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Autophagy in inherited peripheral neuropathies: Focus on the small heat shock protein HSPB1

Autofagie in de erfelijke perifere neuropathieën: Focus op de kleine heat shock proteïne HSPB1

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen: Biochemie en Biotechnologie aan de Universiteit Antwerpen.

te verdedigen door

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Lucretius, De Rerum Natura, Book I. 250

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Summary

Inherited peripheral neuropathies (IPNs) are genetically heterogeneous disorders affecting mainly the peripheral nervous system and with over 1500 mutations in more than 80 affected genes discovered so far. IPNs are characterized by length-dependent degeneration affecting the motor and/or sensory nerves and leading to a varied range of severity, from mild motor or sensory impairment to self-mutilation and wheelchair dependency. Given the increasing heterogeneity among IPN-associated genes, recent functional research studying the pathogenic mechanisms of IPNs has focused on identifying common pathomechanisms underlying the IPN pathology. In the last few years several studies have shown a correlation of IPN-associated genes with autophagy. Autophagy, a cellular homeostatic process, is required for the removal of cell aggregates, long-lived proteins and dead organelles in vesicles termed autophagosomes and directing them for lysosomal degradation. Among the genes associated with IPNs are those coding for the small heat shock protein HSPB1, in which mutations can lead to the axonal form of Charcot-Marie-Tooth neuropathy (CMT2) and distal hereditary motor neuropathy (dHMN). HSPB1 is a molecular chaperone with a function in counteracting protein misfolding and aggregation. Several studies have shown that HSPB1 and other members of the small heat shock protein family are capable of modulating other important cellular functions which might explain the pathogenic effects of their mutations in the context of IPNs.

In the first part of this thesis I will discuss the study we performed to monitor the effects of *HSPB1* mutations on autophagy. We show that mutations in *HSPB1*, both targeting its catalytic alpha-crystallin domain and the C-terminus, display decrease in autophagy levels. We also see that knocking-out HSPB1 also impairs the autophagy process, which indicates that it is necessary for the autophagy pathway. We show that while autophagy is disrupted by mutant HSPB1, the proteasome pathway is not affected; suggesting an autophagy-specific effect of these mutations. Interestingly, we confirm the autophagy deficits in patient-derived motor neurons indicating that the impairment of autophagy might be one of the pathomechanisms by which mutations in *HSPB1* lead to peripheral neuropathy.

In the second part of the thesis I describe the interactomics study we performed to have an in-depth view of the interactors of HSPB1 wild-type and mutant in the context of autophagy. Through an extensive proteomic analysis on the different HSPB1 variants (wild type and mutants) we identified interactors associated with the autophagy process. Then after further validation, we reveal the autophagy receptor sequestosome-1 (SQSTM1/P62) as an interactor

of HSPB1. We show that mutant HSPB1 might cause abnormal interaction with P62 affecting its autophagy function. Our study shows that cells expressing mutant HSPB1 have a decreased capacity to form P62 bodies crucial for the P62 function in autophagy, and lead to impairment of phagophore formation.

In conclusion, my PhD thesis presents autophagy impairment as a pathomechanism caused by various mutations in *HSPB1*, and contributes to the view that disruption of autophagy can be a common pathomechanism in IPN. In addition, this work provides an insight into the role of HSPB1 as a regulator of autophagy through interaction with P62 and that mutations in *HSPB1* might affect this interaction. This is in line with recent work on other small heat shock proteins showing that this family of proteins can be active members in the autophagy process.

Samenvatting

Erfelijke perifere zenuwaandoeningen - of hereditaire motorische en sensorische neuropathieën (HMSN) - zijn genetisch heterogene aandoeningen die voornamelijk het perifere zenuwstelsel aantasten, en waarvan tot dusver meer dan 1500 mutaties in meer dan 80 verschillende genen zijn ontdekt. De HMSN worden gekenmerkt door degeneratie van motorische en/of sensorische zenuwen op een lengte-afhankelijke manier. De HMSN zijn klinisch divers, gaande van een milde motorische of sensorische aantasting, tot rolstoelafhankelijkheid en uitzonderlijk zelfverminking. Gezien de niet onbelangrijke genetische en fenotypische heterogeniteit in HMSN, richt het wetenschappelijk onderzoek zich de laatste jaren toe op het identificeren van gemeenschappelijke mechanismen die de HMSN pathologie kunnen verklaren. Eén van die mechanismen is autofagie, een cellulair homeostatisch proces noodzakelijk voor het verwijderen van intracellulaire aggregaten en het uitvoeren van transport van langlevende proteïnen en dode celorganellen via vesikels – autofagosomen – voor lysosomale afbraak. Eén van de genen die geassocieerd wordt met HMSN codeert voor het 'small heat shock' proteïne HSPB1. Mutaties in dit gen veroorzaken een axonale vorm van de ziekte van Charcot-Marie-Tooth (CMT2) of erfelijke distale motorneuropathie (dHMN). Het HSPB1 is een moleculaire chaperone dat het foutief vouwen van proteïnen en de aggregatie ervan tegengaat. Verscheidene studies hebben aangetoond dat HSPB1 en andere 'small heat shock' proteïnen in staat zijn om diverse belangrijke cellulaire functies te moduleren, wat een verklaring kan zijn voor de pathogene effecten van hun mutaties in de context van HMSN.

In het eerste deel van deze thesis bespreek ik de effecten van *HSPB1* mutaties op autofagie. We tonen aan dat mutaties in *HSPB1*, zowel in het katalytische alfa-crystalline domein als in de C-terminus, de flux in autofagie kan verstoren. Daarnaast zien we dat het uitschakelen van HSPB1 (via knock-out) ook het proces van autofagie aantast, wat aangeeft dat HSPB1 hier een noodzakelijke functie uitoefent. Hoewel HSPB1 mutanten het proces van autofagie verstoren, wordt degradatie van doeleiwitten door het proteasoom niet beïnvloed, wat suggereert dat deze mutaties een specifiek effect hebben op autofagie. We hebben het verstoren van autofagie bevestigd in motorische zenuwcellen gedifferentieerd uit geïnduceerde pluripotente stamcellen van patiënten met *HSPB1* mutaties, wat erop wijst dat de verstoring van autofagie één van de onderliggende mechanismen zou kunnen zijn bij HSPB1 gerelateerde perifere zenuwaandoeningen.

In het tweede deel van mijn thesis beschrijf ik hoe we op zoek zijn gegaan naar de interactiepartners van HSPB1, zowel voor het wild type als mutant HSPB1, en in de context van autofagie. Door een uitgebreide proteoom analyse op de verscheidene HSPB1 varianten, konden we interactiepartners identificeren die geassocieerd zijn met autofagie. Na verdere validatie ontmaskerden we de autofagie-receptor sequestosoom-1 (SQSTM1/P62) als een interactiepartner van HSPB1. We tonen aan dat mutaties in *HSPB1* leiden tot een mogelijke abnormale interactie met P62, en met een verstoring van autofagie als gevolg. Ons onderzoek toont aan dat cellen die mutant HSPB1 tot expressie brengen een verminderde capaciteit hebben om 'P62-bodies' te vormen, wat essentieel is voor de functie van P62 in autofagie en aanleiding geeft tot een verminderde vorming van fagoforen.

In mijn proefschrift besluit ik dat het verstoren van autofagie door mutant HSPB1 een mogelijk onderliggend mechanisme is dat de pathologie van dHMN kan verklaren. Bovendien geeft dit werk een inzicht in de rol van HPSB1 als een regulator van autofagie door interactie met P62, en oppert het dat mutaties in *HSPB1* deze interactie kunnen beïnvloeden. Dit is in lijn met recent onderzoek uitgevoerd op andere 'small heat shock' proteïnen, en dat aantoont dat deze proteïne familie actieve actoren in het autofagieproces kunnen zijn.

RATIONALE and AIMS:

Inherited peripheral neuropathies (IPNs) represent a genetically heterogeneous group of disorders with mutations in more than 80 different genes linked to the disease. Among these genes are those coding for the small heat shock protein HSPB1, a member of the molecular chaperone family of small heat shock proteins. In addition to their molecular chaperone function, members of this family of proteins have been linked to various cellular pathways. In this thesis I tried to understand common pathomechanisms underlying IPNs and how, despite its ubiquitous nature, mutations in *HSPB1* can cause disorders specific to the nervous system.

To attempt to follow up on these questions, in **Chapter 1** I present an extensive literature study proposing the impairment of the autophagy pathway as a common pathomechanism among IPNs. The higher sensitivity of neurons, especially those of the peripheral nervous system to disruptions in autophagy, might help understand how mutations in ubiquitously expressed genes can lead to chiefly neuropathic disease phenotypes. In **Chapter 2**, I discuss the mammalian family of small heat shock proteins (HSPBs) focusing on their role in proteostasis and neurodegeneration.

In **Chapter 3**, the goal was to discover whether mutations affecting different regions of HSPB1 lead to impairment of autophagy. I confirmed that deficits in autophagy are caused by mutations in *HSPB1* and I validated these results in different cell models including patient derived motor neurons. In addition, the results from this chapter pointed out to a role for HSPB1 in autophagy. Therefore, **Chapter 4** was aimed at understanding the binding partners of HSPB1 in the context of autophagy. Since previous studies on HSPBs showed that they can work with different binding partners and affect pathways including autophagy, I explored if HSPB1 is behaving in a similar fashion. I saw that HSPB1 binds to the autophagy regulator P62 and that the binding of mutant HSPB1 can have adverse effects on the function of P62 and ultimately on autophagy.

In the final chapter of my thesis, **Chapter 5,** I compiled all the findings from the different chapters and linked them to the broader picture of the field of IPN and small heat shock proteins.

CHAPTER 1

Autophagy as an emerging common pathomechanism in inherited peripheral neuropathies

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ABSTRACT:

The inherited peripheral neuropathies comprise a growing list of genetically heterogeneous diseases. With mutations in more than 80 genes being reported to cause inherited peripheral neuropathies, a wide spectrum of functional consequences is expected to follow this genotypic diversity. Hence the search for a common pathomechanism among the different phenotypes has become the holy grail of functional research into inherited peripheral neuropathies. During the last decade, studies on several affected genes have shown a direct and/or indirect correlation with autophagy. Autophagy, a cellular homeostatic process, is required for the removal of cell aggregates, long-lived proteins and dead organelles from the cell in double-membraned vesicles destined for the lysosomes. As an evolutionarily highly conserved process, autophagy is essential for the survival and proper functioning of the cell. Recently, neuronal cells have been shown to be particularly vulnerable to disruption of the autophagic pathway. Furthermore, autophagy has been shown to be affected in various common neurodegenerative diseases of both the central and the peripheral nervous system including Alzheimer's, Parkinson's, and Huntington's diseases. In this chapter we provide an overview of the genes involved in hereditary neuropathies which are linked to autophagy and we propose the disruption of the autophagic flux as an emerging common pathomechanism. We also shed light on the different steps of the autophagy pathway linked to these genes. Finally, we review the concept of autophagy being a therapeutic target in inherited peripheral neuropathies, and the possibilities and challenges of this pathway-specific targeting.

