



# Optineurin dysfunction in amyotrophic lateral sclerosis: why so puzzling?

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**Abbreviations:**

ABIN – A20 binding and inhibitor of NF- $\kappa$ B  
ALS – amyotrophic lateral sclerosis  
CC – coiled-coil  
CYLD – cylindromatosis tumor suppressor protein  
HACE1 – HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1  
Htt – huntingtin  
IFNs – interferons  
I $\kappa$ B – inhibitor of  $\kappa$ B  
IRF3 – interferon regulatory factor 3  
LC3 – microtubule-associated protein 1A/1B-light chain 3  
LIR – microtubule-associated protein 1A/1B-light chain 3-interacting region  
MEF – mouse embryonic fibroblasts  
NEMO – nuclear factor  $\kappa$ B essential modulator  
NF- $\kappa$ B – nuclear factor kappa-light-chain-enhancer of activated B cells  
NTG – normal tension glaucoma  
Optn – optineurin  
PD – Parkinson's disease  
RIPK1 – receptor interacting protein kinase 1  
SOD1 – superoxide dismutase 1  
TBK1 – Tank-binding kinase 1  
TDP-43 – TAR DNA-binding protein 43  
TNF – tumor necrosis factor  
UBAN – ubiquitin-binding domain in ABIN proteins and NEMO  
ZF – zinc finger domain

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## Abstract

*Mutations in optineurin have been linked to amyotrophic lateral sclerosis (ALS) a decade ago, but its exact role in the neurodegenerative process is still unclear. As a lysine 63 (K63)- and methionine (M1)-linked polyubiquitin-binding protein, optineurin has been reported to act as an adaptor in inflammatory signaling pathways mediated via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and interferon regulatory factor 3 (IRF3), as well as in membrane-associated trafficking events including autophagy, maintenance of the Golgi apparatus, and exocytosis. Other studies have demonstrated its role in other processes such as regulation of mitosis, transcription, necroptosis and apoptosis. However, many of the reported effects in cell models have been proven difficult to reproduce in optineurin animal models, demonstrating the challenges of extrapolation between model systems. Knowing that multifunctional proteins present a “nightmare” for researchers, to help navigating through this field, we address the most common controversies, open questions, and artefacts related to optineurin and its role in pathogenesis of ALS and other neurodegenerative diseases.*

## INTRODUCTION

The optineurin protein has initially been discovered in 1998 as a second adenoviral E3-14.7K-interacting protein, and was thus termed FIP-2. Since then, optineurin has been reported to participate in an unusually wide number of cellular functions including inflammatory signaling, vesicular trafficking, maintenance of the Golgi apparatus, autophagy, regulation of transcription, mitosis, apoptosis and necroptosis (reviewed in (1, 2)). It takes part in these processes by interacting with a large number of proteins via its ubiquitin-binding domain, microtubule-associated protein 1A/1B-light chain 3 (LC3)-interacting region (LIR), Tank-binding kinase 1 (TBK1)-interacting domain and others, and is thus considered to be an adaptor protein. Via its coiled-coil (CC) domains it oligomerizes with self, TBK1 and perhaps other proteins. Optineurin function is regulated by posttranslational modifications, most importantly phosphorylation by TBK1, ubiquitination by HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) and deubiquitination by cylindromatosis tumor suppressor protein (CYLD) (3–6). To reflect its many functions and cellular partners, its name has been changed several times, finally settling on optineurin, for optic neuropathy inducing, because of the optineurin mutations reported in primary open angle glaucoma patients (7). Of note, optineurin mutations are found specifically in normal tension glaucoma (NTG), a subset of glaucoma that arises without an increase

in intraocular pressure. In addition, optineurin mutations distinct from those previously found in NTG have been subsequently found in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (8–10). Moreover, polymorphisms resulting in lower level of optineurin expression have been found in Paget's disease of the bone and Crohn's disease (11, 12). Various optineurin patient mutations were proposed to act by loss-of-function, haploinsufficiency, gain-of-function or dominant negative effects, suggesting that the regulation of cellular processes by optineurin is complex, and might differ in various cell types and/or pathogenic processes. We will address common open questions, controversies and artefacts related to optineurin in pathogenesis of neurodegeneration, mostly focusing on ALS, in which more than 40 optineurin mutations have been reported thus far (1, 2).

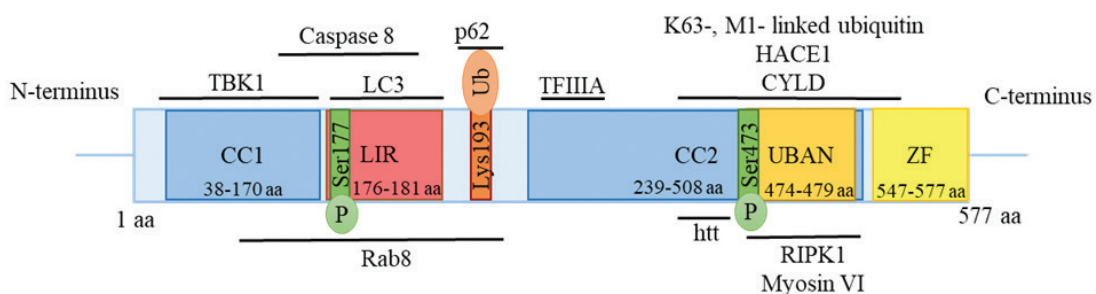
### “Nomen est omen”?

