Human TBK1: A Gatekeeper of Neuroinflammation

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Abstract

The importance of TANK binding kinase-1 (TBK1), a multimeric kinase that modulates inflammation and autophagy, in human health has been highlighted for the first time by the recent discoveries of mutations in *TBK1* that underlie amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), normal tension glaucoma (NTG) or childhood herpes encephalitis (HSE). Gain-of-function mutations in *TBK1* are associated with NTG, whereas loss-of-function mutations result in ALS/FTD or in HSE. In light of these new findings, we review the role of *TBK1* in these seemingly unrelated, yet allelic diseases, and discuss the role of TBK1 in neurological diseases. This discovery has the potential to significantly increase our understanding of the molecular basis to these poorly understood neurological disorders.

TBK1 At Multiple Crossroads

TBK1 (tumour necrosis factor (TNF) receptor associated factor NF-κB activator (TANK)-binding kinase 1), also known as NAK or T2K, has recently attracted the attention of human geneticists, immunologists and neurologists alike for its critical role in central nervous system (CNS) pathology. It is an ubiquitously expressed serine-threonine kinase, belonging to the 'non-canonical IκB kinases (IKKs)', recognized for its critical role in regulating type I interferon (IFN) production [1]. TBK1 is involved in the activation of various cellular pathways leading to IFN and pro-inflammatory cytokine production following infection [1], autophagic degradation of protein aggregates or pathogens [2–4], and homeostatic cellular functions such as cell growth and proliferation [5]. The genetics field has experienced an increased pace of discovery owing to the advances in sequencing technologies, which has begun to reveal a number of new genetic etiologies underlying various diseases. The recent discoveries of TBK1 heterozygous mutations in multiple human diseases has demonstrated the non-redundant role of this multifaceted protein in the CNS in particular [6–11] (Figure 1). Here we review the pleiotropic role of TBK1 in light of new discoveries of human germline *TBK1* mutations underlying neuroinflammatory diseases, including herpes simplex encephalitis (HSE), amyotrophic lateral sclerosis (ALS), frontal temporal lobe dementia (FTD) and normal tension glaucoma (NTG). The discovery comes either as part of a series of the first genetic etiologies defining a disease (HSE) or after a period of stagnant gene discovery (ALS, NTG). This finding suggests the involvement of new molecular pathways in disease pathogenesis which can lead to a better understanding of the causal mechanism underlying these neurological disorders. Furthermore,

knowledge gained from this can be used to develop new more effective therapies for these neurological disease with currently limited treatment options.

TBK1 in Inflammatory Pathways

TBK1 was first identified as a TANK interacting protein in mouse [12] with a role in controlling NF-κB-mediated responses as demonstrated by HEK293T cells co-transfected with TBK1 and NF-kB promoter luciferase reporter [13]. However, in contrast to canonical IKKs (IKKα and IKKβ) that control NF-κB activation, the non-canonical IKKs (TBK1 and IKKƐ) have since been found to play a more important role in the activation of transcription factors of the IFN-inducing interferon regulatory factor (IRF) family [14]. Indeed, TBK1 has been shown to play a key role in multiple cellular pathways, particularly inflammation and autophagy. Consequently, TBK1 sits at the crossroad of multiple inflammatory pathways, including NF-κB, and multiple IFN-inducing pathways.

Pattern recognition receptors (PRRs) such as toll-like (TLRs), retinoic acid-inducible gene I (RIG-I)-like (RLRs), and cytosolic DNA receptors all play important roles in the recognition of invading pathogens leading to IFN production (Figure 2). The engagement of these innate immune sensors by their cognate ligands, such as LPS, double stranded RNA (dsRNA) or DNA, results in the production of cytokines which alert neighboring cells (including immune cells) of danger and foreign invasion, subsequently promoting the early events of defense against infection. Engagement of TLR3 by dsRNA recruits its adaptor TRIF (TIR-domain-containing adaptor-inducing

89 interferon-β), eventually activating TBK1, found complexed with NAK-associated
90 protein 1 (NAP1) and IKKε (see Figure 1). Activated TBK1 phosphorylates IRF3 leading
91 to its homodimerisation and translocation to the nucleus where they drive the
92 expression of antiviral type-I and type-III IFNs (IFNα/β/λ) [1,15,16]. Apart from
93 membrane-bound TLRs, cytosolic RLRs (RIG-I, melanoma differentiation-associated 5
94 (MDA5) activated by viral RNA, [17–19] and cytosolic DNA receptors (cyclic guanosine
95 monophosphate–adenosine monophosphate synthase (cGAS), stimulator of IFN
96 genes (STING)) activated by dsDNA [20], all activate downstream TBK1 and induce IRF3
97 and in some cases IRF7, [21–23] . Finally, another DEAD (Asp-Glu-Ala-Asp)-box
98 helicase 3, X-linked protein (DDX3X) has also been shown to directly interact with TBK1
99 in RAW264.7 murine macrophages following DNA and viral RNA recognition, thus
100 leading to IFNβ production [24] (summarised in Figure 2).

101

102 ***TBK1 in Autophagy***

103 Recent studies have described TBK1 as an important player in yet another critical
104 cellular function, autophagy. Autophagy is an evolutionarily conserved homeostatic
105 process of self-degradation that contributes to the maintenance of cell function at
106 critical times by balancing sources through the turnover of long-lived proteins and
107 organelles, and also, in the clearance of intracellular pathogens [25]. Autophagy is
108 achieved by directing bulk cargo, such as protein aggregates, for degradation and/or
109 recycling in lysosomes. It is a highly regulated process that is orchestrated by a variety
110 of autophagy-related proteins (ATGs) such as beclin-1 (ATG6) that functions upstream
111 of the pathway as an autophagy promoter (reviewed in [26,27]). Traditionally thought

112 to be a non-selective process, it has been increasingly found to recognize specific
113 cargo. This specificity is mediated by recruitment of autophagy receptors such as
114 optineurin, p62, nuclear dot protein 52 kDa (NDP52) and neighbour of BRCA1 gene 1
115 (NBR1) [3,27–30] (Figure 2). These proteins bind simultaneously to ubiquitin residues
116 on target cargo via their ubiquitin binding domain, and to phosphatidyletholamine-
117 conjugated microtubule-associated protein light chain 3 (LC3-II) proteins which are
118 found on the inner leaflet of a forming autophagosomal membrane [27]. For post-
119 mitotic cells such as neuronal cells, autophagy is an essential survival mechanism by
120 which toxic proteins are eliminated, as they are not able to dilute these proteins by
121 mitosis [31,32]. A direct role of TBK1 in recycling protein aggregates has been shown
122 via its role in phosphorylating the autophagy receptor optineurin [33]. TBK1 co-
123 localised with optineurin and cell aggregates in an *in vitro* model of protein
124 aggregation in HeLa cells as well as in a SOD1 transgenic mouse model of ALS [33].
125 TBK1 has also been found to play a role in the autophagic elimination of invading
126 intracellular pathogens such as *Salmonella*, *Mycobacteria*, and herpes simplex virus-1
127 (HSV1) in human and murine cell lines [2–4].