1. Introduction:

Disruption of intracellular homeostasis is at the base of most pathological conditions. Cellular health and function often relies on the maintenance of protein homeostasis by assuring that structurally abnormal proteins do not accumulate in cells, causing aggregate formation and organelle damage. The function of proteostasis is accomplished by two systems, the proteasome and the autophagy system. While the proteasome targets ubiquitinated and short-lived proteins, autophagy can degrade long-lived proteins and damaged organelles. Three main subtypes of autophagy have been identified: microautophagy, chaperone mediated autophagy (CMA), and macroautophagy. Microautophagy involves the degradation of cytosolic material inside the lysosome by direct lysosomal invagination (Mijaljica et al., 2011). CMA is a specific autophagic pathway where chaperones target proteins containing the KFERQ motif to the lysosome for degradation (Cuervo et al., 1995). Unlike CMA, macroautophagy (the main focus of this review), is a bulk degradation process characterized by the formation of double membrane vesicles, autophagosomes, which engulf cytoplasmic material and degrade their contents by fusing with the lysosomes (Yang and Klionsky, 2010b). Disruption of the macroautophagy pathway can lead to failure to clear out misfolded proteins and dead organelles, or increased accumulation of autophagic structures and has been linked to neurodegeneration (Frake et al., 2015). Mutations in key autophagy regulating genes have been shown to cause neurodegeneration (Kyöstilä et al., 2015; Saitsu et al., 2013). Furthermore, mouse models with neuron-specific knock-out of autophagy genes frequently develop neurodegeneration (Komatsu et al., 2006). Interestingly, some of these mouse models display features of peripheral neuropathy as evident by decline in motor performance on the rota-rod, limb-clasping, and paw placement tests (Hara et al., 2006). The link between disruption at the gene level and the development of peripheral neuropathy is the main feature of inherited peripheral neuropathies (IPNs). IPNs are a genetically heterogeneous disease population with over 80 affected genes discovered so far (Baets et al., 2014; Timmerman et al., 2014). While the clinical presentation of IPN patients is rather common, with length-dependent degeneration affecting the motor and/or sensory nerves, the variety of associated genes has produced various molecular phenotypes (Pareyson et al., 2014; Weis et al., 2016). Recently, more and more studies have indicated the involvement of autophagic impairment in IPN causing-mutations, and important roles for IPN related genes in autophagy. While the evidence linking IPN associated genes to autophagy can be direct or indirect, impairment of autophagy presents as

an important contributor to the neuropathic phenotype, given the vulnerability of neurons, especially those of the peripheral nervous system to disrupted cellular recycling and clearance. Identifying a common pathomechanism among the different IPN-related genes would provide a great therapeutic potential for targeting these neuropathies. This chapter discusses the advances made so far regarding the cellular and molecular mechanisms behind the different forms of IPN due to impairment of the autophagic pathway.

2. Autophagy:

Macroautophagy, hereafter referred to as autophagy, is a homeostatic cellular process by which protein aggregates and cellular organelles are targeted, degraded, and recycled (**Figure 1**). Autophagy is a multi-step process consisting of: induction, nucleation of the isolation membrane (phagophore), elongation and expansion of the phagophore into a closed double-membraned autophagosome, lysosomal docking and fusion, and degradation of autophagic cargo. The different autophagy steps, explained below, are governed by a wide array of proteins and protein complexes, most notably the group of proteins encoded by the autophagy-related (*ATG*) genes (Parzych and Klionsky, 2014).

2.1. Induction of autophagy:

The initiation of autophagy is regulated by the ULK complex composed of ULK1 and ULK2 (unc-51 like autophagy activating kinase 1/2), and their stable interactors RB1CC1 (RB1 inducible coiled-coil 1) and ATG13. The autophagy initiation complex is under the control of the nutrient-sensing, mechanistic target of rapamycin complex 1 (MTORC1), a serine-threonine kinase. During nutrient-rich conditions MTORC1 binds to and phosphorylates ULK1/2 inhibiting their kinase activity. Removal of MTORC1 from the ULK1/2 complex, by nutrient starvation for example, allows for the activation of ULK1/2 and the proceeding of the autophagic process (Hosokawa et al., 2009).

2.2. Phagophore nucleation:

The membrane nucleation step involves the conversion of phosphatidylinositol (PtdIns) to phosphatidylinositol 3-phosphate (PtdIns3P) by the kinase complex Class III PtdIns3K. This complex consists of phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3/VPS34), beclin 1 (BECN1), and phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4/VPS15) (Petiot et al., 2000). PtdIns3P is a signal for recruiting ATG proteins in the nucleation site, by recognizing and binding to PtdIns3P. BECN1 moderates the coupling of the PtdIns3K complex with various proteins, and via its binding partners serves as a checkpoint that can inhibit or

upregulate autophagy. Among the binding partners are ATG14, UVRAG (UV radiation resistance associated), and apoptosis regulator BCL2 proteins. BCL2 binding suppresses autophagy (Pattingre et al., 2005). UVRAG-binding targets the nucleation machinery to the endosomal membranes or the cell surface and can up or downregulate autophagy depending on its other binding partners (Yang and Klionsky, 2010b). ATG14 binding on the other hand targets the nucleation to the omegasome (an ER-resident precursor of the autophagosome where formation of autophagic vacuoles ensues) (Axe et al., 2008).

2.3. Elongation and expansion:

Two ubiquitin-like conjugation systems are at the heart of the expansion of the phagophore into an autophagosome. The first system concerns the formation of ATG12-ATG5-ATG16L1 complex. ATG12 is covalently conjugated to ATG5 via the E1 activating enzyme ATG7 (Tanida et al., 1999) and the E2 conjugating enzyme ATG10 (Shintani et al., 1999). The ATG12-ATG5 complex then binds ATG16L1 through ATG5. The second ubiquitin-like conjugation reaction involves the lipidation of ATG8 (LC3) to ATG8-Phospatidylenolamine (LC3II). This first requires the C-terminal cleavage of LC3 by the protease ATG4. The cleaved protein (LC3I) is then processed by the E1 activating enzyme ATG7 and the E2 conjugating enzyme ATG3 yielding LC3II. LC3II remains associated with the phagophore and the mature autophagosomes until its degradation in the lysosome (Kabeya, 2000). This association makes LC3II a good marker for the study of the autophagic activity in cell and animal models. Since lipid synthesis does not occur at the phagophore, delivery of membrane from other locations in the cell is necessary for the elongation step. The task of membrane recruitment is served by ATG9, the only transmembrane protein in the ATG family. Mainly localized to the *trans*-Golgi network and the late endosomes, autophagy activation drives the trafficking of membrane delivering-ATG9 to the sites of autophagosome formation (Mari et al., 2010). Lipid delivery supplied by ATG9 allow for the elongation of the phagophore into a fully closed autophagosome.

2.4. Fusion:

The complete autophagosome eventually moves to and fuses with a lysosome becoming an autolysosome and/or with an endosome forming an amphisome. The transport of autophagosomes to the lysosomes depends on microtubules. The mechanism of docking at and fusing with the lysosome is not well understood (Chen and Klionsky, 2011), but it is thought

to involve UVRAG, RAB7 GTPase and syntaxin-17 (STX17) of the SNARE machinery (Jäger et al., 2004; Jiang et al., 2014; Liang et al., 2008).

2.5. Degradation:

Upon the fusion of the autophagosome with the lysosome, the autophagic cargo is degraded by various lysosomal hydrolases and proteases. The degradation products, including metabolites, amino acids and fatty acids, are recovered and reutilized by the cell.

Fig1. The different steps of the autophagic pathway

The autophagic pathway consists of several steps. The initiation step is governed by the initiation complex which is under the control of the nutrient-sensing MTORC1 complex. After the initiation step, the nucleation of early autophagic membranes is controlled by the nucleation complex. The formed phagophore then undergoes elongation to become a fully closed, double-membraned autophagosome. This step involves 2 conjugation systems and the delivery of membranes via ATG9-containing vesicles. The completed autophagosome then fuses with a lysosome becoming an autolysosome where its cargo is subjected to lysosomal degradation.

