase 7</i>
MAVS	<i>Mitochondrial Antiviral Signaling Protein</i>
MDA5	<i>Melanoma differentiation-associated protein 5</i>
Mis	Mismatch
MLKL	<i>Mixed Lineage Kinase Domain-Like Pseudokinase</i>

MN	Motor neurons
mRNA	messenger ribonucleic acid
mTOR	<i>Mammalian target of rapamycin</i>
mTORC	<i>Mammalian target of rapamycin complex</i>
NAD+	<i>Nicotinamide adenine dinucleotide</i>
NAIP	<i>NLR Family Apoptosis Inhibitory Protein</i>
NAMPT	<i>Nicotinamide phosphoribosyltransferase</i>
NAP1	<i>Nucleosome Assembly Protein 1</i>
NDP52	<i>Calcium-binding and coiled-coil domain-containing protein 2</i>
NEMO	<i>NFκB essential modulator</i>
NF	Neurofilament
NFκB	<i>Nuclear factor-kappa B</i>
NLR	<i>Nod-like receptors</i>
NLRP	<i>Nucleotide-binding oligomerization domain</i>
NMDA	<i>Acide N-methyl-D-aspartique</i>
NMN	<i>Nicotinamide mononucleotide</i>
NR	<i>Nicotinate riboside</i>
NSC34	<i>Neuroblastoma x Spinal Cord-34</i>
O-GlcNac	<i>O-linked β-N-acetylglucosamine</i>
OGT	<i>O-GlcNAc transferase</i>
OPTN	<i>Optineurin</i>
p62	<i>Sequestosome 1</i>
PAMP	Pathogen associated molecular pattern molecules
PARP	<i>Poly (ADP-ribose) polymerases</i>
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI3K-cIII	<i>Phosphatidylinositol-4,5-Bisphosphate 3-Kinase class III complex</i>
PP2A	<i>Protein phosphatase 2</i>
PRR	Pattern recognition receptors
PUFA	<i>Polyunsaturated fatty acid</i>
PUR-a	<i>Purine Rich Element Binding Protein A</i>
QUIN	<i>Quinolinic acid</i>
RAPTOR	<i>Regulatory-associated protein of mTOR</i>
REE	Resting energy expenditure
RIG-I	<i>Retinoic acid-inducible gene I</i>
RIPK	<i>Receptor-interacting serine/threonine-protein kinase</i>
RLR	<i>RIG-I like receptor</i>
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SARM1	<i>Sterile Alpha aand TIR Motif Containing 1</i>
SDD	<i>Scaffold dimerization domain</i>
SINTBAD	<i>TANK-binding kinase 1-binding protein 1</i>
SIRT	<i>Sirtuin</i>
SMCR8	<i>Smith-Magenis Syndrome Chromosome Region candidate 8</i>
SOD1	<i>Superoxide dismutase type 1</i>
SPG11	<i>Spastic paraplegia 11</i>

SQSTM1	<i>Sequestosome 1</i>
STAT1	<i>Signal transducer and activator of transcription 1</i>
STAT2	<i>Signal transducer and activator of transcription 2</i>
STING	<i>Stimulator of interferon genes</i>
TAK1	<i>Mitogen-activated protein kinase kinase kinase 7</i>
TANK	<i>TRAF Family Member Associated NFKB Activator</i>
TARDBP	<i>TAR DNA binding protein</i>
TBK1	<i>TANK-binding kinase 1</i>
TCA cycle	<i>Tricarboxylic acid cycle</i>
TDP43	<i>TAR DNA-binding protein 43</i>
TLR	<i>Toll-like receptors</i>
TNF	<i>Tumor necrosis factor</i>
TRAF3	<i>TNF Receptor Associated Factor 3</i>
TRIF	<i>TIR-domain-containing adapter-inducing interferon-?</i>
TUBA4A	<i>Tubulin Alpha 4a</i>
Tyr	<i>Tyrosinase</i>
UBQLN2	<i>Ubiquilin 2</i>
UDP-GlcNAc	<i>Uridine diphosphate-N-acetylglucosamine</i>
ULD	<i>Ubiquitin like domain</i>
ULK	<i>Unc-51 Like Autophagy Activating Kinase</i>
UMP	<i>Uridine monophosphate</i>
UPR	<i>Unfolded protein response</i>
VAPB	<i>VAMP Associated Protein B</i>
VCP	<i>Valosin Containing Protein</i>
WB	Western blot
WDR41	<i>WD repeat domain 41</i>
WT	wild type
YBH3	<i>Yeast BH3 domain-containing protein</i>

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