Optineurin has been discovered as an interacting partner to various cellular proteins, hence its many names. It was initially characterized upon being fished out in a yeast-two-hybrid screen with an adenoviral E3-14.7K-interacting protein as a bait (13). Next, it was pulled out from a database search for proteins homologous to nuclear factor  $\kappa$ B essential modulator (NEMO), and was named NEMO-related protein. It harbors 53% homology to NEMO, which is most prominent in its ubiquitin-binding C-terminus (14). In 2000, optineurin was termed transcription factor IIIA-interacting protein upon being identified as an interacting partner of transcription factor IIIA, which in eukaryotic cells activates transcription of 5S RNA (15). The name optineurin was finally adopted in 2002 when its mutations were found to cause a hereditary NTG (7). Since then, optineurin has been mapped as interacting partner of many other proteins, and its mutations and polymorphisms have shown prom-

inent roles in other diseases (1). Although its current name does not accurately represent all the roles that optineurin has and/or all the diseases that its mutations or polymorphisms are associated with, it bears reference to the first disease that it was linked to.

### Optineurin protein-protein interactions

Optineurin is a non-enzymatic protein that harbors several functional domains and interacts with a large number of proteins (16). It has an LC3 and TBK1 binding regions in its N-terminus, and an ubiquitin-binding domain in the C-terminus (Figure 1). In addition, CC domains cover a large part of optineurin (around 70%), and mediate its self-oligomerization and oligomerization to other proteins harboring CC domains. Optineurin regulates protein degradation, cell trafficking and signal transduction through binding linear lysine 63 (K63)- and methionine (M1)-linked ubiquitin. The ubiquitin-binding domain of optineurin is highly homologous to several regulators of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway: a positive regulator NEMO and negative regulators A20 binding and inhibitor of NF- $\kappa$ B (ABIN) proteins, and is thus named ubiquitin-binding domain in ABIN proteins and NEMO (UBAN) (17). The high specificity for linear ubiquitin is provided by the zinc finger domain (ZF), located distally to UBAN (18). Some of the ubiquitinated proteins that optineurin binds are receptor interacting protein kinase 1 (RIPK1), which controls NF- $\kappa$ B activation, and myosin VI, a motor protein important in vesicle trafficking and autophagy. During autophagy, optineurin also recognizes ubiquitinated intracellular bacteria, protein aggregates and damaged mitochondria (3, 19, 20). To function as an autophagy receptor, optineurin bridges ubiquitinated cargo to autophagosomes via its LIR domain (3). Notably, LIR is fully functional only upon



**Figure 1. Schematic representation of optineurin domains and interacting partners.** *N-terminus of optineurin possesses coiled-coil domain 1 (CC1) that interacts with TBK1, LC3-interacting region (LIR) that harbors Ser177, which is phosphorylated by TBK1 to enhance binding between optineurin and LC3. Optineurin ubiquitination is mediated by an ubiquitin ligase HACE1, which ubiquitinates optineurin on Lys193. This posttranslational modification is required for p62 binding to optineurin. Coiled-coil 2 (CC2) domain is crucial for self-oligomerization of optineurin, which is important for binding to linear ubiquitin. Ubiquitin-binding domain (UBAN), important for binding polyubiquitinated cargo including intracellular bacteria, protein aggregates and damaged mitochondria, is located at the C-terminus. UBAN is also important for binding RIPK1, regulator of the NF- $\kappa$ B pathway, and myosin VI, a motor protein important in vesicle trafficking and autophagy. Zinc finger domain (ZF) maps distally to UBAN. Phosphorylation on Ser473 enhances optineurin binding to ubiquitin. Rab8 and caspase 8 bind to the N-terminus, transcription factor IIIA (TFIIIA) to the intermediate region, and CYLD deubiquitinase and huntingtin (htt) to C-terminus of optineurin. Remark: the domain sizes are not drawn to scale to accommodate their designations.*

phosphorylation of Ser177 by TBK1. The complexity of the interaction between optineurin and TBK1 is also seen in the signaling pathway necessary for IFN- $\beta$  production, whereby their stable interaction is inducible upon cell activation by pathogen associated molecular patterns. This process was reported to be bipartite: the two proteins initially associate via N-terminal CC1 domain of optineurin and C-terminal CC2 of TBK1, and this is stabilized upon TBK1 ubiquitination, which allows its binding to optineurin UBAN domain (4, 21). Phosphorylation of optineurin by TBK1 may also be required for stabilization of this interaction. Similarly, self-oligomerization of optineurin via the CC2 domain, was found to be crucial for binding of linear ubiquitin (22).

The role of optineurin self-oligomerization has been debated for a long time because it could potentially lead to protein aggregation. Native gel electrophoresis suggested that endogenous optineurin forms hexamers (23). Moreover, oxidative stress has been proposed to trigger cross-linking of optineurin oligomers (24). However, optineurin ALS mutations were not detected in aggregates, suggesting that it does not act by gain-of-function i.e. toxicity. In contrast, immunohistochemical analysis of sporadic ALS cases, showed aggregation of wild-type optineurin in skein-like inclusions and round hyaline inclusions in the spinal cord, suggesting that optineurin appears to be a general marker for ALS (25). Optineurin presence in aggregates was demonstrated in several other neurodegenerative diseases including Alzheimer's, Parkinson's (PD) and Huntington's disease. The role of wild-type optineurin in these aggregates has not been conclusively clarified.