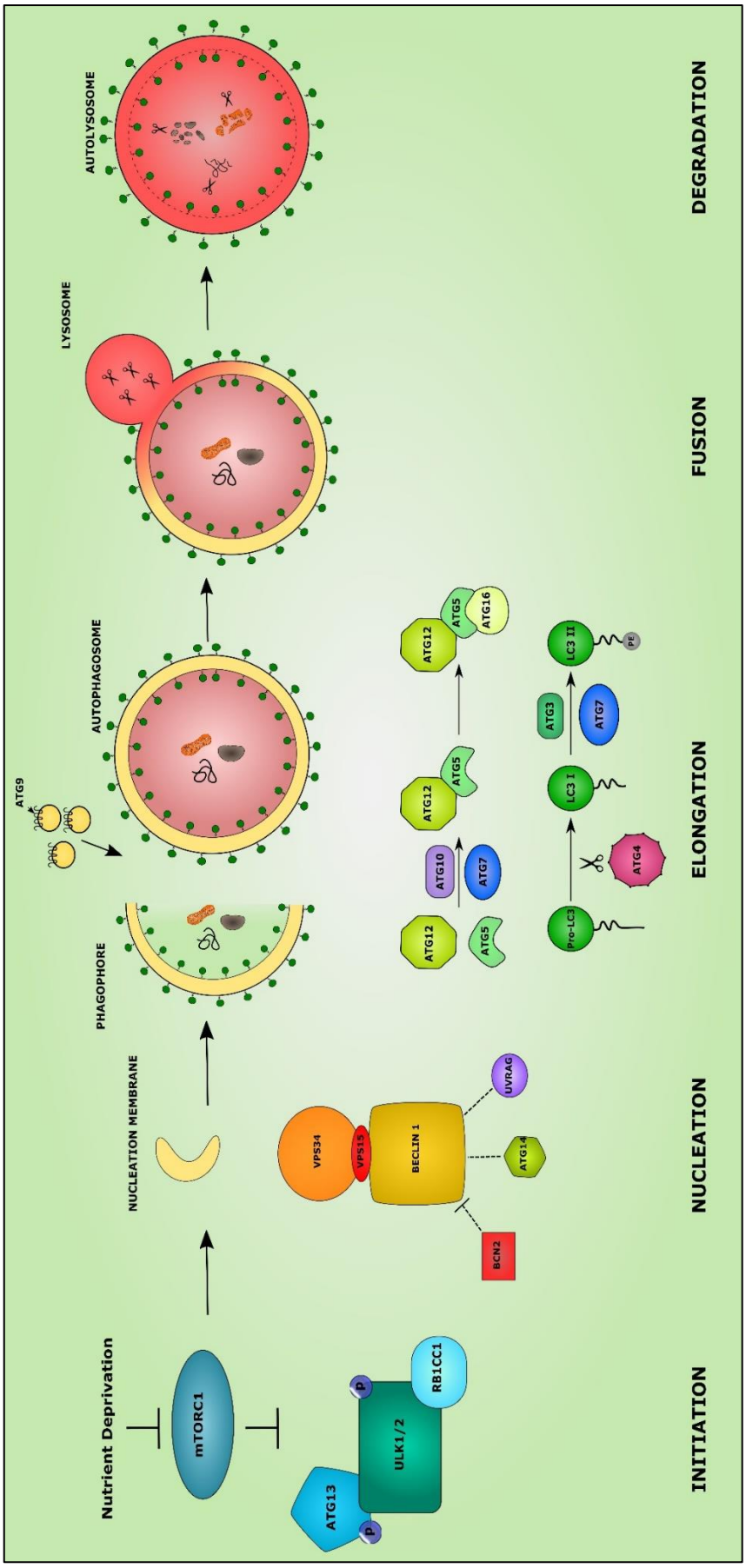

Fig1. The different steps of the autophagic pathway

2.6. Autophagy in neurons:

The morphological features that distinguish neurons from other cells are their post-mitotic nature, highly polarized structure, and extended cytoplasm into axons and dendrites that can stretch far from the cell body. The latter feature is more exaggerated in neurons supplying the peripheral nerves. The spatial compartmentalization of neurons makes them prone to aggregation and accumulation of dead organelles and misfolded proteins. Autophagy therefore forms an essential homeostatic process for neurons. Knocking out key autophagy genes, such as ATG7, in mouse neurons leads to neurodegeneration (Komatsu et al., 2006). Defective autophagy has also been linked with numerous neurodegenerative diseases (Frake et al., 2015). The high susceptibility of neurons to autophagic impairment could explain why mutations in ubiquitously expressed genes can cause neuron-specific pathology in inherited neuropathies.

3. Inherited Peripheral Neuropathies (IPNs):

Inherited peripheral neuropathies are a genetically heterogeneous group of disorders affecting the peripheral nerves. Depending on the affected nerves, IPNs are classified as Charcot-Marie-Tooth (CMT or HMSN) if motor and sensory nerves are affected. If sensory and/or autonomic nerve dysfunction predominates, the neuropathy is termed HSAN and distal hereditary motor neuropathy (dHMN) if motor deficits are the most prevalent (Pareyson et al., 2014).

3.1. Clinical and inheritance pattern classification:

CMT, the most common form of hereditary neuropathies affects about 1:2500. Patients with CMT share a common clinical presentation of slowly progressive muscle wasting and weakness ascending from the feet to reach the thighs and hands, reduced tendon reflexes, skeletal deformities, and sometimes sensory loss (Harding and Thomas, 1980). In many cases, the age of onset is in the first to second decade, although infantile, early-onset and late-onset forms exist. CMT is further classified on the prevalence of myelin or axonal involvement and according to the mode of inheritance. CMT1, the demyelinating subgroup is associated with reduced nerve conduction velocities (NCV) to less than 35 m/s. CMT2, the axonal subgroup on the other hand, only shows slightly reduced NCV but with reduced compound muscle action potential amplitudes (CMAPs). An intermediate CMT subgroup exists with NCV between 35 and 45 m/s. The mode of inheritance further subdivides CMT neuropathies into CMT4 for recessive demyelinating CMT, CMT2R for recessive axonal CMT, CMTDI and CMTRI for

dominant and recessive intermediate CMT respectively, and CMT-X for the X-linked CMT (Stojkovic, 2016).

The **HSAN** disorders are characterized by sensory deficits and the involvement of the autonomic dysfunction. Five types of HSAN are acknowledged depending on the inheritance pattern and the sensory abnormality (Thomas, 2005). HSAN type I is autosomal dominant with variable motor involvement. HSAN types II to VI are autosomal recessive and further classified according to the sensory involvement and the clinical presentation ranging from osteomyelitis in type II to respiratory difficulties in type VI. HSAN types III, IV, and V show congenital onset. HSAN with spastic paraplegia, also a recessive form of HSAN, presents as a sensory neuropathy with mild spastic paraplegia (Rotthier et al., 2012).

The **dHMN** are classified into seven subtypes depending on the age of onset, inheritance pattern, and the distribution of the deficits (Harding and P.K. Thomas, 1980). The dHMN type I, II, V, and VII are autosomal dominant, while types III, IV and VI are autosomal recessive and the X-linked dHMN has an X-linked inheritance. Minor sensory abnormalities are frequent to many dHMN forms, and an overlap with axonal CMT (CMT2), where mutations in the same gene can cause either phenotypes, is common. The phenotypic characteristics of dHMN are very diverse with the pathology involving other organs such as the vocal cords or the respiratory system (Rossor et al., 2012).

4. Gene function classification:

Hereditary peripheral neuropathies show overlap on the genetic level with mutations in the same gene leading to different clinical subtypes. This has recently lead to attempts in classifying IPNs based on the gene function involved in neuropathy (Vallat et al., 2016). While many genes associated with neuropathy have a known function in neuronal maintenance, development, or myelination, several other genes revealed functions that were unrecognized or indirect to the caused neuropathy (Weis et al., 2016). The categorization based on gene function further highlighted the heterogeneity of inherited neuropathies. Hence the search for a common pathomechanism among the different causal genes is starting to become a focal point in the hope of better understanding the pathology of inherited neuropathies and ultimately designing efficient therapeutic approaches. We believe that this classification is of great help for a better understanding of the molecular consequences of the disease-causative mutations. In the following sections, we shed light on the involvement of inherited peripheral neuropathy-

associated genes in the different steps of the autophagy pathway presenting autophagic impairment (**Figure 2**) as an emerging common pathomechanism (Summarized in **Table 1**).

4.1. IPN genes and Autophagy initiation:

PMP22 (peripheral myelin protein 22):

The PMP22 is a myelin glycoprotein which plays an important role in the formation and maintenance of compact myelin hence participating in the formation of the myelin sheath (Li et al., 2012). Autophagy is also involved in myelination of sciatic nerves by regulating the structural plasticity of Schwann cells (Jang et al., 2015). Mutations in *PMP22* are responsible for more than 50% of all inherited neuropathies. Duplication of *PMP22* causes the most common demyelinating neuropathy, CMT1A (Lupski et al., 1992; Timmerman et al., 1992). Point mutations in *PMP22* cause CMT1E neuropathy, while a heterozygous deletion causes hereditary neuropathy with liability to pressure palsies (HNPP) (Nicholson et al., 1994; Russo et al., 2011). CMT1A and CMT1E patients show variable clinical severity but the main features of the diseases include slowly progressive weakness and atrophy of the distal limb muscles, *pes cavus*, and reduced or absent deep tendon reflexes. In the *Trembler* CMT1E mouse models carrying a missense mutation in *Pmp22*, the mutant protein colocalizes with lysosomal markers (Notterpek et al., 1997). In the C22 mouse model of overexpression of PMP22, resembling the CMT1A phenotype, aggregate formation is seen in affected nerves. This aggregation occurs together with a reduced proteasomal activity and association of the aggregates with autophagosomes (Fortun et al., 2006). Since autophagy and the proteasome can play compensatory roles in maintaining cellular homeostasis, autophagy seems to play a part in the pathomechanism of CMT1 neuropathic mouse models. With reduced proteasomal activity, basal autophagy on its own might not be enough to clear out all the aggregating mutant proteins, but induction of autophagy by external methods can aid in the degradation of toxic products. Indeed, autophagy induction in neuronal cultures from CMT1A mouse models via nutrient deprivation or rapamycin treatment decreases the aggregate formation. Autophagy induction also improves PMP22 trafficking leading to an enhancement of remyelination and indirectly increasing the expression of myelin proteins and the abundance and length of myelin internodes (Madorsky et al., 2009; Rangaraju et al., 2010). Suppression of a key autophagy protein, ATG12 by siRNA, abolishes these effects, indicating that the phenotypic improvements are mediated by autophagy and that an intact autophagy pathway is required for proper remyelination (Rangaraju et al., 2010)

Affected Autophagy Step/Type	**IPN-associated Gene**	**Clinical Phenotype**
Initiation	*PMP22* (peripheral myelin protein 22)	CMT1A, CMT1E, HNPP
	VAPB (VAMP associated protein B)	late onset SMA, ALS8
	WNK1 (WNK lysine deficient protein kinase 1)	HSAN-IIA
	BSCL2 (Seipin)	dHMN-V
	LRSAM1 (leucine rich repeat and sterile alpha motif containing 1)	CMT2P
	TRPV4 (transient receptor potential cation channel subfamily V member 4)	CMT2C, congenital distal SMA
	NDRG1 (N-myc downstream regulated 1)	CMT4D
	DNAJB2/HSJ1 (DnaJ heat shock protein family (Hsp40) member B2)	AR-dHMN
	NEFL (neurofilament light)	CMT2E, CMT1F
Autophagosome formation and expansion	*TrkA (NTRK1)* (neurotrophic receptor tyrosine kinase 1)	HSAN-IV, CIPA
	DNM2 (Dynamin2)	DI-CMTB
	KIF1A (kinesin family member 1A)	HSAN-IIC
	LITAF (lipopolysaccharide-induced TNF factor)	CMT1C
	SH3TC2 (SH3 domain and tetratricopeptide repeats 2)	CMT4C
	TECPR2 (tectonin beta-propeller repeat containing 2)	HSAN-III, HSP
Transport of autophagosomes	*DCTN1* (Dynactin subunit 1)	dHMN-VIIb
	DST (Dystonin)	HSAN-VI
Lysosomal fusion and degradation	*RAB7* (member RAS oncogene family)	CMT2B
	CCT5 (chaperonin containing TCP-1 subunit 5)	HSNSP
	FIG4 (FIG4 phosphoinositide 5-phosphatase)	CMT4J
	MTMR2; MTMR13 (myotubularin-related proteins)	CMT4B1, CMT4B2
	VCP (valosin containing protein)	CMT2Y, ALS8, IBMPFD
	HSPB8 (heat shock protein B8)	CMT2L, dHMN-I
Mitophagy	*MFN2* (mitofusin 2)	CMT2A
	GDAP1 (ganglioside induced differentiation associated protein 1)	CMT4A, AR-CMT2, CMT2K
Reticulophagy	*FAM134B* (family with sequence similarity 134 member B)	HSAN-IIB

Table 1. Summary of IPN-associated genes, their clinical phenotype and the step they impact in the autophagy pathway.