128 The role of TBK1 in selective autophagy has been extensively studied in *Salmonella*
129 where it associates with optineurin and NDP52 in targeting ubiquitinated *Salmonella*
130 for autophagic clearance (Figure 2) [2,34]. NDP52 is thought to act upstream of
131 optineurin by directing TBK1 into the vicinity; TBK1 is then able to phosphorylate
132 optineurin. TBK1 is also involved in autophagic clearance of *Mycobacterium*
133 *tuberculosis* in RAW264.7 murine macrophages where it has been shown to
134 phosphorylate the autophagy receptor p62, enhancing its binding to

polyubiquitinated bacteria [4]. Moreover, TBK1 is particularly crucial for the maturation of the autophagosome into the hydrolytic autophagolysosome leading to degradation of p62 and its affiliated cargo [4]. Autophagy is also critical in HSV1 infections, demonstrated by the virus' ability to inhibit host autophagy through two virally-encoded products US11 and ICP34.5 [35–37]. And, although TBK1 has not been directly implicated in HSV1-mediated autophagy, the virally-encoded autophagy antagonist ICP34.5 has been shown to bind and inhibit TBK1 in a mouse model of HSV1 infection [38]; this interaction has been been suggested to play a role in limiting the propagation and dissemination of HSV1 to the CNS (35]. Hence, TBK1 has been implicated in pathogen clearance via autophagy contributing to cell-autonomous immunity. The two TBK1-regulated processes, autophagy and IFN signaling are not mutually exclusive as their crosstalk has been reported. Upon HSV1 infection, cGAS was shown to bind Beclin-1 leading to the suppression of IFN production, and a simultaneous increase in the autophagosomal clearance of cytosolic viral DNA in mice bone marrow-derived macrophages (BMDMs) [39]. Similarly, mouse BMDMs were shown to induce type-I IFN following mycobacterial infection as well as trigger autophagic clearance of the pathogen in a TBK1 dependent manner via cGAS [40]. Although mouse models of TBK1 deficiency have contributed to our fundamental understanding of TBK1 function, particularly in immune signaling (see Box 1), they have not been predictive of the human phenotypes associated with human *TBK1* mutations as neurological phenotypes were not assessed.

TBK1 Variants in Human Diseases

158 *Mutations in Human TBK1 Predispose to HSE: Impairment in IFN Production*

159 Herpes simplex encephalitis (HSE) is a devastating neurological disease caused by
160 HSV1 infection of the CNS. HSV1 is a neurotropic dsDNA alphaherpesvirus usually
161 causing asymptomatic or benign disease in the general population. With an incidence
162 of 1-2 individuals per million annually, HSE is a sporadic and rare manifestation of
163 HSV1 infection [41]. Peak incidence of HSE follows a bimodal curve, affecting children
164 between three months-six years of age, coincident with the time of primary HSV-1
165 infection, and adults over 50 years of age, probably due to reactivation of latent HSV1
166 infection [42]. It is thought to reach the CNS through the nasal or oral epithelium via
167 the olfactory or trigeminal nerves [43]. It exerts a wide spectrum of clinical features
168 ranging from necrosis of brain tissue, fever, altered behavior and disturbed
169 consciousness usually in the absence of viremia. Standard current treatment of
170 acyclovir has greatly improved survival rates of HSE patients, although survivors tend
171 to suffer from lifelong neurological sequelae characterized by global developmental
172 delay, intellectual deficiencies, seizures and motor skill disturbances [42,44,45]. HSE
173 has never been associated with any particularly neurovirulent strain of HSV1, and
174 hence it had been a rare idiopathic complication of HSV1 infection until the
175 identification of single gene defects in the TLR3-IFN pathway, including autosomal
176 dominant TBK1 deficiency [46].

177 Isolated childhood HSE can be caused by at least seven different genetic etiologies of
178 the TLR3-IFN pathway. These include autosomal recessive (AR) UNC93B1, autosomal
179 dominant (AD) and AR TLR3, AD and AR TRIF, AD TRAF3, AD TBK1, and AD IRF3
180 deficiencies, reflecting the importance of IFN production in defense against HSV1

infection (Figure 2) [6,47–52]. For both AD and AR defects however, the clinical penetrance of HSE is incomplete, as healthy family members have also found to carry HSE-causing mutations [6,47–50,52]. This is consistent with HSE being almost invariably sporadic, with only four multiplex families reported since 1941 [6,48,50]. There is however, complete penetrance of the mutations at the cellular level. For instance, functional studies of fibroblasts or induced pluripotent stem cell (iPSC)-derived neuronal cells derived from these patients have revealed a common defect in antiviral type-I and type-III IFN production. However, IFN responses have shown to be intact in these patient cells, underscoring the importance of IFN production in clearing HSV1 infection [53,54].

TLR3 signaling has also been studied in cells from patients with HSE. Endosomal TLR3 recognizes dsRNAs [55], produced during the HSV1 life cycle [56,57], triggering the production of anti-viral type-I and type-III IFNs (IFNα/β, IFNλ) (Figure 2). These IFNs are essential in controlling viral infection and establishing an anti-viral state by activating various host mechanisms that inhibit viral propagation and spread, such as translational arrest, and the induction of apoptosis [35,58]. Surprisingly, despite having impaired TLR3-mediated IFN production by their fibroblasts, these patients are otherwise healthy and are not susceptible to other viral infections, presumably because of the presence of intact and protective TLR3-independent IFN signalling mediated by cytosolic receptors such as RLRs [17].

Two different heterozygous missense *TBK1* mutations were also found in two unrelated European children with HSE (p.G159A and p.D50A respectively) (Figure 1,

Table 1). Both heterozygous mutations occur in the kinase domain of the protein; however, one produces its effect in a dominant negative fashion (p.G159A) whilst the other is dominant by haploinsufficiency (p.D50A) [6]. The patient carrying the G159A mutation developed HSE at 7 years of age and subsequently developed epilepsy and cognitive disabilities [6]. The patient carrying the D50A mutation developed HSE at 11 months of age and suffered from obesity as well as cognitive and motor dysfunctions thereafter [6]. Despite normal protein and mRNA expression, the G159A mutant allele produced a kinase-dead TBK1. And, in terms of IFN signaling, the G159A mutation led to impairment of IRF3 phosphorylation, resulting in lack of IFNβ and IFNλ production but normal IL-6 production upon TLR3 stimulation of patient dermal fibroblasts *in vitro* [6]. Because overexpression of this mutant allele in control human fibroblasts (with endogenous wild type TBK1) led to blocked IFN production, this suggested that the impaired signaling occurred due to the dominant negative effect of the mutant allele over the wild type allele. The D50A mutant allele however, exhibited poor expression at both protein and mRNA levels and hence, loss of kinase activity. Despite this, it showed normal poly I:C responsiveness in fibroblasts as demonstrated by normal IRF3 activation and IFNβ, IFNλ, and IL-6 production [6]. It was therefore concluded that the D50A allele is dominant due to haploinsufficiency. Autophagy function was not tested in these patients. Of note, both patients' fibroblasts presented intact RLR-mediated IFN production, suggesting that TBK1 function was unaffected downstream of the cytosolic PRRs [6]. However, fibroblasts from both patients were unable to control HSV1 or VSV infections, suggesting that a functional TBK1-dependent TLR3-IFN pathway was necessary for limiting viral replication [6]. It should be noted that one

cannot rule out other, as yet to be defined mechanisms that might also potentially contribute to HSE pathology (Box 2).

TBK1 Variants Can Predispose Individuals to ALS, ALS-FTD, or FTD: Implications for Aberrant Autophagy

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or Charcot's disease, is a typically adult-onset neurodegenerative disease characterized by progressive muscle wasting which is usually fatal [59]. First described in 1869 by Jean-Martin Charcot [60], it has a an incidence of 1-2 per 100, 000 adults per year typically affecting individuals of 50-60 years old [59]. Approximately 90% of ALS cases are sporadic and the remaining 10% are familial [59]. Parental consanguinity does not seem to be higher than in the general population. ALS is associated with progressive loss of upper and lower motor neurons that lead to weakening and atrophy of muscles, paralysis and eventually death mostly due to respiratory failure typically within 2 to 3 years after diagnosis [61]. Neuropathological features include extensive degeneration of motor neurons in anterior roots of the spinal cord and brainstem, corticospinal tract and loss of large pyramidal neurons residing in the primary motor cortex. Another hallmark feature is the presence of protein aggregates in degenerating neurons, most of which are ribonuclear proteins, such as transactive response (TAR) DNA-binding protein 43 (TDP-43). Proposed pathophysiological mechanisms of ALS include oxidative stress [62], impaired mitochondrial functions [63], perturbed axonal transport that lead to accumulation of organelles [64] and neuroinflammation that is triggered by motor neuron degeneration [65].