### **Optineurin cellular localization**

Optineurin is a widely expressed cytoplasmic protein. Many studies confirmed that optineurin is prominently present in the perinuclear region around the Golgi apparatus (26). One of the proposed candidates accountable for driving this localization is huntingtin (htt), an adaptor protein preferentially located at the Golgi apparatus. Mutations of htt caused by polyglutamine repeats take part in intracytoplasmic inclusions in Huntington's disease, a neurodegenerative disease characterized by involuntary movements and various cognitive and behavioral defects. In cells harboring mutated htt, optineurin binding to the htt and Golgi was reduced (27). This led to impaired recruitment of Rab8 to the Golgi apparatus, and partial blockade of the Rab8/optineurin-mediated secretion, while Rab8 was still bound to optineurin but not at the right location. Furthermore, upon activation of pathogen recognition receptors by viral sensing, optineurin was reported to serve as a recruitment platform for TBK1 activation at the Golgi apparatus (4). Prerequisite for TBK1 recognition by optineurin was TBK1 polyubiquitination on conserved lysines 30 and 401. Interestingly, this pathway is apparently hijacked by viruses because NS3 protein

of the Bluetongue virus acts as a decoy for optineurin, thereby diminishing TBK1 recruitment to the Golgi apparatus and its subsequent activation and downstream signaling.

Only one optineurin patient mutation, which harbors a two base pair AG insertion changing the open reading frame (691\_692insAG), localizes to the nucleus (28). ALS-linked ubiquitin-binding mutations Q398X and E478G show abnormal diffuse cytoplasmic distribution in non-vesicular manner and elicit Golgi fragmentation in motor neuron-like NSC-34 cells (28, 29). Wild-type optineurin also colocalizes with ubiquitinated cellular cargo destined for degradation, whereas many ubiquitin-binding mutants fail to do so (3). However, ubiquitin-binding mutants do not completely lose their ability to bind to cargo so it is possible that they partially retain their normal location by piggybacking to endogenous optineurin or some of its protein partners (19, 30). Some uncertainties about optineurin location remain because immunostaining patterns depend on antibodies, tags and washing procedures. For example, adding glycine in a washing step that has been used previously for antigen retrieval, can result in uncovering new epitope(s) and thus different immunostaining patterns (26). Thereby, a diffuse, cytoplasmic distribution of optineurin was present when cells were washed in glycine, whereas without that step, the cytoplasmic staining was low and optineurin was located in the perinuclear area overlapping with the Golgi marker GM130. Other inconsistencies in optineurin staining in overexpression models could also be attributed to supraphysiological level of binding to ubiquitin, p62 and other targets.

### **Optineurin as one of many amyotrophic lateral sclerosis-linked genes**

ALS, also known as Lou Gehrig's disease, is a heterogeneous neurodegenerative disease characterized by the death of motor neurons at three levels: the primary motor cortex, brainstem and spinal cord (31). In up to 50% of patients it can also affect cognition and behavior by targeting neurons in the frontal and temporal cortex (32). Loss of motor neurons leads to skeletal muscle weakness, problems in swallowing and talking, which rapidly progress to complete muscle paralysis and death, most often due to inability to breathe (31). ALS has a complex genetic background (32, 33). Only 10% of cases are familial, although the genetic component is high even in sporadic cases. The discrepancy in the frequency of familial and genetic cases is due to *de novo* mutations, incomplete penetrance and misdiagnosis, among others. Despite an enormous progress in finding ALS-linked mutations, which encompass more than 30 genes so far, understanding their mechanism of action has proved to be a great challenge. One of the most prominent features of ALS is protein aggregation but its direct mechanistic link to neuronal dysfunction is debated (34). Other proposed mech-

anisms include impaired aggregate disposal via autophagy or proteasomes, defective RNA metabolism/stress granule formation, mitochondrial dysfunction, enhanced excitotoxic death, impaired nucleocytoplasmic transport, cytoskeletal and axonal defects, impaired DNA repair, and defects in vesicle trafficking (32, 35). Moreover, neuronal dysfunction is accompanied by inflammatory processes and proliferation of glial cells (32). In this respect it is perhaps not surprising that the direct link to disease is unclear for optineurin mutations as well. As an adaptor in inflammatory signaling, autophagy, and exocytosis, optineurin could contribute to neurodegeneration in several of the above-mentioned pathways.

Around 40 optineurin mutations have been found in hereditary and sporadic ALS patients. The majority are point mutations, including both missense and nonsense mutations. Most of them are in ubiquitin-binding region or CC domains, suggesting that binding of ubiquitin, oligomerization, and protein-protein interactions are responsible for the disease pathogenesis (26). Because mutated optineurin was not detected in aggregates, as discussed above, it is thought that optineurin mutations give rise to the loss of its functions rather than new toxic functions (1). The first optineurin mutations with proven pathogenicity were a homozygous null mutation of exon 5, a homozygous nonsense Q398X and a heterozygous E478G missense point mutation (8). Homozygous mutations of exon 5 and a Q398X nonsense mutation that encodes for a premature stop codon suggest that disease occurs when both alleles are affected leading to loss of complete protein or its ubiquitin-binding region.

### Cellular and animal models for optineurin research

Some of the initial approaches in studying protein function include protein-protein interactions in yeast or bacterial systems, often followed by overexpression studies in cellular models. All of these have been used in optineurin research. Very often the cell line studied is selected based on its ease of transfectability, availability, and not on its relevance for a particular disease. Those include HEK293 and HeLa cells, which might be problematic when analyzing potential cell-specific effects. Furthermore, various ALS or glaucoma patient mutations, as well as artificial mutations that include constitutively active variants, point mutations in relevant domains which lead to loss-of-function or dominant negative effects, have been used in optineurin overexpression studies. However, overexpressed proteins do not necessarily replicate the interactions and/or oligomerization patterns of proteins expressed at physiologic levels. In particular, because more than 70% of optineurin is predicted to form CC domains, it has a high propensity for oligomerization or self-oligomerization. Oligomerization, combined with overexpression that makes proteins supersaturated, would thus facilitate non-physiologic aggregation of optineurin (36).