VAPB (VAMP associated protein B and C):

VAPB belongs to a family of ER-anchored proteins and plays a role in Golgi-mediated transport, membrane trafficking and neurotransmitter release (Lev et al., 2008). Recently the tethering complex formed by VAPB (ER) and PTPIP51 (mitochondria) has been shown to regulate the induction of autophagy through a role in mitochondria-ER calcium delivery which acts on BCL2-Beclin 1 interaction (Gomez-Suaga et al., 2017). Dominant mutations in *VAPB* lead to complex and atypical forms of inherited motor neuron disease (Nishimura et al., 2004). In a VAPB/ALS8 knock-in mouse model, mutant VAPB translocates from the ER to the autophagosome where it is degraded (Larroquette et al., 2015). The mislocalisation of VAPB causes ER stress and can lead to loss of VAPB functions in the ER. Absence of VAPB in the ER abolishes the VAPB-PTPIP51 tethering complex. Loosening of the ER-mitochondria contacts by loss of VAPB-PTPIP51 can lead to overstimulation of autophagy (Gomez-Suaga et al., 2017). The dysregulated autophagy may have adverse effects on neuronal homeostasis and might be a prime pathological sign in VAPB-associated neuropathies.

WNK1 (WNK lysine deficient protein kinase 1):

WNK1 is a serine/threonine kinase with the canonical function of regulating ion transport across cell membranes (Moriguchi et al., 2005). Truncating mutations in *WNK1* cause hereditary sensory and autonomic neuropathy type II (HSAN-II) (Rivière et al., 2004). HSAN-II is characterized by absence of pain sensations and patients suffer from ulcero-mutilating form of neuropathy. Little is known about the non-canonical functions of WNK1, but a role in autophagy was discovered recently. WNK1 exerts an inhibitory effect on basal and starvation-induced autophagy by interacting with UV Radiation Resistance-Associated Gene (UVRAG), a component of the main autophagy complex PI3KC3. WNK1 interaction reduces the activity of PI3KC3 and alters the phosphorylation status of ULK1 leading to inhibition of autophagy. Knocking-down WNK1 by siRNA in different cell lines leads to increased activation of autophagy (Gallolu Kankanamalage et al., 2016). WNK1 appears to play a regulatory role in autophagy by inhibiting the process, therefore truncating mutations in *WNK1* causing neuropathy might hinder its autophagy-related function and lead to toxic increase in autophagic flux that can have deleterious effects contributing to the neuropathology.

BSCL2 (Seipin):

The *BSCL2* gene encodes the protein seipin, a transmembrane protein that resides in the ER (Ito et al., 2008). The exact function of seipin remains elusive, though a role in lipid

homeostasis and adipogenesis has been suggested (Cartwright and Goodman, 2012). Null mutations in *BSCL2* are responsible of lipodystrophy, dominant mutations on the other hand cause distal hereditary motor neuropathy (dHMN-V) and Silver syndrome. dHMN-V patients present with uncharacteristic prominent hand muscle wasting and weakness early in the course of the disease, and mild to severe plasticity of the lower limbs (Windpassinger et al., 2004). Neuropathy-causing *BSCL2* mutations have been shown to affect the glycosylation sites of BSCL2 and lead to the accumulation of the unfolded protein in the ER (Windpassinger et al., 2004). It has been confirmed that different mutations in *BSCL2* can lead to the formation of perinuclear aggregates (Hsiao et al., 2016). In a neuropathic mouse model expressing mutant seipin exclusively in neurons (seipinopathy), seipin presents in intracellular aggregates. In addition, motor neurons show accumulation of autophagy marker LC3II together with a fragmented Golgi apparatus phenotype (Guo et al., 2013). The accumulation of autophagosomes was also confirmed by electron microscopy indicating that overstimulation of autophagy by mutant seipin leads to signs of degeneration (fragmented Golgi). The increased activation of autophagy was also confirmed in cell models expressing mutant seipin (Fan et al., 2015). Together these studies show that disrupted autophagy is a hallmark of seipin-related neuropathy.

LRSAM1 (leucine rich repeat and sterile alpha motif containing 1):

LRSAM1 is a leucine-rich repeat protein and an E3 ubiquitin ligase. LRSAM1 is involved in cell adhesion and cargo sorting during receptor endocytosis (Amit et al., 2004). Dominant negative mutations in *LRSAM1* cause CMT2P neuropathy (Weterman et al., 2012). CMT2P patients display features of the axonal CMT2 including mild lower-limb sensorimotor neuropathy, foot deformities, and hammer toes (Weterman et al., 2012). Early studies reported a function for LRSAM1 in endocytosis and retrovirus budding (Amit et al., 2004). Later it was shown to recognize, ubiquitinate and guide several bacteria strains to autophagy (bacteriophagy) (Huett et al., 2012). LRSAM1 is also a potential interactor of LC3 family protein GAPARAPL2 (GABA type A receptor-associated protein) (Ng et al., 2011), and is also involved in the autophagy pathway via interaction with PHD finger protein 23 (PHF23), a negative regulator of autophagy (Wang et al., 2014). A role for LRSAM1 in the general autophagic pathway is evident by the fact that LRSAM1 overexpression increases autophagic flux by increasing the formation of LC3-GFP puncta in cultured cell lines, while silencing it causes a decrease in autophagic flux (Wang et al., 2014). LRSAM1 appears to play a regulatory

role in the activation of autophagy probably through the interaction with other signaling proteins. Though the exact connection between LRSAM1, interactors and general autophagy remains unclear, loss of function mutations may disrupt the role of LRSAM1 in autophagy.

TRPV4 (transient receptor potential cation channel subfamily V member 4):

TRPV4 is a member of the TRP cation channels, and has an important role in sensing temperature, osmotic pressure and mechanic stimuli (Köttgen et al., 2008). Mutations in *TRPV4* cause CMT2C, a hereditary motor and sensory neuropathy with diaphragm and vocal cord paresis (Dyck et al., 1994). Alterations in TRPV4 can have toxic consequences in neuronal cells due to changes in calcium concentrations (Deng et al., 2010). TRPV4 can induce autophagy through the AKT-pathway and potentially via regulation of calcium levels and osmotic pressure (Zhan et al., 2015). Furthermore, inhibition of TRPV4 by siRNA leads to inhibition of autophagy (Zhan et al., 2015). Therefore, dysregulation of autophagy is a potential pathomechanism in TRPV4 associated neuropathies, as a direct effect of mutant TRPV4 or as one of the neurotoxic consequences due to the disruption in calcium concentrations by TRPV4 mutants.

NDRG1 (N-myc downstream regulated 1):

NDRG1 is mainly involved in cyto-protective stress response through regulation of p53 protein (Chen et al., 2010). In addition, it plays a role in immunity, development, differentiation and an important role in cancer (Fang et al., 2014). Mutations in *NDRG1* cause CMT4D neuropathy characterized with severe reduction in nerve conduction velocities, skeletal and foot deformities and sensory loss (Kalaydjieva et al., 2000). NDRG1 inhibits basal and ER stress-induced autophagy via suppression of protein kinase-like endoplasmic reticulum kinase (PERK)/eIF2α axis (Sahni et al., 2014). Upregulation of NDRG1 has also been shown to initiate BNIP3 and Beclin-mediated autophagy (Han et al., 2014). Autophagy regulation seems to be a part of the cyto-protective function of NDRG1. It may therefore play a role in fine-tuning autophagy levels by acting on different autophagy inducing or inhibiting pathways. Though a link between CMT4D-causing *NDRG1* mutations and autophagy is yet to be established, mutations in *NDRG1* might influence autophagy regulation by NDRG1.

DNAJB2 (DnaJ heat shock protein family (Hsp40) member B2, HSJ1):

HSJ1, a co-chaperone protein, is involved in binding ubiquitylated chaperone clients and their loading onto the Hsc70 chaperone, thus preventing aggregation and aiding proteasome sorting

(Westhoff et al., 2005). HSJ1 consists of two isoforms with distinct intracellular localization. HSJ1a is cytoplasmic and nuclear, while HSJ1b localizes to the ER (Westhoff et al., 2005). In addition to its function in the proteasome system, HSJ1 has been shown to be involved in the recruitment of the autophagy marker protein LC3 to damaged mitochondria aiding in mitochondrial autophagy (mitophagy) (Rose et al., 2011) Recessive mutations in *HSJ1* affecting both isoforms can cause CMT2T neuropathy or purely motor AR-dHMN (Gess et al., 2014). HSJ1a, the cytoplasmic isoform has been shown to enhance autophagy and decrease aggregation (Novoselov et al., 2013; Sanchez et al., 2016). Furthermore, deletion mutations in HSJ1b isoform, have been shown to lead to neurodegeneration and irregular increase in the autophagy marker LC3 due to increase in HSJ1a expression (Sanchez et al., 2016). These studies present HSJ1 as a moderator of autophagy and proteasome activity and show that mutations in *HSJ1* could disrupt this balance, leading to impaired clearance of ubiquitylated proteins and autophagy.

NEFL (neurofilament light):

NEFL is part of the axoskeleton (neurofilaments) of large myelinated axons of the central and peripheral nerves. The main role of neurofilaments is maintaining fiber caliber and subsequently the conduction velocities of myelinated axons (Perrot et al., 2008). Mutations in *NEFL* can cause CMT2E/1F neuropathy with distal weakness and wasting of the lower limbs, and occasional cerebellar dysfunction, tremor, and hearing loss (Mersiyanova et al., 2000; Pareyson et al., 2014). CMT causing NEFL mutations have been shown to cause aggregate formation in cell culture and *in vitro* models. Activation of protein kinase A (PKA), alleviates the aggregation phenotype (Sasaki et al., 2006). PKA is a known regulator of autophagy mostly exerting an inhibitory effect through phosphorylation of ATG13 an essential protein in the Ulk1 autophagy-initiation complex (Stephan et al., 2009), and of LC3 (Cherra et al., 2010). Therefore, the aggregation phenotype of NEFL might be a by-product of abnormality in the autophagy pathway which is restored by the activation of PKA. Furthermore, NEFL has been shown to interact with the PI3-phosphatase MTMR2 (Previtali et al., 2003). This interaction might be essential for the potential role of MTMR2 in autophagy through its regulation of endosomal vesicle trafficking or through its interaction with MTMR13, a direct regulator of autophagosome-lysosome fusion (See section 4.4).