Furthermore, 15% of ALS patients develop cognitive abnormalities reminiscent of frontotemporal lobar dementia (FTD), and 15% of FTD patients have features of ALS [66]. Recent studies that have looked at CNS tissues of FTD and ALS patients have proposed that ALS and FTD form part of the same disease spectrum with common underlying features such as the presence of the TDP-43 proteins that accumulate in the cytoplasm of neurons [67]. Currently, there are no effective therapies available for this debilitating and lethal disease.

Many genes associated with ALS pathogenesis have been identified, including superoxide dismutase 1 (*SOD1)*, TAR DNA-binding protein 43 (*TDP-43)*, FUS RNA-binding protein (*FUS)*, Alsin *(ALS2)*, Ubiquilin-2 (*UBQLN2)*, Optineurin (*OPTN)*, Sequestosome 1 (*SQSTM1)*, Valosin-containing protein (*VCP)*, and chromosome 9 open reading frame 72 (*C9orf72)* amongst many others, although these collectively account for less than one third of all ALS cases [68–71] (reviewed in[72]). These genes were all identified initially in familial forms of ALS through linkage studies, and then, further found in sporadic cases. In ALS patients, these mutations are all typically mono-allelic, with the exception of some forms of disease including *SOD1, OPTN*, *FUS* and *ALS2* mutations amongst others [70,73–76]. Protein aggregates are a hallmark of the disease, comprised by proteins which are encoded by genes linked to causing ALS, e.g. *SOD1, TARDBP, FUS*. These protein aggregates can be stained with antibodies against two autophagy receptors previously mentioned, p62 and optineurin, which have also been implicated in the pathogenesis of ALS (Figure 2) [73,77]. Further evidence implicating autophagy as a putative pathogenic mechanism for ALS, came from a report by Cirulli *et al.* identifying for the first time, *TBK1* as a new ALS-

susceptibility gene in a whole exome sequencing (WES) study of 2,869 ALS patients and 6, 405 controls, along with two other autophagy genes *OPTN* and *SQSTM1* (encoding optineurin and p62 respectively) [7]. Although none of the *TBK1* variants found were functionally assessed in this study, heterozygous *TBK1* mutations were found to be significantly enriched in patients when compared to controls (1.099% of cases and 0.194% of controls). In particular, *TBK1* mutations were bioinformatically predicted to constitute 'loss-of-function' (LoF) mutations, including nonsense, splice site, frameshift, and deletions in *TBK1*, (the latter were 10-fold more prevalent in patient cases) [7] (Table 2).

This finding was further supported by Freischmidt *et al*., who identified genome-wide enrichment of *TBK1* mutations in 252 familial ALS patients [8]. WES of 13 European Caucasian families diagnosed with ALS or ALS-frontotemporal dementia (FTD) identified 8 heterozygous LoF classes of variants in *TBK1*. These mutations were assessed for optineurin binding as well as their ability to induce IFNβ signaling (IRF3 activation and IFN induction) in HEK293T cells (Figure 1, Table 1). These LoF mutations were shown to have no mRNA or protein expression, consistent with haploinsufficiency (Figure 1, Table 1). Specifically, patients' cells (lymphoblastoid cell lines, keratinocytes, or fibroblasts), heterozygous for four of these variants, (Y185X, I450KfsX15, T77WfsX4, A417X), exhibited 50% reduced expression of *TBK1* at the mRNA and/or protein levels [8]. Furthermore, HEK293T cells expressing two of the other mutations (T320QfsX40, V479EfsX4) showed no allele-specific expression of the TBK1 protein. A number of these LoF variants was tested for optineurin binding and IFN induction in 293 cells, which showed complete impairment of both TBK1-related

functions (Table 1). These variants were therefore reported to exert their effect via haploinsufficiency and determined to be causative. The p.690-713del variant, despite producing a TBK1 protein product, had a 24 amino acid deletion in the C-terminal CCD2 domain, specifically at the optineurin binding site, resulting in impaired binding to optineurin. The causative LoF mutations (Figure 1), p.Y185X, p.I450KfsX15, p.T77WfsX4, p.A417X, p.T320QfsX40, p.V479EfsX4, p.690-713del (R440X was not assessed), resulted in either haploinsufficiency or loss of CCD2 function and were found in ALS-FTD patients (approximately 50% of cases presented significant cognitive disabilities, often progressing towards FTD), as seen with other familial forms of the *C9orf72* mutation, extending the TBK1 phenotype to include FTD [8]. The clinical penetrance of these mutations was high, with 33 out of 40 carriers harboring *TBK1* mutations over the age of 60 years old, presenting ALS [8].

In addition, Freischmidt *et al.* reported 9 missense mutations and 1 in-frame deletion in ALS, ALS-FTD patients. Although missense mutations were not found to be enriched in their genetic analysis, *in vitro* assays (optineurin binding and IFN induction in 293 cells) on a selection of these mutations showed impaired TBK1 function, however the authors suggest further experiments to determine pathogenicity of these missense mutations (Figure 1, Tables 1 and 2) [8]. One particular missense variant located in the CCD2 domain, E696K, resulted in a failure of TBK1 to bind optineurin following co-immunoprecipitation in HEK293T cells. This mutation as well as the E643del mutation, also seen in Freischmidt et al, were identified as causative in a separate study of isolated FTD cases, and showed reduced expression in patient post-mortem cerebellar tissue and lymphoblast cells respectively [9,11]. Additional mutations in French ALS,

ALS-FTD, and isolated FTD patient cohorts, as well as a Chinese ALS patient have been subsequently reported (Table 2) [78,79]. Disease-causing mutations as reported in the literature are shown in Figure 1, whereas Tables 1 and 2 list variants of unknown pathogenicity that have been molecularly characterized, or not, respectively. In summary, these studies have now provided a link between *TBK1* and other previously identified ALS genes *SQSTM1* and *OPTN*, to autophagy, suggesting that this is an important cellular regulatory mechanism, which, when dysfunctional, can contribute to neurodegeneration, as observed in ALS disease. Full functional characterization of these *TBK1* mutations in context of autophagy function, using autophagy flux assays for example, will be necessary to unequivocally prove autophagy dysregulation. It will be interesting to see if IFN signaling is also impaired in these diseases as autophagy has been shown to regulate IFN responses [39,40] (Box 2).

TBK1 Duplications and Predisposition to Glaucoma: Gain-of-function Mutations

Glaucoma is the leading cause of adult-onset blindness with a prevalence of 1.86% in the US in adults over 40 years old; it is a neurodegenerative disease affecting the retinal ganglion cells of the optic nerve, usually resulting in irreversible ocular damage [80,81]. Glaucoma can be classified into two subtypes; primary open angle glaucoma (POAG), characterized by high intraocular pressure causing damage to the optical nerves, and normal tension glaucoma (NTG), associated with normal intraocular pressure (IOP) [82,83]. Single-gene heterozygous mutations underlying both types of glaucoma have been described, and are thought to account for 5% of all cases [84]. Heterozygous nonsense mutations in the myocilin gene (*MYOC*), a protein found in

342 the trabecular meshwork and the ciliary body of the eye thought to regulate IOP, are
343 known to cause POAG with relatively high penetrance (98.6%) and recent reports
344 describe familial and sporadic NTG patients to harbour heterozygous mutations in
345 *OPTN* [84]. Mutant *OPTN* (p.E50K) has been shown to form aggregates of insoluble
346 protein in neuronal cells derived from NTG patient's iPS cells, thus leading to cell death
347 [83]. TBK1 has also been found to interact with mutant E50K OPTN protein,
348 contributing to insolubility of the latter, and consequently, to NTG pathology [83].
349 Moreover, familial analysis of NTG patients has revealed several highly penetrant copy
350 number variants encompassing a chromosome 12 region, and inclusive of *TBK1* (Figure
351 1, Tables 1 and 2) [10]. This heterozygous duplication has been associated with higher
352 *TBK1* transcription levels in skin fibroblasts derived from the patients, suggesting a
353 TBK1 gain of function underlying glaucoma [10]. Hence NTG *TBK1* mutations present
354 a different genetic etiology than that which has been observed in TBK1 deficiency
355 models underlying HSE, ALS, ALS-FTD, or FTD. Furthermore, this gene duplication has
356 since been observed in other cohorts of NTG patients (Table 2) [85,86]. The original
357 study also reported three missense heterozygous TBK1 variants in the patient cohort
358 (p.S151F, p.L306I, p.V464A) although they remain of unknown pathogenicity (Table
359 2) [10].