Therefore, it is possible that some optineurin patient mutations that were reported to aggregate in *in vitro* systems do not behave the same *in vivo*. It must also be noted that some cellular functions are particularly difficult to study. For example, when studying autophagy, one has to be aware of a number of potential artefacts that could lead to data misinterpretation, which will be discussed later.

The first attempts to analyze optineurin function *in vivo* were in zebrafish. Morfolino-induced optineurin silencing in mutated superoxide dismutase 1 (SOD1<sup>G93A</sup>) model, harboring the most researched aggregation-prone ALS patient mutation, resulted in cell death, motor axonopathy, but no motor phenotypes were noted (19). After that, several optineurin mouse models were designed to mimic the pathogenic ALS-linked mutations (Table 1). The first developed was the mouse knock-in model harboring a D477N optineurin (Optn<sup>D477N</sup>) point mutation, which mimics the E478G mutation found in ALS patients and lacks the ubiquitin-binding activity (37). In the same line was the optineurin truncation 1-470 (Optn<sup>470T</sup>), which lacks whole ubiquitin-binding domain (UBAN and ZF), and the distal part of CC2 domain, and as such perhaps best mimics Q398X truncation in humans (38). Another model, an N-terminal deletion (Optn $\Delta$ <sup>157</sup>) that lacks the TBK1-binding region, was designed to study the relevance of optineurin interaction to TBK1 (21). Finally, several groups generated and/or analyzed optineurin deficiency mouse models (Optn<sup>-/-</sup>), which best mimic null mutations of optineurin in ALS patients (39–41). Optineurin mouse models have shown surprisingly little functional defects, if any. One group reported axonal degeneration and defects in vertical rearing activity (40), but this was not confirmed in a follow-up study (42). Analyses of cellular functions in primary cells from optineurin animal models will be further discussed in the subsequent specialized chapters.

Given the poor overlap between optineurin models and patients, one could pose a question whether mouse models are relevant in optineurin or ALS research in general. To answer this, we would argue that the absence of an ALS-like motor phenotype in optineurin models is perhaps not surprising given that mice live on average 30-fold shorter than humans, and do not replicate many of the age-related phenomena present in humans (43). Indeed, the optineurin deficiency and insufficiency mouse models substantially differ from classical toxic prion-like ALS models (44). For example, the first and the most used ALS transgenic model carries the human several-fold overexpressed aggregate-prone mutation SOD1<sup>G93A</sup>, and exhibits early motor neuron death, neuroinflammation and subsequent paralysis (45). However, all preclinical studies for ALS therapies were performed in these mice, and so far most of them failed (44, 46). Although attractive, this may not be an adequate model of ALS, particularly not for its sporadic form. For this reason, the emerging ALS models in other ALS-linked genes are highly sought after (44). These models will likely be valuable

**Table 1.** *Optineurin mouse models.*

Mouse model	Domain/mutation	Related human mutation	Functional readouts in primary cells or <i>in vivo</i>
Optn <sup>D477N</sup>	Point mutation in ubiquitin-binding region → lacks the ubiquitin-binding activity	E478G	<ul style="list-style-type: none"> <li>✓ impaired TBK1 activation, IRF3 phosphorylation and IFN-<math>\beta</math> production</li> <li>✓ reduced phosphorylation of OPTN<sup>D477N</sup> by TBK1</li> <li>✗ no direct differences in NF-<math>\kappa</math>B activation</li> </ul>
Optn <sup>470T</sup>	Truncation of ubiquitin-binding region and the distal part of CC2 → lacks the ubiquitin-binding activity; lower expression level	Q398X	<ul style="list-style-type: none"> <li>✓ impaired TBK1 activation, IRF3 phosphorylation and IFN-<math>\beta</math> production</li> <li>✓ disbalance in gene expression of pro- and anti-inflammatory factors</li> <li>✗ no direct differences in NF-<math>\kappa</math>B activation</li> </ul>
Optn $\Delta$ <sup>157</sup>	N-terminal deletion → lacks TBK1-binding activity	/	<ul style="list-style-type: none"> <li>✓ impaired TBK1 activation, IRF3 phosphorylation and IFN-<math>\beta</math> production</li> <li>✓ reduced phosphorylation of Optn<math>\Delta</math><sup>157</sup> by TBK1</li> </ul>
Optn <sup>-/-</sup>	Total protein deletion → complete loss-of-function	Null mutations	<ul style="list-style-type: none"> <li>✓ impaired TBK1 activation, IRF3 phosphorylation and IFN-<math>\beta</math> production</li> <li>✓ mice more susceptible to Salmonella infection</li> <li>✓ increased necroptosis in oligodendrocytes and mouse embryonic fibroblasts</li> <li>✓ mildly increased proinflammatory cytokines <i>in vivo</i> (in unmanipulated mice)</li> <li>✗ no direct differences in NF-<math>\kappa</math>B activation</li> </ul>

even if the impact of a particular mutation is not seen in unmanipulated mice, because they could in turn be used to analyze the cumulative effects of other ALS-predisposing factors, both environmental and genetic. This is likely particularly important for loss-of-function models, which are less likely to exhibit functional defects than transgenic (overexpression) models.