4.2. IPN genes and autophagosome formation and expansion:

TrkA (NTRK1) (neurotrophic receptor tyrosine kinase 1):

TrkA, a nerve growth factor (NGF) receptor, is essential for neuronal survival and regulation of neuronal death (Miller and Kaplan, 2001). TrkA has been associated with regulating the interplay between autophagy and apoptosis mainly in cancer cells (Dadakhujaev et al., 2009; Hansen et al., 2007). Overexpression of TrkA leads to an increase in the amount of ATG5-ATG12 conjugates and ultimately to an increase in autophagy marker LC3II (Dadakhujaev et al., 2009). Mutations in *TrkA* cause HSAN-IV neuropathy with congenital insensitivity to pain (CIPA) marked by absence of reaction to noxious stimuli and self-mutilating behavior (Indo et al., 1996). Recently, mutant TrkA has been shown to cause accumulation of autophagosomes. This accumulation proved to be a result of aberrant activation of autophagy and oversaturation of the lysosomes rather than a deficit in autophagosome-lysosome fusion. Neurons expressing TrkA mutant show increased cell toxicity and dystrophic neurites as a result of autophagosomes accumulation by the abnormal autophagy activation (Franco et al., 2016).

DNM2 (Dynamin2):

DNM2 is one of the three isoforms of dynamin. DNM2 is a mechano-GTPase involved in endocytosis, Golgi function and vesicle trafficking (González-Jamett et al., 2013). DNM2 has been shown to play essential roles in autophagy. One of its main roles has been the autophagic lysosomal reformation, supplying nascent protolysosomes and hence maintaining the autophagic flux (Klionsky et al., 2016; Schulze and McNiven, 2014). Recently, DNM2 has been shown to interact with another protein Bif-1 to mediate the generation of Atg9-containing vesicles delivering Atg9 to autophagosome formation sites and promoting the formation of autophagosomes (Takahashi et al., 2016). Dominant mutations in *DNM2* have been linked with the intermediate form of CMT (DI-CMTB) with nerve conduction velocities ranging from normal to 25 m/s (Züchner et al., 2005). A mutant DNM2 mouse model shows signs of neuropathy and a decreased autophagic flux indicated by lower levels of LC3II expression and P62-positive bodies (a selective autophagy marker also known as sequestosome1 (SQSTM1)) (Durieux et al., 2012). These studies suggest that mutations in *DNM2* leading to neuropathy affect its function in the formation of autophagosomes and present autophagy impairment as a pathomechanism of DNM2-linked neuropathy.

KIF1A (kinesin family member 1A):

KIF1A is a kinesin motor protein essential for the transport of vesicles in neuronal axons in anterograde fashion (Lo et al., 2011). Mutations in *KIF1A* can cause hereditary sensory and autonomic neuropathy type 2 (HSAN-II) (Rivire et al., 2011). HSAN-II patients suffer from frequent occurrence of unrecognized injuries and fractures of hands and feet due to loss in all peripheral sensations including pain and temperature. Autonomic symptoms include severe feeding problems in infants and common gastroesophageal reflux. Recently, it was shown that KIF1A controls the localization of ATG-9 and regulates the spatial distribution of autophagosomes in developing neurons of *C. Elegans* (Stavoe et al., 2016). Maintaining ATG9 trafficking is essential for proper autophagosome formation (Lamb et al., 2016). The effects on autophagy in mutant KIF1A patients or cell/mouse models remains to be studied, but it is not inconceivable that impairment of autophagy due to disruption in ATG9 trafficking in KIF1A mutants is one of the pathomechanisms of neuropathy due to *KIF1A* mutations.

LITAF (lipopolysaccharide-induced TNF factor):

LITAF, has been mainly linked with inflammatory functions, namely the secretion of cytokines such as TNF upon lipopolysaccharide stimulation (Xiaoren Tang, Daniel Metzger, Susan Leeman, 2007). It has also been implicated in tumor suppression pathways and p53-induced apoptotic pathway (Liu et al., 2012; Zhou et al., 2011). LITAF seems to have different functions in different cell types. A role for LITAF in the positive regulation of autophagy has been shown in B cells. LITAF regulates LC3 expression and colocalizes with autophagosomes in B cells. Furthermore, overexpression of LITAF stimulates autophagy while silencing LITAF decreases the autophagic flux in these cells (Bertolo et al., 2013). Mutations in *LITAF* cause the dominant demyelinating CMT1C neuropathy (V.A. Street et al., 2003). In HEK293 cell models expressing CMT1C associated LITAF mutant protein, the mutant proteins cause mislocalization of LITAF from early endosomes to the cytosol, destabilize LITAF and cause it to be aggregation prone. The aggregating mutant LITAF is then degraded by the proteasome and by autophagy (Lee et al., 2011). In neuroblastoma cell lines expressing wild-type (WT) and mutant forms of LITAF, CMT-causing LITAF mutants are mislocalized to the mitochondria, while the WT form traffics through the secretory pathway to the late endosome/lysosome (Lacerda et al., 2014). Late endosomes and lysosomes are essential for supplying the maturing autophagosome and for degrading the lysosomal content (Murrow et al., 2015). The trafficking function of LITAF might be related to its effect on autophagic flux,

mislocalisation of mutant LITAF can therefore have a negative effect on the integrity of the endo/lysosomes and consequently on the autophagic process.

SH3TC2 (SH3 domain and tetratricopeptide repeats 2):

SH3TC2 encodes the Src homology 3 domain and tetratricopeptide repeats 2 protein. Little is known about the function of SH3TC2. However, the several motifs it contains suggest protein-protein interaction functions. Recessive mutations in *SH3TC2* cause a form of demyelinating neuropathy, CMT4C, characterized with severe scoliosis (Senderek et al., 2003b). Wild-type SH3TC2 has been shown to localise to recycling endosomes in rat Schwann cells, while the CMT-causing mutant forms mistarget SH3TC2 away from the recycling endosomes (Roberts et al., 2010). SH3TC2 interacts with the small GTPase Rab11.The SH3TC2/Rab11 interaction is disrupted in mutant SH3TC2 in cultured cell lines (Roberts et al., 2010). Interestingly, knock-out of SH3TC2 in a transgenic mouse model decreases the expression of Rab11 in nerves of these mice (Stendel et al., 2010). Rab11 has been described as a positive regulator of autophagy. ULK1 and ATG9 localize in part to Rab11 positive recycling endosomes. Rab11-dependent vesicular transport from the recycling endosomes contributes to the forming autophagosomes and regulates starvation induced autophagy (Longatti et al., 2012). So by disrupting Rab11 interaction and expression levels and mistargeting SH3TC2 away from the recycling endosomes, *SH3TC2* CMT-associated mutations could disrupt the contribution of recycling endosomes to autophagosome formation.

TECPR2 (tectonin beta-propeller repeat containing 2):

TECPR2 was initially identified as human ATG8-interacting protein (Behrends et al., 2010). TECPR2 possesses an LC3-interacting motif (LIR) through which it binds to LC3B and LC3C family proteins. TECPR2 LIR-dependent binding leads to its association with cellular trafficking components such as HOPS and SEC24D. This interaction ultimately maintains functional ER exit sites (ERES) and efficient ER export. TECPR2 depletion causes a decrease in ERES number and a delayed ER export. Maintaining a functional ERES provides a scaffold for autophagosome formation (Stadel et al., 2015). Mutations in *TECPR2* leading to the translation of a truncated and unstable version of TECPR2 cause a form of hereditary spastic paraparesis (HSP) (Oz-Levi et al., 2012) and hereditary sensory and autonomic neuropathy (HSAN-III) (Heimer et al., 2016). The latter presents with intellectual disability, spasticity, and chronic respiratory disease. Skin fibroblasts from TECPR2-related HSP patients show a

decreased number of LC3 and P62 proteins marking a decreased autophagic flux (Oz-Levi et al., 2012). These fibroblasts also show delayed ER export (Stadel et al., 2015), an indication that the function of TECPR2 in maintaining ER export and the formation of early autophagosome intermediates is disrupted by HSP-causing mutations, presenting autophagy disruption as a likely pathomechanism in HSP neuropathy.

4.2. IPN genes and autophagosome transport:

DCTN1 (Dynactin subunit 1):

DCTN1 is the largest subunit of the dynactin complex. The dynactin complex has been associated with a large range of cellular functions including mitosis, ER-Golgi transport, lysosomes and endosomes movement as well as interacting with dynein for cargo transport (Schroer, 2004). Mutations in *DCTN1* disrupt axonal transport and lead to hereditary motor neuropathy (dHMN-VIIb) with breathing difficulty due to vocal fold paralysis and progressive facial weakness (Puls et al., 2003). DCTN1 knock-down shows motor neuron degeneration associated with autophagosomes accumulation due to impaired transport of autophagosomes along the axons (Ikenaka et al., 2013). Similarly, in a mouse model of mutant DCTN1, abnormal accumulation of autophagosomes has been shown in motor neurons and linked to the motor neuropathy phenotype (Wiesner et al., 2015). This presents autophagy impairment as a pathomechanism leading to motor neuropathy for *DCTN1* mutations.

DST (Dystonin):

The dystonin gene encodes several dystonin protein isoforms which are cytoskeletal cross-linking proteins that can interact with different organelles, microtubules and protein complexes (Ferrier et al., 2013). Mutations in *DST* cause hereditary sensory and autonomic neuropathy (HSAN-VI) also known as familial dystonomia and presenting with alacrima, depressed deep tendon reflexes, and lingual fungiform papillae (Edvardson et al., 2012). In a mouse model of dystonia, mutant DST disrupts the autophagic process as evident by the accumulation of LC3 and P62 proteins. This impairment is attributed to the failure of autophagosome-lysosome fusion suggesting that dystonin is required to transport the autophagosomes to the lysosomes for maturation into autolysosomes (Ferrier et al., 2015). Interestingly, expression of the non-mutated form of dystonin restores the autophagy deficits revealing that the autophagy deficit is a direct effect of the mutated dystonin (Ferrier et al., 2015).

4.4. IPN genes and lysosomal fusion and degradation:

RAB7 (member RAS oncogene family):

RAB7 is a small GTPase functioning in vesicular trafficking, more specifically in the transport from early to late endosomes and from late endosomes to lysosomes (Stenmark, 2009). RAB7 is recruited to autophagosomes and is required for the fusion of autophagosomes and lysosomes (Jäger et al., 2004). Mutations in *RAB7* cause CMT2B neuropathy characterized by distal muscle wasting and weakness, and frequent foot ulcers and infections (Verhoeven et al., 2003). Dominant negative mutations retard the recruitment of RAB7 to autophagosomes, and prevent the progression of autophagy by impairing autolysosome formation (Gutierrez et al., 2004).