360

361 ***TBK1: One Gene, Multiple Diseases. Molecular Basis to Disease Pathogenesis***

362 It comes as no surprise that *TBK1* would be important for human health, as it is highly
363 conserved evolutionary as well as in the general population (only 1 commonly
364 occurring missense variant has been reported in 66,000 WES individuals Exome

Aggregation Consortium (ExAC) [87]). HSE, ALS, FTD, and NTG are diverse diseases caused either by infection, or protein aggregate accumulation in neuronal cells. Of course, the identified genes associated with these diseases explain only a proportion of all patient cases, suggesting that further genetic heterogeneity is present. Indeed, these diseases share heterozygous mutations in *TBK1*, an essential multifunctional kinase participating in two distinct pathways: innate immune inflammatory signaling (TLR3-IFN pathway) and autophagy. So what potential mechanisms render this gene responsible for such clinically-distinct pathological conditions?

TBK1 Domain-specific Mutations

Mutations in a single gene can give rise to different phenotypes due to domain specific mutations which determine modular impairment of a multimeric protein. Examples of this are not uncommon and include the *STAT1* deficiencies [88]. Interestingly, none of the HSE and NTG mutations have been found in ALS, isolated FTD or ALS-FTD patients, although identical mutations have been observed in the latter three diseases. The HSE mutations were shown to occur exclusively in the kinase domain, resulting in allele-specific impairment of IFNβ induction [8]. This may possibly suggest that the kinase domain is particularly important for effective IFN production. (Figure 1, Table 1). In contrast, Freischmidt *et al.* reported CCD2 domain mutations in *TBK1*, impairing optineurin binding but maintaining normal IFNβ promoter activation suggesting that TBK1 autophagy function may play a protective role in ALS and FTD [8]. As such, *TBK1* mutations affecting domain specificity might represent an underlying factor contributing to differential phenotypes in these diseases.

Subcellular Localization and Tissue Specificity of TBK1

On a similar note, mutations affecting specific protein interactions could affect subcellular localization of TBK1, which might potentially affect disease manifestation. In that regard the subcellular localization of TBK1 has been shown to determine its role in different pathways [89]. For example, one study reported that TBK1 could interact with each of its adaptors TANK, SINTBAD, and NAP1 in a mutually exclusive manner, such that TBK1 activation following viral infection was TBK1-TANK-dependent and specifically occurring in perinuclear compartment, whereas TBK1-NAP1 co-localized with autophagosomes in HeLa cells [89]. Hence, it is conceivable that mutations which alter the spatial distribution of TBK1 in a cell might be connected to altered cellular phenotypes that are manifested in different disease pathologies. Moreover, despite its ubiquitous expression, TBK1 may have cell type-specific roles favoring specific signaling pathways. This has been difficult to determine, as most functional assays have been carried out on leukocytes or fibroblasts, as opposed to the relevant CNS cells affected in these disease-types. In light of the fact that CNS cells selectively utilise autophagy over IFN signaling during viral infections, such putative tissue specificity might play a larger role than previously thought [90]. Consequently, intrinsic spatial localization characteristics combined with domain and tissue specificity might play a role in how various *TBK1* mutations within the same gene are manifested in different diseases.

TBK1 Mutation Type

It is possible to consider that the type of *TBK1* mutation (loss-of-function (LoF), gain-of-function (GoF), dominant negativity, haploinsufficiency) might also play a role in determining disease type. NTG is a GoF model of TBK1 pathogenicity, due to *TBK1* duplications. In contrast, ALS and FTD have been largely associated with LoF heterozygous mutations resulting in haploinsufficiency. By inference, a moderate reduction of *TBK1* expression (~50%) by haploinsufficiency, due to residual expression from the wild type allele, could presumably affect TBK1-dependent autophagy function. On the other hand, a missense HSE-causing *TBK1* mutation has been shown to result in a dominant negative effect on the wild type allele, leading to impaired IFN signaling, and suggesting that very low overall levels of functional TBK1 could impact the IFN signaling pathway [6]. The difference in absolute levels of TBK1 due to its respective mutations might be responsible, or capable of modulating the outcome for such observed differences in cellular phenotypes. This may suggest that different mutations may have different thresholds of effective TBK1 function, which could result in disparate diseases.

Further Implications for HSE/ALS Pathogenesis

HSE, ALS-FTD and NTG have not previously been proposed as having a similar disease spectrum, however we propose that their common genetic etiology raises questions about a possible shared pathogenesis and implications for new treatment avenues (Figure 3). This may be due to TBK1's niche role, possibly determined by tissue

specificity, in CNS inflammation. Despite sharing 'TBK1' features with HSE, ALS and FTD, NTG presents a GoF TBK1 model of which sets it apart from the LoF TBK1 model of other diseases. In light of the evidence discussed here suggesting a shared disease-causing gene, why do we not see co-occurrence of these diseases? There are no reports on the co-occurrence of HSE and ALS in the same individual. This may be because both HSE and ALS are exceedingly rare events (HSE 1-2/1million/yr; ALS 2/100,000/yr) such that the co-occurrence of disease would be highly unlikely, especially when incomplete penetrance is a feature of both diseases. TBK1's involvement in ALS is progressive (accumulation of protein aggregates) resulting in the manifestation of disease pathology whereas in HSE, exposure and infection by HSV1 is necessary in order to reveal a phenotype. Furthermore, the HSV1 seropositivity rate among adults ranges from 40-87%, contributing to this reduced penetrance [91]. However, as the age of onset for ALS-FTD is much higher than that of HSE, which peaks in early childhood, it would be of interest to carefully follow long-term outcomes of HSE patients, as ALS symptoms may have yet to manifest. Prior to the advent of acyclovir in the 1980s, HSE patients would not have survived and therefore we do not have any long-term follow-up. Additionally, the fitness of patients post-HSE is reduced, with mortality rates up to 30% and over 50% suffering from severe sequelae, such that they may never reach age of onset for ALS [42,44]. Testing for the presence of protein aggregates in CNS samples from HSE patients would reveal whether a similar pathology is observed. Of note, both reported HSE patients with TBK1 deficiency were also found to have developed cognitive impairment and/or motor disabilities subsequent to HSE [6]. HSE patients with other TLR3-IFN deficiencies (not TBK1) would probably have low risk of developing ALS if the molecular defect of HSE

456 is truly restricted to IFN signaling and is autophagy independent. Testing HSV1
457 serology in all ALS-FTD patients, in particular those with *TBK1* mutations may be
458 informative. The reciprocal experiments of testing autophagy and IFN signaling in HSE
459 vs. ALS/FTD patient cells might help address how similar HSE and ALS/FTD disease
460 states are, as currently, these TBK1 functions in patient cells have not been fully
461 explored in any of the studies discussed here.