It is notable that neither animal- nor patient-induced-pluripotent stem cell models are available for most optineurin patient mutation missense mutations. For this reason, they are still analyzed only in cellular models. For example, some mutations including R96L, E478G and V295F were analyzed in functional studies by *in vitro* overexpression (28, 47). Altogether, *in vivo* models are considered more relevant for addressing physiologic functions of particular genes, whereas the *in vitro* research is valuable for initial characterizations of molecular mechanisms. However, limitation of each model must be taken into account.

### Inflammatory signaling

The first reported mutations in optineurin were proposed to trigger excessive inflammatory responses, which is an attractive hypothesis for neurodegeneration (8). Indeed, ever since activated microglia and astrocytes were reported to inflict collateral damage to neighboring neurons in ALS and other neurodegenerative diseases (48, 49), it was unclear if this was exclusively an aftermath of neuronal damage or if glial cells could also be the primary trigger of neuronal death. Optineurin and several of its interacting proteins such as TBK1 and RIPK1, are key regulators of innate immune responses. Importantly, mutations in TBK1, which result in its haploinsufficiency have also been recently described in ALS patients, opening a possibility that innate immune responses could participate in ALS initiation and/or progression (50, 51).

### Optineurin in NF- $\kappa$ B signaling pathway

Activation of the NF- $\kappa$ B pathway via Toll-like receptors or other pathogen or damage sensors leads to proinflammatory cytokine secretion (52). Inflammatory signaling is highly regulated to orchestrate the appropriate magnitude of response to an individual threat. Thus, various feedback loops either amplify or block signaling in immune cell subsets to avoid, on one hand, suboptimal responses incapable of clearing the damage, as well as exaggerated responses leading to autoimmunity and/or autoinflammation. Due to its high homology to NEMO, optineurin was initially proposed to block the NF- $\kappa$ B pathway by competitively binding to polyubiquitinated RIPK1 (14, 53). RIPK1 normally gets ubiquitinated upon pathogen or damage sensing, which allows docking of NEMO, a regulatory component of the I $\kappa$ B kinase complex. I $\kappa$ B kinase complex then gets activated and phosphorylates the inhibitor of  $\kappa$ B (I $\kappa$ B), which leads to its degradation and allows NF- $\kappa$ B translocation to the nucleus, thus triggering the secretion of proinflammatory cytokines such as interleukin 1 $\beta$  and tumor necrosis factor (TNF). For these reasons, the possibility that hyperactivation of the NF- $\kappa$ B could trigger neurodegeneration was studied as a potential mechanism of action of optineurin mutations.

*In vitro* studies showed that overexpressed optineurin inhibited NF- $\kappa$ B activation by competing with NEMO in HEK293 cells (53). Conversely, overexpression of patient ALS mutation E478G, which is deficient in ubiquitin binding, was incapable of NF- $\kappa$ B inhibition in NSC-34 and neuroblastoma Neuro2A mouse cell lines (8, 54). Unlike these results in cell lines though, the investigations in primary murine cells from several models of optineurin deficiency and insufficiency (Optn<sup>470T</sup>, Optn<sup>D477N</sup> and Optn <sup>$\Delta$ 157</sup>), showed no influence of optineurin on the acti-

vation of NF- $\kappa$ B pathway and subsequent TNF production in various primary cells including macrophages, dendritic cells, mouse embryonic fibroblasts (MEFs), and osteoclasts (4, 21, 38, 39). Optineurin was also dispensable for TNF production in neonatal microglia (16). Since optineurin is upregulated by the NF- $\kappa$ B pathway itself (55), it was also tested if it was a late regulator of this pathway, but the analyses done in macrophages and microglia were negative as well (16, 38). All of these reports strongly suggested that the initial characterization of optineurin function in cell lines by overexpression and silencing was an artefact of the experimental system, perhaps due to non-specific binding and transfection artefacts. However, a recent study suggested that ALS mutation E478G led to enhanced NF- $\kappa$ B activation and nuclear translocation in *Optrn*<sup>-/-</sup> MEFs (56). When the same mutation was delivered to mouse motor cortex via a lentiviral vector, it led to neuronal death, accompanied with microglia and astrocyte activation. Similarly, a minor increase in expression of inflammatory genes was reported in an optineurin deficiency model, although this was reported to be secondary to increased necroptosis (40). Therefore, because of these contradictory data, the role of optineurin in the NF- $\kappa$ B pathway is still unclear. Several possibilities could explain for these discrepancies. Firstly, given the potential distinct cell-specific effects, it is possible that optineurin regulates this pathway only in selected cell subsets. Therefore, the effects of optineurin mutations should be tested in a broad panel of cell model systems. Secondly, it is possible that the stimuli used on the primary cells do not represent the dangers faced by neurons or glia in the CNS. For this reason, it would be necessary to use the agents proven to elicit neurotoxicity and/or study neuron:glia co-cultures or otherwise mimic the CNS microenvironment. Thirdly, optineurin could have distinct effects in mouse and human cells. Finally, although it was initially presumed that optineurin deficiency and putative loss-of-function mutations have the same effects, this might not be the case. It is possible that point mutations or truncations act as dominant-negative and/or toxic gain-of-function mutations. Therefore, to understand if indeed optineurin regulates the NF- $\kappa$ B pathway, it will be crucial to study these possibilities.