CCT5 (chaperonin containing TCP-1 subunit 5):

A subunit of the TCP-1 containing chaperonin complex (CCT), an ATP-dependent chaperone responsible for folding unfolded proteins including actin and tubulin. Recessive mutations in *CCT5* cause a mutilating hereditary sensory and autonomic neuropathy with spastic paraplegia (HSNSP) (Bouhouche et al., 2006). Studies have shown that CCT can prevent mutant huntingtin (htt) aggregation *in vitro* (Darrow et al., 2015). Recently, it was shown that the function of CCT in preventing aggregation of mutant proteins is mediated by autophagy (Pavel et al., 2016). CCT is required for lysosomal biogenesis and functioning, and for autophagosome-lysosome fusion, possibly via interaction with cytoskeleton proteins. Disruption of CCT integrity by knock-down or by mutations in *CCT5* disrupts autolysosome formation and cargo degradation (Pavel et al., 2016).

FIG4 (FIG4 phosphoinositide 5-phosphatase):

FIG4 is a phospholipid phosphatase responsible for the generation and turnover of the PtdIns(3,5)P2 phosphoinositide (Di Paolo and De Camilli, 2006). Mutations in *FIG4* cause Yunis-Varon syndrome, familial epilepsy with polymicrogyria, and the severely demyelinating Charcot-Marie-Tooth type 4J neuropathy (CMT4J) (Chow et al., 2007; Katona et al., 2011; Vaccari et al., 2015). In a FIG4 deficient neuropathic mouse model, sensory and motor neurons as well as Schwann cells seem to be affected (Katona et al., 2011; Vaccari et al., 2015). Neurons show decrease of PtdIns(3,5)P2 levels, a sign of impaired FIG4 enzymatic activity, and enlargement of late endosomes and lysosomes. Enlargement of late endosomes and lysosomes

can hinder the fusion capacity of autophagosomes with lysosomes. Interestingly, these mice show accumulation of autophagic markers LC3II and P62 in their sciatic nerve. This accumulation is independent of an increase in the autophagic flux (Ferguson et al., 2010; Vaccari et al., 2015). Thus abolishing the enzymatic activity of FIG4 by missense mutations or haploinsufficiency seems to affect the progression of autophagic degradation by preventing the fusion between autophagosomes and the then enlarged late endosomes/lysosomes (Vaccari et al., 2015).

MTMRs (myotubularin-related proteins):

MTMRs are PI3-phosphatases consisting of catalytically active or inactive members. Mutations in *MTMR2* and *MTMR13* (also called SET-binding factor 2) cause the demyelinating CMT4B1 and CMT4B2 neuropathies respectively with early-onset glaucoma (Azzedine et al., 2003). MTMR2 is a catalytically active phosphatase which dephosphorylates phosphatidylinositol 3-phosphate (PI(3)P) and bisphosphate PI(3,5)P2. MTMR13 on the other hand is an inactive phosphatase but it has been shown to associate with MTMR2 (Robinson and Dixon, 2005). MTMR2 has been linked with regulation of the late endocytic pathway and vesicular transport through its putative substrate PI(3,5)P2 (Bolino et al., 2004; Lenk and Meisler, 2014). MTMR13 on the other hand acts as a RAB21 guanine exchange factor (GEF) required for fusion of autophagosome with the lysosome. MTMR13 GEF activity is induced upon starvation and it promotes the trafficking of VAMP8 to the lysosome where it is needed to mediate fusion with the autophagosome (Jean et al., 2015). Autophagy has not yet been studied in the context of CMT4B-causing mutations in *MTMRs* but the roles of both MTMR2 and MTMR13 in autophagy and their functional association with each other presents autophagy impairment as a possible common pathomechanism in mutant MTMRs leading to neuropathy.

VCP (valosin containing protein):

VCP is an AAA+ ATPase associated with diverse cellular activities including the ATP-dependent remodeling of proteins to unfold them or extract them from cell structures or binding partners (Erzberger and Berger, 2006). Mutations in *VCP* cause a multisystem degenerative disease consisting of inclusion body myopathy, Paget's diseases and frontotemporal dementia (IBMPFD) (Kimonis et al., 2000), familial ALS (Shaw, 2010) and can also lead to CMT2Y neuropathy with distal muscle weakness and atrophy and length-dependent sensory loss (Gonzalez et al., 2014). VCP also plays a role in lysosomal homeostasis and the lysosomal damage response. Lysosomal damage induced by lysomotropic reagents triggers a concomitant

recruitment of VCP to lysosomes. VCP then cooperates with a set of cofactors to drive the degradation of ruptured lysosomes (Papadopoulos et al., 2016). Damaged lysosomes accumulate in cells expressing mutant VCP showing that its role in clearing out damaged lysosomes is compromised by neuropathy-causing mutations. VCP mutant knock-in mouse model shows accumulation of LC3II positive structures in myoblasts (Nalbandian et al., 2013). Furthermore, knock-down of VCP leads to accumulation of autophagosomes due their failure to mature into autolysosomes (Ju et al., 2009). A high percentage of the accumulated autophagic vesicles contain ubiquitin-positive structures. VCP has been shown to be critical for the maturation of ubiquitin-containing autophagosomes into autolysosomes under basal conditions and under proteasome inhibition. This function of VCP in maturation of autophagosomes seems to be impaired by neuropathy-causing mutations (Lee et al., 2010).

HSPB8 (heat shock protein B8):

HSPB8 belongs to the family of small heat shock proteins, ATP-independent chaperones that aid the folding of misfolded proteins by ATP-dependent chaperones (Holmgren, 2012). In addition to its function as a molecular chaperone, HSPB8 is involved in several other stress signaling functions such as removal of aggregates through chaperone assisted selective autophagy (CASA) (Carra et al., 2008a). CASA involves the recognition of substrate proteins by a complex of chaperones and co-chaperones including HSPB8 and P62 and its subsequent loading onto autophagosomes. Mutations in *HSPB8* cause CMT2L and dHMN-IIa neuropathies typically presenting with paresis of the extensor muscles of the big toe and then the feet (Irobi et al., 2004; Tang et al., 2005). Expression of mutant HSPB8 in cultured cells induces aggregate formation (Irobi et al., 2004). Transcriptional induction of wild-type HSPB8 in mouse models of motor neuron disease aids in the clearance of misfolded proteins and aggregates (Crippa et al., 2016). Interestingly, cells from neuropathic patients carrying HSPB8 mutations also show deficits in lysosomal delivery of autophagosomes (Kwok et al., 2011). Taken together, these studies indicate that intact HSPB8 is necessary for aggregate clearance, possibly through CASA. Disruption of this role by mutations in *HSPB8* contribute to the neuronal pathogenicity.

4.5. IPN genes involved in selective autophagy: Mitophagy, Reticulophagy (ER-phagy)

FAM134B (family with sequence similarity 134 member B):

FAM134B is a transmembrane protein localized to the cis-golgi and predominantly expressed in the sensory and autonomic ganglia. Mutations in *FAM134B* cause hereditary sensory and autonomic neuropathy HSAN-IIB leading to impaired nociception, autonomic dysfunction and severe mutilations (Kurth et al., 2009). FAM134B interacts with LC3/GABARAP through an LC3-interacting motif at its C-terminal domain. This interaction is essential for selectively directing part of the ER to autophagosomes (reticulophagy). Mutations causing sensory neuropathy disrupt the LC3-interacting motif and consequently the FAM134B-LC3 binding. FAM134B knock-out mice develop sensory neuropathy and degeneration of sensory neuronal axons. Deficit in reticulophagy in these mice but not general autophagy disrupts ER homeostasis leading to ER expansion and inhibition of ER turnover that ultimately leads to degeneration of sensory neurons (Khaminets et al., 2015).

MFN2 (mitofusin 2):

MFN2 was identified as a transmembrane mitochondrial GTPase required for mitochondrial fusion (Santel and Fuller, 2001). Mutations in *MFN2* cause CMT2A neuropathy (Züchner et al., 2004) and are one of the most frequent CMT2-causing mutations (OMIM 609260, CMT2A ≈ 35% of CMT2). CMT2A presents as a severe predominantly motor neuropathy or motor accompanied with profound proprioception loss. In addition to its role in mitochondrial fusion, MFN2 plays multiple roles including regulation of cell survival, cell proliferation, ER stress and autophagy (reviewed in (Schrepfer and Scorrano, 2016)). *MFN2* deletion has been shown to impair starvation-induced autophagy by disrupting the MAM (mitochondria-associated membranes), a described site for autophagosome formation (Hailey et al., 2010; Hamasaki et al., 2013). *MFN2* deletion in HeLa cells has also been shown to affect cell proliferation due to autophagy impairment (Ding et al., 2015). Motor neurons derived from induced pluripotent stem cells (iPSCs) obtained from CMT2A patients show increase in mitophagy and mitochondrial depletion and increased expression of *PINK1*, *PARK2* and BNIP3, known triggers for autophagic degradation of mitochondria (Rizzo et al., 2016). In a MFN2 KO mouse model, sarcopenia is seen in correlation with impaired autophagy in the muscle, accumulation of damaged mitochondria, and activation of an adaptive mitophagy pathway (Sebastián et al., 2016). Taken together, MFN2 seems to play an essential role in autophagosome formation, possibly through maintenance of mitochondrial membranes and MAMs. This role is impaired

in MFN2 deficiency and in CMT2A-causing mutations, leading to autophagy impairment and disruption of balance between mitophagy and general autophagy.

GDAP1 (ganglioside induced differentiation associated protein 1):

GDAP1 is an integral protein of the outer mitochondrial membrane formed of 2 glutathione-S-transferase domains (Huber et al., 2016). Dominant and recessive mutations in *GDAP1* cause demyelinating (CMT4A), or axonal (AR-CMT2 or CMT2K) neuropathies (Baxter et al., 2002; Senderek et al., 2003a; Sivera et al., 2010). GDAP1 is required for the regulation of the mitochondrial network and mitochondrial integrity (Niemann et al., 2005). In a knock-out mouse model of GDAP1 that recapitulates the neuropathic phenotype of CMT4A, disruption of the mitochondrial network and mitochondrial calcium homeostasis is evident in neurons and associates with accumulation of autophagic vesicles (Barneo-Muñoz et al., 2015). Mitochondrial defects can lead to abnormal increase in targeting mitochondria to autophagic degradation (mitophagy). In addition, both mitochondrial interconnectivity and calcium homeostasis are essential for the proper functioning of autophagy (Gomez-Suaga et al., 2017). It remains unclear whether the increased accumulation of autophagosomes in neurons of CMT4A mouse models is a by-product of increased mitophagy or defects in the general autophagic flux. Nonetheless, autophagic dysregulation does seem to be a feature of the pathology caused by CMT-causing *GDAP1* mutations and might explain the variety of clinical phenotypes presented by these mutations ranging from myelinating to axonal CMT and from dominant to recessive.