462

463 ***Concluding Remarks***

464 Advances in sequencing technologies have begun to reveal a growing number of single
465 gene variants that can underlie a diverse range of diseases [92–94]. These types of
466 studies will undoubtedly reveal further novel genetic models to explain disease
467 pathogenesis. In fact, mutations associated with a particular disease, which are found
468 in other atypically presenting diseases, would have been overlooked if it were not for
469 large-scale sequencing studies. The concept that mutations in a single gene can cause
470 a broad spectrum of disorders has been well documented [95,96]. This effect might
471 be mediated through different mechanisms, including i) mutations occurring in
472 domain-specific regions of a given multimeric protein, ii) qualitative differences
473 resulting from a certain type of mutation, or iii) subcellular localization/tissue
474 specificity. Human partial TBK1 deficiency results in
475 neuroinflammatory/neurodegenerative disorders of the CNS such as HSE, ALS, ALS-
476 FTD, whereas TBK1 GoF results in NTG. These conditions are probably a consequence
477 of dysregulated autophagy (ALS, FTD, NTG) or of impaired IFN signaling (HSE). The
478 surprisingly important role of this protein in the CNS, particularly its role in autophagy,

is consistent with other reports that post mitotic cells such as neurons depend on autophagy to deal with inflammation and cell survival following infection [90]. Not only does this suggests a common underlying disease etiology but also raises more questions about the pathogenesis of these diseases (see Outstanding Questions). Despite the exciting and unexpected finding of TBK1 involvement in these diseases, further studies to confirm the pathogenic mechanism underlying TBK1 defects in context of neuroprotection and neuroinflammation are needed to fully appreciate its role in disease (See Outstanding Questions). Any further knowledge gained form this can be applied to ameliorate treatment options for these debilitating diseases, particularly focusing on the neuroprotective aspects of intervention as current treatment for HSE, ALS, FTD and NTG are limited (Box 3). Any lessons learnt in one of the TBK1-disease could be extended to the other, which can lead to beneficial advances in all TBK1-neuroinflammatory diseases.

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769

Box 1. Mouse models of TBK1 deficiency

TBK1 is highly conserved in mammals, with human TBK1 protein sharing 99% homology with its mouse ortholog [13]. However, characterization of TBK1 function *in vivo* remains a major challenge, as homozygous deletion of *TBK1* in mice results in embryonic lethality at embryonic day 14.5 due to severe hepatic tissue loss and apoptosis [97]. However, mice homozygous for a truncated allele ($TBK1^{\Delta/\Delta}$) are viable, with minimal expression of truncated TBK1, which lacks kinase activity [98]. Macrophages from these mice have shown reduced IRF3 DNA-binding activity and IFNβ induction upon LPS induction. Heterozygous mice with one truncated allele ($TBK1^{\Delta/+}$) are also viable although their immunological response to infection has not been studied [98]. Much of our understanding of TBK1 function in viral infections and upon stimulation with the synthetic analog of dsRNA, polyinosinic:polycytidylic acid (poly I:C) *in vitro,* has mainly come from observations in TBK1-deficient ($TBK1^{-/-}$) mouse embryonic fibroblasts (MEFs) or macrophages exhibiting impaired IFN responses (IFNβ/α) or IFN-induced responses such as IP-10 (IFN-gamma-inducible protein 10) and Mx1 [38,99–101]. However, the autophagy function in TBK1 deficient mouse models has yet to be characterized.

Box 2. Additional putative TBK1 aberrations: Dysfunctional autophagy in HSE? IFN impairment in ALS-FTD?

HSE in patients with AD TBK1 deficiency has been attributed to impaired type-I and type-III IFN production, similar to other HSE-causing genes of the TLR3-IFN signaling

792 pathways [46]. It would be of interest to further test whether autophagy defects are
793 observed in these HSE patients. Although no mutations occur in the CCD2 domain of
794 *TBK1*, which is particularly important for autophagy, the dominant negative HSE
795 mutation has shown overall functional reduction in TBK1 which may also affect its
796 autophagy function. Furthermore, the haploinsufficient *TBK1* mutation in HSE, despite
797 exhibiting moderate reduction (~50%) in protein levels in heterozygous cells, has not
798 shown an impairment of the IFN pathway, even though the patient's cells were shown
799 to be susceptible to viral infection [6]. This might suggest that other TBK1 pathways
800 could be affected. Assessing the role of autophagy is particularly relevant in the
801 context of HSV1 infections because it has been shown to be critical in controlling HSV
802 infection in post-mitotic neuronal cells [90]. Beyond TBK1-deficient HSE patients,
803 whether or not this may reveal a general feature of HSE disease remains to be
804 explored. A selection of the *TBK1* mutations identified in ALS-FTD patients has been
805 assessed for IFN signaling, presenting either complete impairment (T320QfsX40,
806 I450KfsX15, V479EfsX4, R47H, M559R) or reduced IFNβ induction (R357Q) in patient
807 cells (Table1). This suggests that these mutations may affect antiviral responses,
808 although this parameter has not been specifically tested. Moreover, these mutations
809 have been tested in an allele-specific manner, overexpressing the tagged mutants in
810 HEK293T cells, but not in the context of an endogenous WT allele. Hence, the true
811 effect of the mutation in heterozygosity has not been determined. Whether such IFN
812 impairment could also contribute to ALS-FTD pathogenesis is a possibility that has not
813 been explored either. Nevertheless, other studies using mouse models of ALS or *in*
814 *vitro* studies that looked at the expression and effects of type I IFNs on CNS-resident
815 cells have demonstrated a pleiotropic role of type I IFNs in neuronal survival, in which

816 they could confer protection or be detrimental to these cells, suggesting that IFN could
817 be relevant to ALS pathogenesis [102–104]. Furthermore, optineurin-TBK1 complexes
818 have been implicated in the regulation of IRF3-IFN responses following dsRNA or viral
819 infections, suggestive of a possible crosstalk between the two different TBK1
820 pathways in disease [20,39,105,106]. Of note, these TBK1 variants have not been
821 tested for their role in NF-κB signaling which may also potentially affect
822 neuroinflammation [107]. In fact, in NTG, the role of abnormal NF-κB signaling due to
823 TBK1 duplications has been proposed as a pathogenic mechanism [10]. As such, the
824 impact of TBK1 on other pathways may also contribute to disease in the context of
825 heterozygous mutations.

826 ***Box 3. Implications For Treatment Avenues***

827 Studies on HSE used to be hindered by the fact that this primarily childhood disease is
828 lethal which made it difficult to trace the transmission of the underlying genes, until
829 the advent of acyclovir [42,44]. Acyclovir is a nucleoside analogue with proven efficacy
830 of inhibiting HSV-1 DNA replication which has significantly reduced mortality [108].
831 Unfortunately survivors still suffer from neurological sequelae and
832 neuroinflammation [42,44]. ALS and FTD both have no cure, and current treatments
833 involve palliative care with variable success. Riluzole (Rilutek©) is the only FDA-
834 approved drug that can delay ventilator dependence by few months for ALS patients
835 although its mechanism is unknown [109]. Glaucoma patients rely on prostaglandin
836 analogues or surgical procedures to relieve symptoms [110]. Broad effect treatment
837 such as autophagy inducer rapamycin has been shown to be a promising ALS drug
838 candidate [111]. However, given the implication of TBK1 in these diseases, perhaps

839 exploring TBK1 as a more defined target of novel therapeutics such as TBK1 activators
840 would be a solution. As ALS, FTD and glaucoma are progressive diseases; there is an
841 urgent need for neuroprotective treatments. HSE would on the other hand, benefit
842 from treatments aimed at decreasing neuronal death or neuroinflammation
843 associated with infection.