### **Optineurin in interferon regulatory factor 3 signaling pathway**

Type I interferons (IFNs), including IFN- $\beta$  that is expressed in all cells, are essential for elimination of viruses and certain bacteria (57, 58). Their production is regulated by the transcription factor IRF3, which is activated downstream of the same pathogen or damage sensors that trigger NF- $\kappa$ B activation. Activation of IRF3 is mediated by TBK1, which activates IRF3 by phosphorylation. After Toll-like receptor activation, optineurin interacts with TBK1 at the Golgi apparatus, which allows autophosphorylation of TBK1. It was initially reported that optineurin is a negative regulator of IFN- $\beta$  expression induced by Sendai

virus and dsRNA in HEK293 cells (59), but various studies in primary cells from optineurin loss-of-function mouse models have shown the opposite result in response to various viral and pathogen-mimicking agents like lipopolysaccharide and polyinosinic:polycytidylic acid. For example, in primary cells including embryonic fibroblasts and various innate immune cells (macrophages, dendritic cells, microglia, osteoclasts and NK cells) optineurin deficiency or mutations compromising its ubiquitin-binding function resulted in impaired TBK1 activation and subsequent IFN- $\beta$  production (4, 16, 21, 37–39). Notably, diminished IFN- $\beta$  signaling in microglia led to a disbalance in gene expression of several pro- and anti-inflammatory factors, including IRF7, NOS2, CXCL10, CXCL1, and IL-10 (16). Therefore, the discrepancy exists in cell lines and primary cells, similar to the results in the NF- $\kappa$ B pathway. Notably, the *in vivo* relevance of the TBK1 pathway still needs to be assessed before final conclusions are made. IFN- $\beta$  has not thus far been linked to ALS, IFN- $\beta$  has not thus far been linked to ALS, and if confirmed, it would be a new pathogenic mechanism in ALS.

### **Necroptosis**

Necroptosis is a caspase-independent form of programmed cell death, which results in the release of cellular components and triggers inflammation (60). ALS mouse models and patient autopsies exhibit various necroptosis markers in the affected tissues (40, 42). Ito *et al.* showed that necroptosis markers, RIPK1, RIPK3 and phosphorylated mixed lineage kinase domain-like pseudokinase (p-MLKL) were upregulated in *Optrn*<sup>-/-</sup> MEFs and oligodendrocytes treated with TNF (40). They have also reported upregulation of necroptosis markers in the spinal cord of SOD1<sup>G93A</sup> mice and experimental RIPK3 deletion resulted in reduction of these markers (40). Necroptosis blockade in *Optrn*<sup>-/-</sup> mice restored axonal myelination, suggesting that necroptosis is directly linked to axonal degeneration. However, the follow-up study done by Deremntazki *et al.*, showed equal myelination in *Optrn*<sup>-/-</sup> and WT mice. Moreover, RIPK3 and p-MLKL were not upregulated in the spinal cords of SOD1<sup>G93A</sup> mice, and RIPK3 deletion did not result in reduction of necroptosis markers. These results argue that necroptosis is an unlikely mechanism for optineurin mutations in ALS (42).

### **Optineurin in membrane-associated trafficking events**

Due to the post-mitotic nature of neurons, these cells are vulnerable to increased accumulation of cellular waste and/or impaired vesicle trafficking (61). Failure of these processes demonstrably leads to neurodegeneration. Macroautophagy (hereafter referred as autophagy) is a highly conserved cellular process in charge of eliminating aggregated proteins, damaged organelles, and invading pathogens via lysosomes (62). The fact that mutations in

optineurin and several optineurin-binding partners implicated in autophagy (TBK1, p62) were found in ALS patients, allows for the possibility that they act by disrupting autophagy during the neurodegenerative process. For example, in a SOD1<sup>H46R</sup> mouse model it was shown that loss of p62, a member of a group of autophagy receptor proteins, could worsen the disease, while p62 overexpression ameliorated the phenotype (63, 64). Additionally, TBK1 recruitment to optineurin was prevented by its ALS-linked E696K mutation (65, 66).

Neurodegeneration is also often characterized by Golgi fragmentation, which may not only be the consequence of the neurodegenerative process, but could also be its trigger (67, 68). Golgi apparatus is an organelle that processes proteins destined for secretion. It is fragmented in up to 50% of motor neurons of sporadic ALS patients (69, 70). Golgi fragmentation in SOD1<sup>G93A</sup> model precedes neuromuscular denervation, axon retraction and inclusion formation (70). Additionally, impaired endoplasmic reticulum homeostasis is present in several neurodegenerative diseases (71). In SOD1<sup>G93A</sup> mouse model of ALS for instance, depletion of Reticulon 4, an ER sculpting protein, accelerates the disease onset and progression (72). So, it is possible that optineurin mutations trigger neurodegeneration by perturbing autophagy and/or protein trafficking. Notably, some optineurin mutations found in glaucoma patients have also been reported to impact autophagy and vesicle trafficking (73–76). Because of their distinct mode of action, which includes autophagy-mediated cell death that was not reported with optineurin ALS mutations, they will not be discussed here, and the readers are referred to several recent reviews (2, 77).

### **Optineurin: a pan-autophagy adaptor?**

Autophagy is a cellular process that removes aggregated proteins, damaged organelles, and invading pathogens via lysosomes (62). Autophagy can be induced by various stresses such as starvation, cytokines and potentially toxic cargo (78, 79). It also occurs constitutively without any additional stimuli, providing an important quality control mechanism. Constitutive (also known as basal) autophagy is particularly important in neurons, because they cannot be replaced nor divide to dilute cellular waste (80–82). Of note, neurons also cannot induce autophagy upon starvation, and have limited capacity to increase autophagy upon neurotoxic stress. Upon induction of autophagy, the toxic cargo is ubiquitinated and transported in specialized vesicles called autophagosomes. Autophagosome then fuses with lysosome to form autophagolysosome where degradation of cargo takes place (83). The transportation of ubiquitinated cargo to autophagosomes is mediated by a group of autophagy receptor proteins (alternatively referred to as adaptors), which also comprise optineurin (1).