Fig2. Effects of the different IPN-associated genes on the autophagy pathway

Inherited peripheral neuropathy associated genes disrupt autophagy at various levels. Several affected proteins including peripheral myelin protein 22, involved in more than 50% of IPNs, disrupt the initiation of autophagy by inhibiting the initiation complex (PMP22, TRPV4, LRSAM1) or overstimulating it (VAPB, WNK1, Seipin, DNAJB2). Mutations affecting mitochondrial proteins can inhibit autophagy by causing an abnormal increase in mitophagy and disrupting the autophagy/mitophagy balance (MFN2, GDAP1) (left inset). Mutant ER-resident FAM134B affects its role in reticulophagy (right inset). The cyto-protective NDRG1 inhibits the initiation complex or stimulates the nucleation complex depending on the physiological triggers. At the nucleation step, mutant TECPR2 disrupts the formation of early autophagic membranes from the ER. The elongation of the phagophore into an autophagosome is disrupted by mutations that affect the supply for forming membranes from late endosomes (LITAF and SH3TC2), and from ATG9-containing vesicles (KIF1A, DNM2). This step can also be overstimulated by mutations in the tyrosine kinase (TrkA) which lead to toxic increase in ATG12-ATG5 conjugates. The transport of autophagosomes to lysosomes is disrupted by mutations affecting cytoskeleton associated proteins such as DCTN1 and DST. Mutations involving RAB7, VCP, HSPB8, and the phosphatases FIG4, MTMR2, MTMR13 block the lysosomal fusion step in autophagy. Mutant chaperone protein CCT5 on the other hand inhibits the degradation step. In addition, several mutant proteins lead to the formation of aggregates which basal autophagy on its own might not cope with (PMP22, Seipin, NEFL, LITAF, HSPB8).

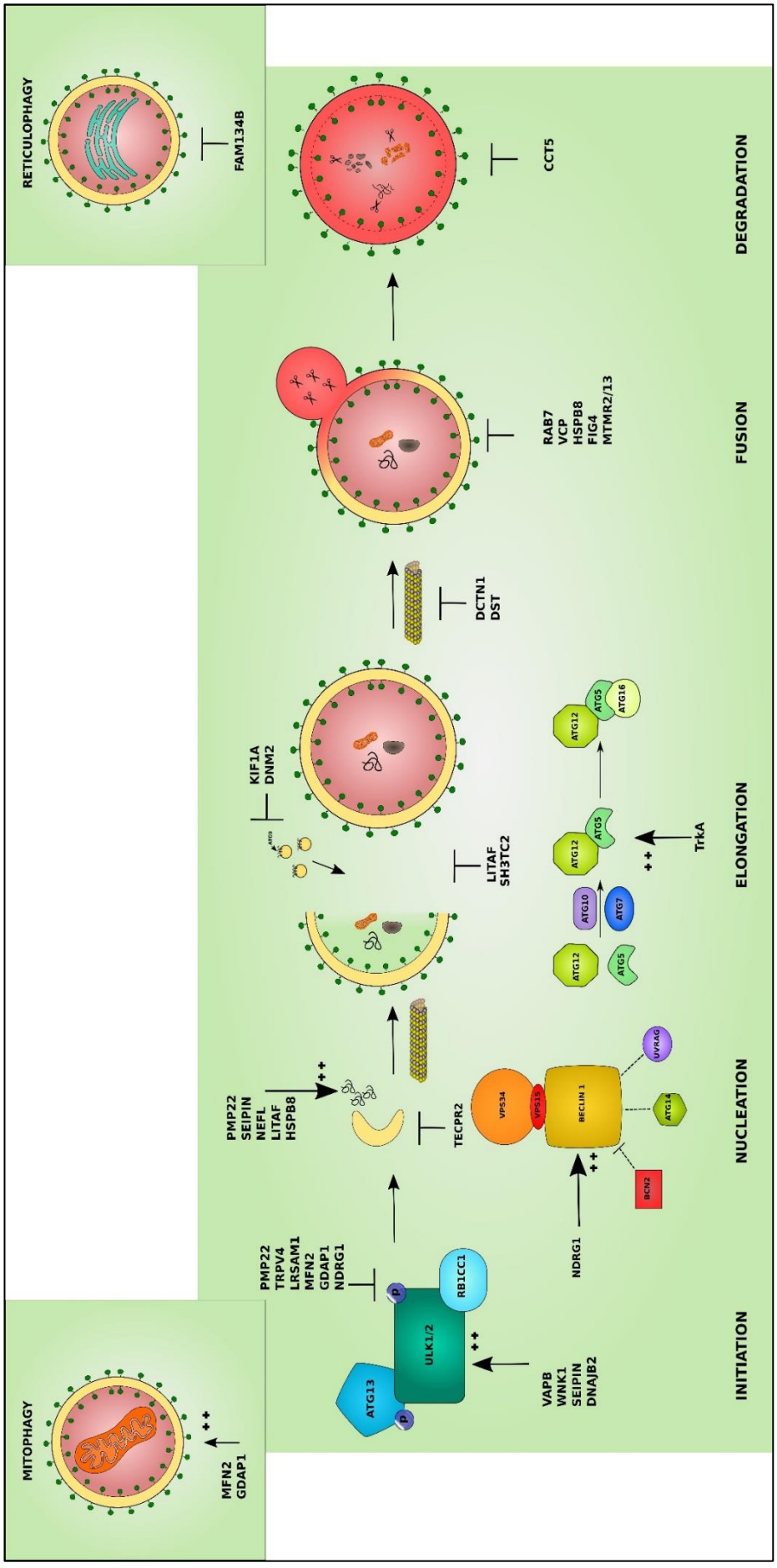

Fig2. Effects of the different IPN-associated genes on the autophagy pathway

5. Autophagy modulation as a therapeutic target in hereditary neuropathies:

So far, finding a treatment for hereditary neuropathies remains a challenge. An important step towards a successful therapy, from both an economic and a pharmaceutical perspective, is finding a common pathomechanism that can be targeted in different neuropathic genotypes. By bridging different neuropathic phenotypes and different causal genes, autophagy impairment emerges as a good candidate for modulation by therapeutic measures. Autophagy modulation as a therapy approach has been used in several more common neurodegenerative disease models. Inducing autophagy has been shown to reduce the severity of the Huntington's disease phenotype and improve clearance of Htt aggregates in mouse and neuronal cell models (Rose et al., 2010). In Alzheimer's disease, accumulation of autophagosomes contributes to the pathology (Ohta et al., 2010). Inhibition of autophagy has been reported as a mode of action in drugs that decrease amyloid-β accumulation in Alzheimer's patients (Lipinski et al., 2010). Accumulation of autophagosomes has also been attributed to defects in beclin-1 expression, hence autophagy-enhancing treatments such as resveratrol and lithium can improve the pathology of Alzheimer's disease (Rahvar et al., 2011; Vingtdeux et al., 2010). In addition, expression of beclin-1 in neuronal cells of transgenic mice improves their Parkinson's disease phenotype by enhancing lysosomal activation (Spencer et al., 2009). Similarly, the mTOR-independent autophagy inducer trehalose improves the clearance of α-synuclein, huntingtin (Sarkar et al., 2007) and SOD1 aggregates (Castillo et al., 2013a). Furthermore, autophagy-modulating compounds are already in use to treat different neurodegenerative diseases and cancers, and are currently at different phases of clinical trials (Reviewed in (Towers and Thorburn, 2016)). Despite its promise, autophagy modulation as a treatment for hereditary neuropathies still presents many challenges. The model of treating mutants that increase autophagic activation with inhibitors of autophagy and those that inhibit autophagy with activators is oversimplified. Many autophagy-modulating drugs can have off-target effects. For example, mTORC1 inhibitors can affect cell metabolism and lipid synthesis in an autophagy-independent manner (Li et al., 2014). Therefore, targeting the specific autophagy step affected by a hereditary neuropathy-causing mutation is a better and more elegant approach to enhance the autophagic status without hindering other vital cellular processes. Certain step-specific approaches already exist and include direct stimulation of autophagy by targeting beclin1 with the alkaloid isorhynchophylline (Lu et al., 2012). Targeting the lysosomal fusion and degradation is also possible using pharmacological activators of lysosomal enzymes such ambroxol (McNeill et al., 2014) or acidic nanoparticles that increase the acidity of the

lysosomes (Baltazar et al., 2012). Non-pharmacological enhancement of autophagy such as caloric restriction or exercise presents another way of overcoming the pharmacological modulation of other cellular pathways. Physical exercise has already been described as a rehabilitating measure in CMT patients (Roberts-Clarke et al., 2016; Vita et al., 2016). Whether improvement of autophagy is a mediator of the positive impact of exercise remains to be investigated, but exercise has been shown to enhance autophagy in the brain (He et al., 2012) and skeletal muscles (Ferraro et al., 2014). Another caveat of autophagy-directed therapy is the difficulty of tracing autophagy *in vivo*. In other words, there is a need for biomarkers that can be used to make sure that the intended therapy is improving the autophagic status in patients in order to alleviate their symptoms. Such markers are as of yet unavailable, but recent advances using mouse models allow following up a treatment's effect on autophagy in mouse tissues (Castillo et al., 2013b). This strategy, combined to behavioral and electrophysiological testing in a neuropathic mouse model can permit the direct correlation between a drug's effect on autophagy and its ultimate modulation of the neuropathic status laying out a strong case for such a drug to enter clinical trials.

6. Conclusions:

The genetic heterogeneity of IPNs and the fact that they are rare disorders, highlights the need for finding common cellular and molecular pathomechanisms among the different disease-linked genes. This review complements recent work in attempting to find shared molecular mechanisms underlying IPNs (Bucci et al., 2012; Prior et al., 2017). The relevance of autophagy as a target pathway stems from the fact that it has been implicated in other disease conditions, mainly neurodegeneration, where autophagy-targeting drugs have already entered clinical trials. In addition, autophagic impairment presents a unique overlap among neuropathies affecting both sensory and motor nerves on one hand, and the myelin sheath and the neuronal axon on the other. While more research is required to get a clearer view of the involvement of autophagy in neuropathic mechanisms, current evidence points out to the emergence of autophagy as a frequently affected pathway in inherited peripheral neuropathy conditions.

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REFERENCES

Ackerley, S., James, P. A., Kalli, A., French, S., Davies, K. E., and Talbot, K. (2006). A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. *Hum. Mol. Genet.* 15, 347–354. doi:10.1093/hmg/ddi452.