844

Figures & Legends

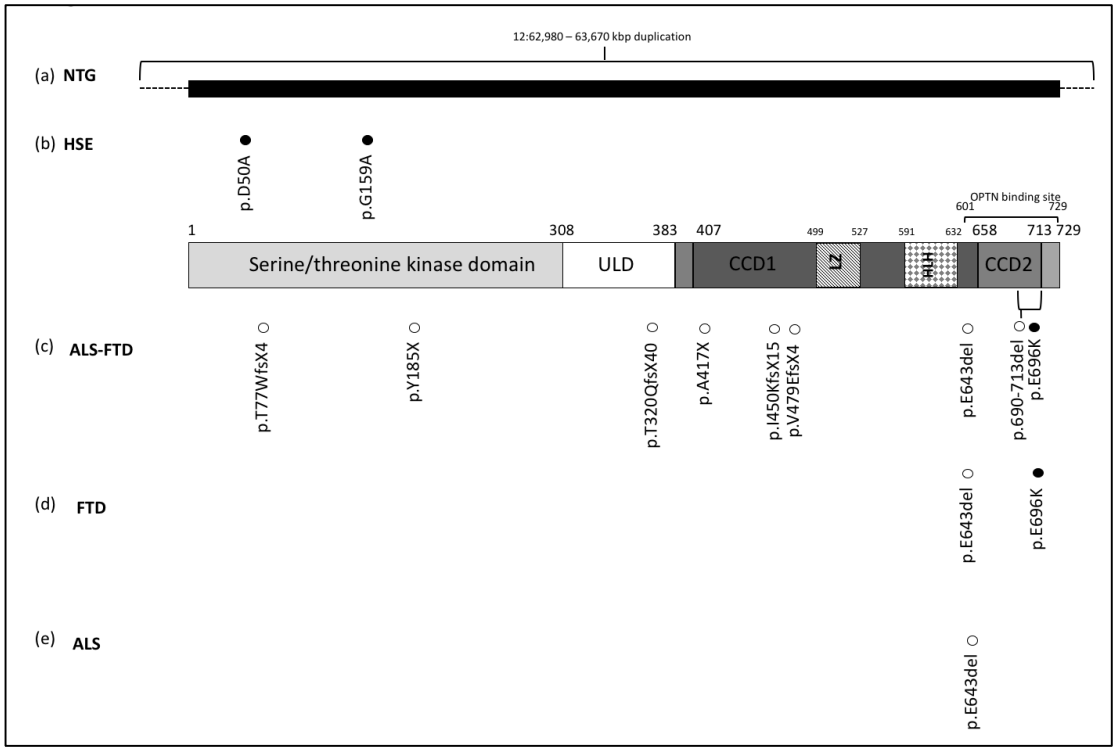
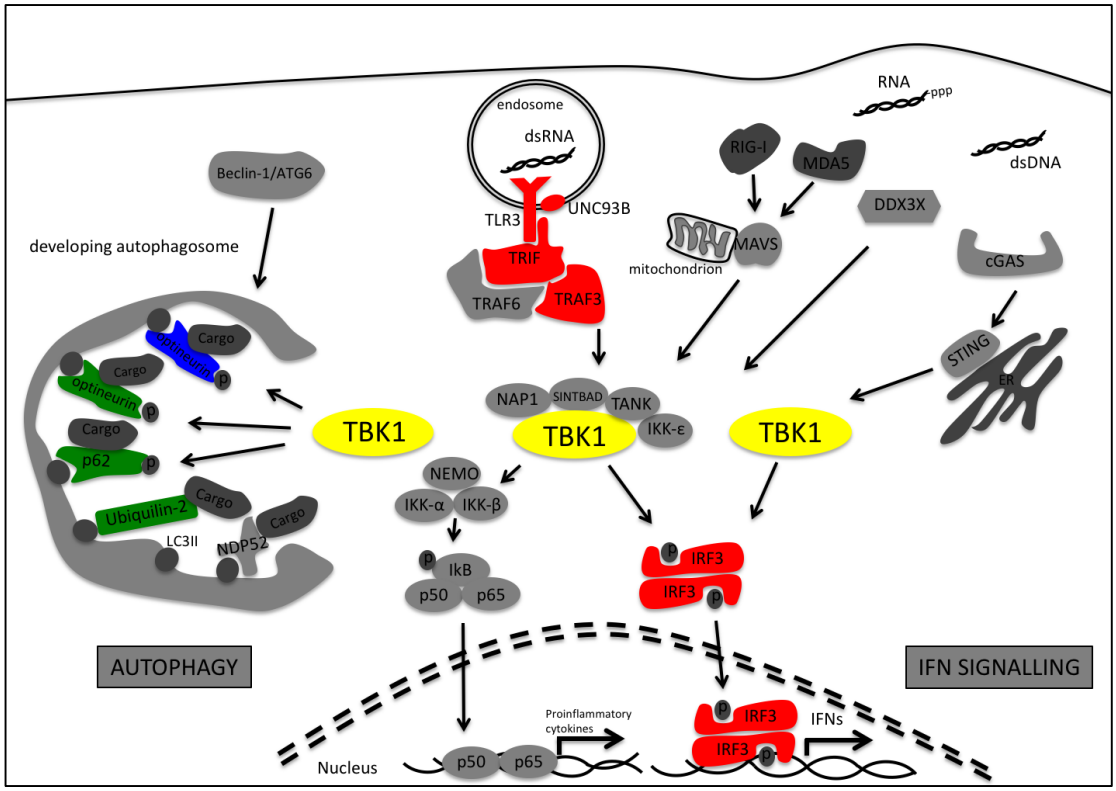


Figure 1 – Disease-causing mutations in human *TBK1*. TBK1 is an 84 kDa, 729 amino-acid protein that is composed of a kinase domain, an ubiquitin-like domain (ULD), and CCD1 (coiled-coiled domain 1) and CCD2. The kinase domain is critical for its activity to phosphorylate its various substrates, such as IRF3 [15], whereas the ULD domain regulates kinase activation and interactions with other proteins of the pathway [112]. The CCD1 domain harbors a leucine zipper (LZ) and helix-loop-helix (HLH) domains which specifically control dimerisation. The C-terminus CCD2 harbors an adaptor-binding motif facilitating the interaction of TBK1 with its adaptors TANK, NAK–associated protein (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD) [89]. Germline human *TBK1* mutations reported in the literature to be disease-causing in **(a)** normal tension glaucoma (NTG), **(b)** herpes simplex encephalitis (HSE), **(c)** amyotrophic lateral sclerosis-frontotemporal dementia ALS-FTD, **(d)** FTD and **(e)** ALS and are shown with

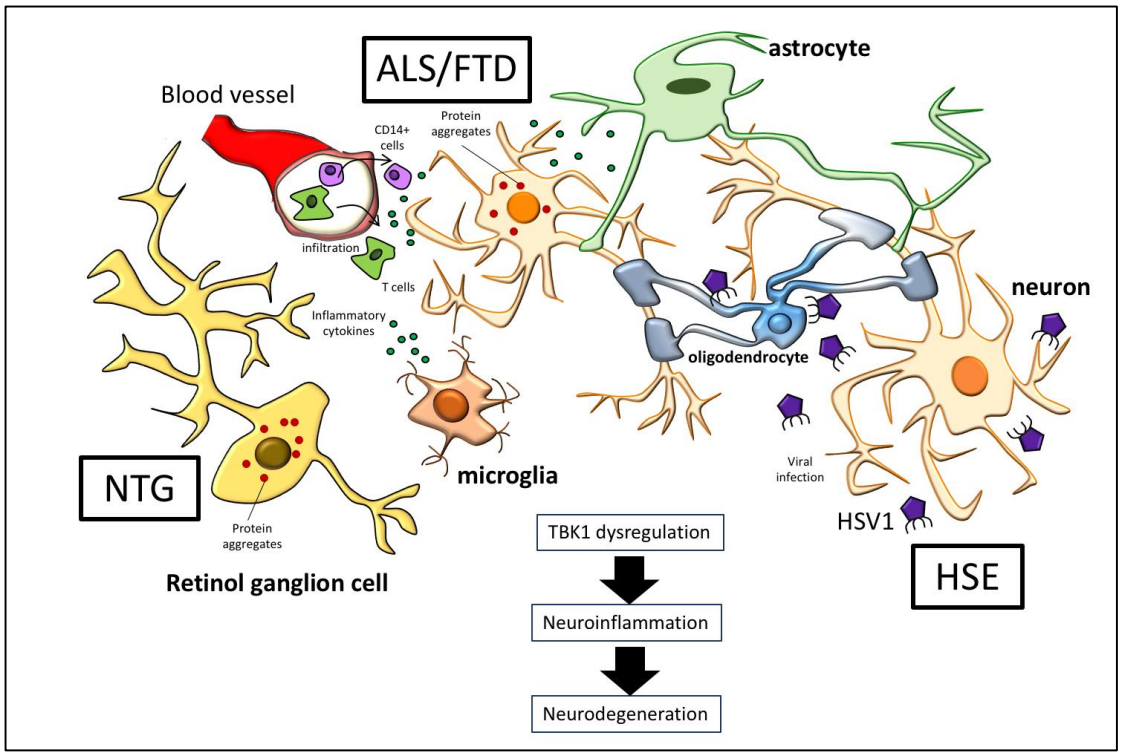
respect to their amino acid position within the TBK1 protein. The black horizontal box in **(a)** indicates duplications in kbp that have been reported to include TBK1. Open circles represent LoF variants; filled circles represent missense variants. (See Table 1).