Optineurin has initially been described as an autophagy receptor that bridges the ubiquitinated intracellular

bacteria to LC3 on autophagosomal membranes (3). Numerous follow-up reports showed that optineurin binds to ubiquitinated mitochondria destined for degradation as well (19, 20, 65, 84). Damaged mitochondria become autophagy cargo upon their ubiquitination by parkin, which is activated by PTEN-induced putative kinase 1. Redundancy with other autophagy receptors, NDP52 and TRAF6-binding protein, was reported for some but not all systems. For example, in the absence of NDP52, optineurin was dispensable for autophagy of mitochondria (mitophagy), but was indispensable for autophagy of bacteria (xenophagy). To enhance its binding to both LC3 and the ubiquitinated cargo, optineurin must be phosphorylated by TBK1 on Ser177 and Ser473 (3, 66). The follow-up research has suggested that in addition to its role in cargo selection, optineurin is implicated in two other distinct stages of autophagy: initiation and maturation. The evidence for its role in autophagy initiation comes from selective mitophagy, whereby optineurin and NDP52 are both recruited to damaged mitochondria via their ubiquitin-binding domains, and participate in recruitment of several autophagy initiating factors including Unc-51 like autophagy activating kinase 1 (20). The role of optineurin in autophagosome maturation was proposed when it was observed that it is required for autophagosomal fusion with lysosome, which is a prerequisite for subsequent fusion with endosomes (85). Optineurin does so by binding to myosin VI, which in turn binds to Tom1-positive endosomes. In addition to optineurin, two other autophagy receptors, NDP52 and TRAF6-binding protein, facilitate the maturation process. It is of note that almost all of these initial reports on the role of optineurin in xenophagy and mitophagy were performed in HeLa cells, necessitating the use of more appropriate models for addressing the role of optineurin in neurodegeneration.

Several cellular models of neurodegenerative disease were used to analyze the role of optineurin in mitophagy and autophagy of abnormal protein aggregates (aggrephagy). Investigation in HeLa cells showed that depletion of optineurin or expression of ALS-associated E478G and Q398X mutations impair parkin-mediated mitophagy by inhibiting LC3 recruitment to damaged mitochondria (20, 86). Optineurin was also shown to act as a cargo receptor in aggrephagy of abnormal protein aggregates like mutated forms of SOD1, htt or TAR DNA-binding protein 43 (TDP-43) (19, 30). However, unlike in mitophagy and xenophagy, a study by Korac *et al.* (19) proposed that optineurin does not need its ubiquitin-binding domain to bind to aggregates. This discrepancy is possibly due to the usage of different experimental models and/or possible formation of optineurin wild-type/mutant dimers in an over-expression system. Several reports also demonstrated that optineurin is necessary for autophagy maturation, as mentioned above. This was further analyzed with ALS patient optineurin mutations. Optineurin mutations E478G and

Q398X disrupted autophagosome formation and degradation of damaged mitochondria in NSC-34 cell line because of their inability to bind to myosin VI (29).

Unfortunately, there are only few reports on the role of optineurin in autophagy in primary cells, and none thus far in cells from optineurin mouse models. The importance of optineurin for the autophagy of protein aggregates characteristic for PD was suggested in wild-type neurons of rat rotenone model of PD, whereby optineurin colocalized with LC3 and  $\alpha$ -synuclein aggregates in the dopaminergic neurons prior and during the manifestation of neurodegeneration (87). In primary mouse microglia, amyloid beta forms complexes with both optineurin and LC3, which could additionally support the role of optineurin in autophagic degradation of neurodegenerative protein aggregates (88). Recent investigation in primary neurons under mild oxidative stress confirmed some of the observations from the cell lines (89). In primary neurons, optineurin colocalized with damaged mitochondria, parkin, and LC3. The transfection of mutant E478G optineurin to wild-type cells led to mitochondrial damage, as assessed by lowered mitochondrial potential and mitochondrial swelling. However, in contrast to HeLa cells, primary neurons needed much more time for the damaged mitochondria to be delivered to the lysosome, demonstrating some discrepancies to the *in vitro* studies. This is the only report in which optineurin-mediated mitophagy was assessed in primary cells. It is still not clear how optineurin affects other types of autophagy and other autophagy steps in these circumstances.

To conclude, it is still not clear if and/or how ALS patient optineurin mutations affect autophagy and if this is relevant to disease pathogenesis. Normally, each autophagy step is tightly regulated, so it would be unusual that optineurin serves as a potential pan-autophagy adaptor throughout this process. It is thus possible that some of the reports with overexpressed patient mutations are experimental artefacts and irrelevant for disease pathogenesis. Alternatively, it is possible that differential expression of autophagy receptors in individual cell subsets accounts for the variability in functional readouts in different systems. If so, it is conceivable that optineurin is rate-limiting for different autophagy steps in different cells. Finally, several general artefacts linked to autophagy investigation could potentially account for data misinterpretation. For example, in patient autopsy samples, autophagy is often evaluated by electron microscopy for the presence of autophagosomes, and by immunohistochemistry, immunofluorescence or immunoblotting for potential accumulation of LC3-II or autophagy receptors/substrates. However, the accumulation of LC3-II or autophagy substrates can mean either an increase in autophagy or a block in autophagosomal degradation. To distinguish between these outcomes, several inhibitors of lysosomal degradation can be used, which is difficult to achieve in mouse models and impossible in patients, thus making the autophagy detection *in vivo* inevitably imprecise.