Key Figure, Figure 2 – Molecular Pathways of TBK1. TBK1 and IKKε function as the non-cannonical IkB kinases downstream of TLRs, RLRs, DDX3X, and DNA receptors leading to the activation of the transcription factors NF-kB (p65/p50) and IRFs (IRF3), resulting in the production of proinflammatory cytokines and antiviral IFNs. TLR3 recognises dsRNA initiating the recruitment of adaptors such as TRIF and TRAF3 (TNF receptor-associated factor 3), which then activate TBK1 found complexed with its interacting proteins NAP1 (NF-kB-activating kinase-associated protein-1), SINTBAD

872 (similar to NAP1 TBK1 adaptor) and TANK. LPS recognition by TLR4 can also recruit
873 TRIF and subsequently TRAF3 which mediates activation of TBK1. Activated TBK1 can
874 then phosphorylate IRF3, leading to its homodimerisation and subsequent
875 translocation into the nucleus where it induces the production of IFNs. Cytosolic RLRs
876 and DDX3X, as well as DNA sensor cGAS signal via TBK1 following recognition of their
877 ligands viral 5'-ppp RNA and DNA respectively. RLRs typically signal via the adaptor
878 MAVS (mitochondrial antiviral-signaling protein; also known as IPS-1, CARDIF or VISA),
879 which activates TBK1. cGAS detects dsDNA and stimulates STING (stimulator of
880 interferon genes) to bind and activate TBK1 directly. TBK1 is also involved in
881 autophagy where it directly phosphorylates the autophagy receptors optineurin and
882 p62, which target cargo to the autophagosome. Ubiquilin-2 can also target
883 ubiquitinated cargo to autophagosomes [113]. Target cargo may be pathogen or
884 ubiquitinated protein aggregates. Proteins which genes have been reported to
885 predispose to diseases are indicated in red, HSE; green, ALS or ALS-FTD; blue, NTG.
886 Yellow denotes TBK1, where all pathways converge.

887



888

889 **Figure 3 – Dynamic interplay between cells in CNS in ALS, FTD, HSE and NTG.** In
890 ALS/FTD, motor neurons accumulate toxic protein aggregates (e.g.: TDP-43 inclusions)
891 which contribute to neurodegeneration. In addition to this, other cells are known to
892 mediate neuroinflammation leading to cell death. Activated microglia and inflitrating
893 monocytes and T cells produce inflammatory cytokines; and astrocytes are shown to
894 downregulate their supportive function contributing to neurodegeneration [65]. In
895 HSE, studies using iPSCs-derived neurons from a TLR3 deficient patient demonstrated
896 that TLR3-dependent cell-intrinsic immunity in neurons and oligodendrocytes are
897 critical in primary infection against HSV1 [54]. In NTG, progressive degeneration of
898 retinol ganglion cells occurs which is poorly understood [114].

899

Trends Box

- HSE, in a subset of children, is caused by impaired antiviral IFN production due to monogenic mutations in the TLR3-IFN signalling pathway, including *TBK1*.
- Due to advances in sequencing technologies, a number of new amyotrophic lateral sclerosis (ALS) or ALS-frontotemporal dementia (ALS-FTD) genes have been identified, five of which are known to be involved in autophagy, *SQSTM1*, *VCP*, *OPTN*, *UBQLN2* and *TBK1*. These mutations are thought to contribute to disease pathogenesis possibly due to impaired autophagy.
- The genetic aetiology of normal tension glaucoma (NTG) has recently been attributed to copy number variants found in chromosome region 12q14, specifically leading to duplications of the *TBK1* gene. This duplication has been found to increase *TBK1* transcript levels, suggesting a gain of function role for*TBK1* in NTG.
- Recent developments in the field of selective autophagy have implicated this evolutionarily conserved process in innate immunity and pathogen clearance, including neuronal cells.

Outstanding questions

- What are the respective roles of IFN and autophagy in human TBK1 disorders? Do they work independently or together to resolve/exacerbate inflammation?
- Neuronal cells are the common cell in human TBK1 disorders, however what other cell types control TBK1-mediated neuroinflammation?
- What is the mediator of tissue damage/neuroinflammation in these human TBK1 diseases? Do they point to the same culprit i.e. protein aggregates? Are protein aggregates a feature of herpes encephalitis?
- Is there evidence for a viral trigger in the development of ALS/glaucoma ? Does IFN play a role in the development of ALS, FTD or glaucoma?
- Could the dissection of TBK1 function provide us with new therapeutic strategies to treat for HSE, ALS, FTD, ALS-FTD or NTG? Common disease pathogenesis focusing on neuroprotective effects or pathways mediated by TBK1 may reveal more effective and targeted therapies for these diseases. Treatment to boost autophagy may be helpful in these diseases, i.e rapamycin to prevent cell toxicity and cell death, or drugs to boost proteasome function in patients with TBK1 deficiencies, so that the proteasomal ubiquitination pathway may help clear out toxic build up of protein aggregates.

Glossary

- **TBK1 (TANK-binding kinase 1):** TBK1 is a kinase that functions downstream of multiple IFN inducing pathways that are activated following pathogen sensing and are mediated by Toll-like receptor 3 (TLR3), RIG-I-like receptors (RLRs) and cytosolic DNA sensors. Following activation, it phosphorylates cytosolic IRF3 or IRF7, which then dimerise and enter the nucleus to activate IFN production.

- **Trigeminal nerves:** Trigeminal nerves are the nerves that innervate the cranium and are responsible for sensory and some motor functions in the face. Following primary infection, HSV1 may take this route to reach the central nervous system to cause acute infection.

- **Pattern recognition receptors (PRRs):** These are innate immune receptors that form the first line of defence against pathogens. They recognise pathogen-associated molecular patterns (PAMPs) that are conserved across groups of pathogens.

- **TAR DNA-binding protein 43 (TDP-43):** This is a nuclear protein that has a role in regulating gene expression. Mutations in its gene *TARDBP* can lead to its accumulation and aggregation in the cytoplasm of motor neurons, which is considered to be the hallmark of ALS and FTD.

- **Frontotemporal lobar dementia (FTD):** is a disease that is characterised by progressive neuronal loss of the frontal and temporal lobes of the brain.

- **STAT1 deficiencies:** Several inborn mutations of human *STAT1* have been

identified that exhibit allelic heterogeneity, different modes of inheritance and variable immunological/clinical phenotypes. AR complete and partial deficiencies predispose to bacterial and viral infections due to impaired IFN-γ, -α/β-mediated immunity; AD deficiency selectively underlies mycobacterial disease due to impaired IFN-γ mediated immunity; AD gain of function STAT1 mutations, found exclusively in the coiled-coil domain of STAT1, give rise to autoimmunity and chronic mucocutaneous candidiasis due to increased IFN--α/β response and impaired TH17 response [96].