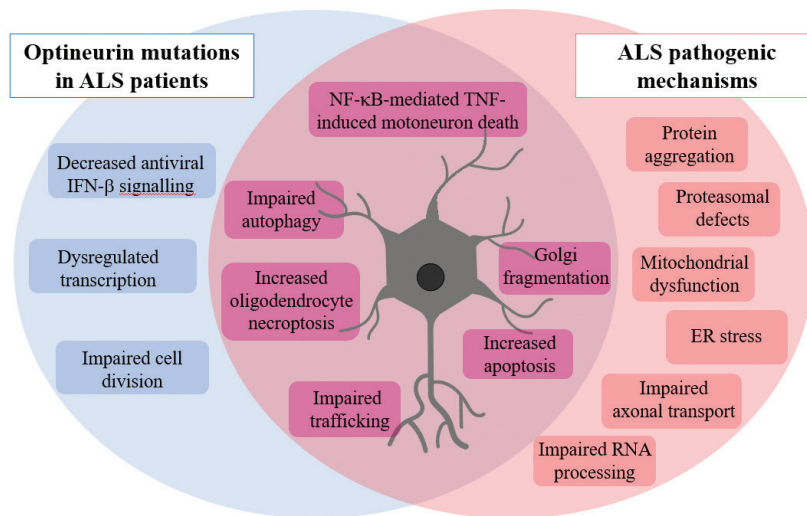
### The role of optineurin in other trafficking events

By binding to myosin VI, a crucial molecule for vesicle trafficking, optineurin was proposed to regulate autophagosomal maturation and maintain normal autophagic flux (85). Additionally, it was demonstrated that in NSC-34 cell line, ALS-linked optineurin mutations E478G and Q398X failed to bind to myosin VI, resulting in Golgi fragmentation and blocking the transport of secretory proteins to plasma membrane (29). A year earlier, van Dis *et al.* (2014) found that Golgi fragmentation in SOD1<sup>G93A</sup> model neuromuscular denervation, axon retraction and inclusion formation (70). However, contrasting the *in vitro* findings and SOD1 overexpressing models, no obvious perturbations of Golgi structure were detected in macrophages of the mice lacking either C- or N-terminus of optineurin (Optn<sup>470T</sup> and Optn<sup>D157</sup> mice) (21, 38). This could suggest an existence of artefacts in *in vitro* models of vesicle trafficking. Therefore, it is still not possible to estimate impact of optineurin mutations on Golgi fragmentation *in vivo*. To clarify this, it will be important to conduct investigations on relevant animal models and determine whether other ALS-linked optineurin mutations have an impact on ER and Golgi apparatus in various CNS cell subtypes.

### CONCLUSIONS

Optineurin is a multifunctional protein involved in various processes including signal transduction, cytokine secretion, vesicle trafficking and autophagy. Many of these processes overlap with the proposed ALS pathogenic mechanisms (Figure 2). Although a decade has passed since its mutations were found to cause ALS, their exact pathogenic mechanism is still elusive. Most mutations map to the C-terminal ubiquitin-binding or CC domains suggesting that defective binding to ubiquitin or oligomerization are responsible for disease pathogenesis. Based on patient and various *in vitro* data in cell lines carrying overexpressed patient mutations or optineurin silencing, one could have expected overt neurological symptoms and various functional defects in optineurin mouse models. However, none of the optineurin deficiency or insufficiency mouse models spontaneously develop ALS. Moreover, investigations on inflammatory signaling, vesicle trafficking and autophagy, have shown some conflicting results in optineurin mouse models compared to the *in vitro* cell lines and patient data. Each of these approaches comes with its own set of potential artefacts. Overexpression studies are particularly problematic because of potential non-physiologic protein-protein interactions and protein aggregation. On the other hand, mouse models, especially those of carrying loss-of-function mutations may not develop symptoms of neurodegeneration because their life span is shorter and other evolutionary differences exist. Alternatively, substantial





**Figure 2. The effects of optineurin patient mutations overlap with ALS pathogenic mechanisms.** Loss of optineurin function has been proposed to result in decreased IFN- $\beta$  signaling, dysregulated transcription of 5S ribosomal RNA, defects in cell division, overactivation of NF- $\kappa$ B signaling and TNF production, blockade of autophagy, increased apoptosis and necroptosis, Golgi fragmentation and disruption in vesicle trafficking. Notably, disruption in six of these cellular processes overlap with the proposed ALS pathogenic mechanisms (marked in the overlapped area), which can also be provoked by mutations in other ALS-linked genes such as C9orf72, SOD1 and TDP-43 (35). Other molecular mechanisms that contribute to ALS pathogenesis, but so far have not been linked to optineurin are listed in the red circle.

redundancy of cellular adaptors in both autophagy and inflammation, could compensate a loss-of-function of individual genes in individual cell types or even in whole-body mouse models. In this case exacerbation of disease phenotype could be expected only under some stressful conditions and/or in the presence of additional mutations. It is also possible that different mutations cause ALS by distinct mechanisms, which could account for the variability in studies with distinct patient mutations. Therefore, future studies should focus on stressful conditions such as danger stimuli or additive mutations, which better mimic the disease pathogenesis. Other desired approaches include optineurin ALS patient derived induced pluripotent stem cells and CRISPR/Cas9-mediated introduction of patient mutations in cells or animal models.

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