Table 1. Molecular characterization of TBK1 variants reported in human diseases.

Type of variant	Mutation (location in a.a. or kbps)	Premature STOP	Expression				Function		Disease	References
			mRNA level		Protein level		Optineurin binding	IFN		
			Allele-specific	Patient cells	Allele-specific	Patient cells				
Frameshift	p.T77WfsX4	Yes	-	Reduced	-	Reduced	-	-	ALS-FTD	[8]
	p.T320QfsX40	Yes	-	-	No	-	Impaired	Impaired	ALS-FTD	[8]
	p.S398PfsX11	Yes	-	Reduced	-	Reduced	-	-	ALS	[11]
	p.I450KfsX15	Yes	-	Reduced	Truncated	Reduced	Impaired	Impaired	ALS-FTD	[8]
	p.V479EfsX4	Yes	-	-	Truncated	-	Impaired	Impaired	ALS-FTD	[8]
	p.S518LfsX32	Yes	-	Reduced	-	Reduced	-	-	ALS	[11]
Deletion	p.D167del	No	-	Normal	-	Normal	-	-	ALS	[11]
	p.G272_T331del	No	-	Reduced	-	Reduced	-	-	FTLD	[11]
	p.E643del	No	-	Normal	-	Reduced	-	-	ALS; FTD; ALS-FTD	[8] [11]
	p.690-713del	No	-	Normal	Normal & truncated	Normal & truncated	Impaired	Normal	ALS-FTD	[8]
Nonsense	p.Y185X	Yes	-	Reduced	-	-	-	-	ALS-FTD	[8]
	p.R117X[†]	Yes	-	Reduced	-	Reduced	-	-	FTD	[9]
	p.A417X	Yes	-	Reduced	-	Reduced	-	-	ALS-FTD	[8]

Missense	p.R47H	No	-	-	Normal	Normal	Normal	Impaired*	ALS-FTD	[8]
	p.D50A	No	-	Reduced	No	Reduced	-	Normal	HSE	[6]
	p.G159A	No	-	Normal	Normal	Normal	-	Impaired	HSE	[6]
	p.R271L	No	-	Normal	-	Normal	-	-	FTD	[11]
	p.K291E	No	-	Normal	-	Normal	-	-	FTD	[11]
	p.L306I	No	-	-	-	Normal	-	-	FTD	[9]
	p.R308Q	No	-	-	Normal	-	Normal	Normal**	ALS-FTD	[8]
	p.H322Y	No	-	Normal	-	Normal	-	-	ALS	[11]
	p.R357Q	No	-	-	Normal	-	Reduced	Impaired***	ALS-FTD	[8]
	p.K401E	No	-	-	-	Reduced	-	-	FTD	[9]
	p.I515T	No	-	Normal	-	Normal	-	-	ALS	[11]
	p.A535T	No	-	Normal	-	Normal	-	-	FTD	[11]
	p.M559R	No	-	-	Normal	-	Impaired	Impaired	ALS-FTD	[8]
	p.M598V	No	-	-	-	Normal	-	-	ALS-FTD	[8]
	p.E696K	No	-	-	Normal	Reduced	Impaired	Normal	ALS-FTD; FTD	[8] [9]
Duplication	12:62, 980 – 63, 670 kbp	-	-	Elevated	-	-	-	-	NTG	[10]

All variants are either novel or have allele frequency of <0.0005% in general population.

Expression: assessed either allele specifically (in transfected cells) or in patient cells (expression of combined WT/mutant levels).

Function: autophagy function was tested by optineurin binding; IFN activation was tested by either IRF3 binding, phosphorylation, or IFNβ promoter induction.

* normal IRF3 binding but impaired IRF3 phosphorylation/IFNβ induction.

** normal IRF3 binding, phosphorylation but reduced IFNβ induction.

*** no IRF3 binding but reduced IRF3 phosphorylation/IFNβ induction.

† patient also carried a heterozygous deletion in *OPTN* exons 13-15

"-" = not determined.

Table 2. TBK1 variants of unknown pathogenicity reported in human diseases.

Type of variant	Mutation (location in a.a. or kbps)	Mutation prediction	Disease	References
Nonsense	p.Q2X	STOP	ALS	[7] [11]
	p.R117X	STOP	ALS	[7]
	p.R357X	STOP	ALS	[7]
	p.R440X	STOP	ALS; ALS-FTD; ALS-dementia	[7] [8] [78]
	p.R444X	STOP	ALS	[7]
	p.Y482X	STOP	ALS-FTD	[78]
	p.S499X	STOP	ALS	[7]
	p.Q655X	STOP	ALS-FTD	[78]
Frameshift	p.T156RfsX6	STOP	ALS-FTD	[78]
	p.T278fs	n.a.	ALS	[7]
	p.L399fs	n.a.	ALS	[79]
	p.V421fs	n.a.	ALS	[7]
	p.T462fs	n.a.	ALS	[7]

	p.D500fs	n.a.	ALS	[7]
	p.E550fs	n.a.	ALS	[7]
	p.Q629fs	n.a.	ALS	[7]
Deletion	p.E640del	n.a.	ALS	[7]
Splice	p.180sp	n.a.	ALS	[7]
	p.331sp	n.a.	ALS	[7]
	p.587sp	n.a.	ALS	[7]
	c.1960-2A>G; p.653sp	n.a.	ALS-FTD	[78]
Missense	p.T4A	Probably damaging	FTD	[78]
	p.L11S	Possibly damaging	ALS	[7]
	p.N22H*	Probably damaging	ALS	[7]
	p.N22D	Probably damaging	ALS	[7]
	p.R25H	Probably damaging	ALS	[7]
	p.G26E	Probably damaging	ALS	[78]
	p.Y105C	Possibly damaging	ALS-FTD	[8]
	p.N129D	Possibly damaging	ALS	[7]
	p.V132E	Probably damaging	ALS	[7]
	p.R134H	Probably damaging	ALS	[7]
	p.R143C	Probably damaging	ALS	[78]
	p.S151C	Probably damaging	ALS	[7]
	p.S151F	Probably damaging	ALS; NTG	[7] [10]
	p.G217R	Probably damaging	ALS	[7]
	p.R228H	Probably damaging	ALS	[7]
	p.I257T	Probably damaging	ALS	[7]
	p.L277V	Benign	ALS	[7]
	p.I305T	Possibly damaging	ALS-FTD	[8]
	p.L306I	Possibly damaging	NTG	[10]
	p.T320I	Benign	ALS	[78]
	p.T331I	Benign	ALS	[7]
	p.T343S	Probably damaging	ALS	[7]
	p.Y394D	Possibly damaging	ALS	[7]
	p.R440Q*	Probably damaging	ALS	[7]
	p.V464A	Benign	NTG	[10]
	p.C471Y	Benign	ALS	[7]
	p.I522M	Possibly damaging	ALS	[7]
	p.A571V	Benign	ALS-FTD	[8]
	p.Q565P	Probably damaging	ALS	[7]
	p.Q581H	Probably damaging	ALS	[7]
	p.M662T	Benign	ALS-FTD	[78]
	p.I710N*	Benign	ALS	[7]
Duplication	12:62, 900 – 63, 680 kbp	n.a.	NTG	[10]
	12:62, 760 – 63, 410 kbp	n.a.	NTG	[10]
	12:63, 060 – 63, 360 kbp	n.a.	NTG	[10]
	12:64,802 – 65 ,099 kbp	n.a.	NTG	[85]
	12:64,830 – 65, 096 kbp	n.a.	NTG	[86]

984

985 Mutation predictions were predicted by online tool PolyPhen-2
986 (http://genetics.bwh.harvard.edu/pph2/index.shtml).

987 n.a.= not applicable.

988 * Mutations also found in controls.

989