Adriaenssens, E., Geuens, T., Baets, J., Echaniz-Laguna, A., and Timmerman, V. (2017). Novel insights in the disease biology of mutant small heat shock proteins in neuromuscular diseases. *Brain* 140, 2541–2549. doi:10.1093/brain/awx187.

Almeida-Souza, L., Asselbergh, B., d'Ydewalle, C., Moonens, K., Goethals, S., de Winter, V., et al. (2011). Small Heat-Shock Protein HSPB1 Mutants Stabilize Microtubules in Charcot-Marie-Tooth Neuropathy. *J. Neurosci.* 31, 15320–15328. doi:10.1523/JNEUROSCI.3266-11.2011.

Almeida-Souza, L., Goethals, S., De Winter, V., Dierick, I., Gallardo, R., Van Durme, J., et al. (2010). Increased monomerization of mutant HSPB1 leads to protein hyperactivity in Charcot-Marie-Tooth neuropathy. *J. Biol. Chem.* 285, 12778–12786. doi:10.1074/jbc.M109.082644.

Amit, I., Yakir, L., Katz, M., Zwang, Y., Marmor, M. D., Citri, A., et al. (2004). Tal, a Tsg101-specific E3 ubiquitin ligase, regulates receptor endocytosis and retrovirus budding. *Genes Dev.* 18, 1737–1752. doi:10.1101/gad.294904.

An, J. J., Lee, Y. P., Kim, D. W., Sohn, E. J., Jeong, H. J., Kang, H. W., et al. (2009). Transduced HSP27 protein protects neuronal cell death by enhancing FALS-associated SOD1 mutant activity. *BMB Rep.* 42, 136–141. doi:10.5483/BMBRep.2009.42.3.136.

Ariosa, A. R., and Klionsky, D. J. (2016). Autophagy core machinery: overcoming spatial barriers in neurons. *J. Mol. Med.*, 1–11. doi:10.1007/s00109-016-1461-9.

Arrigo, A. P. (2017). Mammalian HspB1 (Hsp27) is a molecular sensor linked to the physiology and environment of the cell. *Cell Stress Chaperones* 22, 517–529. doi:10.1007/s12192-017-0765-1.

Arrigo, A. P., and Gibert, B. (2014). *HspB1, HspB5 and HspB4 in human cancers: Potent oncogenic role of some of their client proteins*. doi:10.3390/cancers6010333.

Axe, E. L., Walker, S. a., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., et al. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J. Cell Biol.* 182, 685–701. doi:10.1083/jcb.200803137.

Azzedine, H., Bolino, a, Taïeb, T., Birouk, N., Di Duca, M., Bouhouche, a, et al. (2003). Mutations in MTMR13, a new pseudophosphatase homologue of MTMR2 and Sbf1, in two families with an autosomal recessive demyelinating form of Charcot-Marie-Tooth disease associated with early-onset glaucoma. *Am. J. Hum. Genet.* 72, 1141–53. doi:10.1086/375034.

Baets, J., Jonghe, P. De, and Timmerman, V. (2014). Recent advances in Charcot – Marie – Tooth disease. doi:10.1097/WCO.0000000000000131.

Bailey, C. K., Andriola, I. F. M., Kampinga, H. H., and Merry, D. E. (2002). Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* 11, 515–523. doi:10.1093/hmg/11.5.515.

Baltazar, G. C., Guha, S., Lu, W., Lim, J., Boesze-Battaglia, K., Laties, A. M., et al. (2012). Acidic Nanoparticles Are Trafficked to Lysosomes and Restore an Acidic Lysosomal pH and Degradative Function to Compromised ARPE-19 Cells. *PLoS One* 7, 1–10. doi:10.1371/journal.pone.0049635.

Barneo-Muñoz, M., Juárez, P., Civera-Tregón, A., Yndriago, L., Pla-Martín, D., Zenker, J., et al. (2015). Lack of GDAP1 Induces Neuronal Calcium and Mitochondrial Defects in a Knockout Mouse Model of Charcot-Marie-Tooth Neuropathy. *PLOS Genet.* 11, e1005115. doi:10.1371/journal.pgen.1005115.

Basha, E., Lee, G. J., Breci, L. A., Hausrath, A. C., Buan, N. R., Giese, K. C., et al. (2004). The Identity of Proteins Associated with a Small Heat Shock Protein during Heat Stress *in Vivo* Indicates That These Chaperones Protect a Wide Range of Cellular Functions. *J. Biol. Chem.* 279, 7566–7575. doi:10.1074/jbc.M310684200.

Basha, E., O'Neill, H., and Vierling, E. (2012). Small heat shock proteins and alpha-crystallins: Dynamic proteins with flexible functions. *Trends Biochem. Sci.* 37, 106–117. doi:10.1016/j.tibs.2011.11.005.

Baxter, R. V, Ben Othmane, K., Rochelle, J. M., Stajich, J. E., Hulette, C., Dew-Knight, S., et al. (2002). Ganglioside-induced differentiation-associated protein-1 is mutant in Charcot-Marie-Tooth disease type 4A/8q21. *Nat. Genet.* 30, 21–22. doi:10.1038/ng796.

Behrends, C., Sowa, M. E., Gygi, S. P., and Harper, J. W. (2010). Network organization of the human autophagy system. *Nature* 466, 68–76. doi:10.1038/nature09204.

Bertolo, C., Roa, S., Sagardoy, A., Mena-Varas, M., Robles, E. F., Martinez-Ferrandis, J. I., et al. (2013). LITAF, a BCL6 target gene, regulates autophagy in mature B-cell lymphomas. *Br. J. Haematol.* 162, 621–630. doi:10.1111/bjh.12440.

Bilen, J., and Bonini, N. M. (2007). Genome-wide screen for modifiers of ataxin-3 neurodegeneration in Drosophila. *PLoS Genet.* 3, 1950–1964. doi:10.1371/journal.pgen.0030177.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., et al. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* 171, 603–614. doi:10.1083/jcb.200507002.

Boland, B., and Nixon, R. a. (2006). Neuronal macroautophagy: From development to degeneration. *Mol. Aspects Med.* 27, 503–519. doi:10.1016/j.mam.2006.08.009.

Bolino, A., Bolis, A., Previtali, S. C., Dina, G., Bussini, S., Dati, G., et al. (2004). Disruption of Mtmr2 CMT4B1-like neuropathy with myelin outfolding and impaired spermatogenesis. *J. Cell Biol.* 167, 711–721. doi:10.1083/jcb.200407010.

Bouhouche, A., Benomar, a, Bouslam, N., Chkili, T., and Yahyaoui, M. (2006). Mutation in the epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5) gene causes autosomal recessive mutilating sensory neuropathy with spastic paraplegia.

J. Med. Genet. 43, 441–443. doi:10.1136/jmg.2005.039230.

Bouhy, D., Juneja, M., Katona, I., Holmgren, A., Asselbergh, B., de Winter, V., et al. (2017). A knock-in/knock-out mouse model of HSPB8-associated distal hereditary motor neuropathy and myopathy reveals toxic gain-of-function of mutant Hspb8. *Acta Neuropathol.*, 1–18. doi:10.1007/s00401-017-1756-0.

Bucci, C., Bakke, O., and Progida, C. (2012). Charcot-Marie-Tooth disease and intracellular traffic. *Prog. Neurobiol.* 99, 191–225. doi:10.1016/j.pneurobio.2012.03.003.

Bucciantini, M. (2002). Inherent cytotoxicity of aggregates implies a common origin for protein misfolding diseases. *Nature* 416, 507–511. Available at: http://dx.doi.org/10.1038/416507a.

Capponi, S., Geuens, T., Geroldi, A., Origone, P., Verdiani, S., Cichero, E., et al. (2016). Molecular Chaperones in the Pathogenesis of Amyotrophic Lateral Sclerosis: The Role of HSPB1. *Hum. Mutat.* 37, 1202–1208. doi:10.1002/humu.23062.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., et al. (2010). Identification of the Drosophila ortholog of HSPB8: Implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* 285, 37811–37822. doi:10.1074/jbc.M110.127498.

Carra, S., Brunsting, J. F., Lambert, H., Landry, J., and Kampinga, H. H. (2009a). HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2?? phosphorylation. *J. Biol. Chem.* 284, 5523–5532. doi:10.1074/jbc.M807440200.

Carra, S., Brunsting, J. F., Lambert, H., Landry, J., and Kampinga, H. H. (2009b). HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2α phosphorylation. *J. Biol. Chem.* 284, 5523–5532. doi:10.1074/jbc.M807440200.

Carra, S., Rusmini, P., Crippa, V., Giorgetti, E., Boncoraglio, A., Cristofani, R., et al. (2013). Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368, 20110409. doi:10.1098/rstb.2011.0409.

Carra, S., Seguin, S. J., Lambert, H., and Landry, J. (2008a). HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J. Biol. Chem.* 283, 1437–1444. doi:10.1074/jbc.M706304200.

Carra, S., Seguin, S. J., and Landry, J. (2008b). HspB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 4, 237–239. doi:5407 [pii].

Cartwright, B. R., and Goodman, J. M. (2012). Seipin: from human disease to molecular mechanism. *J. Lipid Res.* 53, 1042–55. doi:10.1194/jlr.R023754.

Castillo, K., Nassif, M., Valenzuela, V., Rojas, F., Matus, S., Mercado, G., et al. (2013a). Trehalose delays the progression of amyotrophic lateral sclerosis by enhancing autophagy in motoneurons. *Autophagy* 9, 1308–1320. doi:10.4161/auto.25188.

Castillo, K., Valenzuela, V., Matus, S., Nassif, M., Oñate, M., Fuentealba, Y., et al. (2013b). Measurement of autophagy flux in the nervous system in vivo. *Cell Death Dis.* 4, e917. doi:10.1038/cddis.2013.421.

Chen, B., Longtine, M. S., Sadovsky, Y., and Nelson, D. M. (2010). Hypoxia downregulates p53 but induces apoptosis and enhances expression of BAD in cultures of human syncytiotrophoblasts. *Am. J. Physiol. Cell Physiol.* 299, C968–C976. doi:10.1152/ajpcell.00154.2010.

Chen, Y., and Klionsky, D. J. (2011). The regulation of autophagy - unanswered questions. *J. Cell Sci.* 124, 161–170. doi:10.1242/jcs.064576.

Cheng, G., Basha, E., Wysocki, V. H., and Vierling, E. (2008). Insights into small heat shock protein and substrate structure during chaperone action derived from hydrogen/deuterium exchange and mass spectrometry. *J. Biol. Chem.* 283, 26634–26642. doi:10.1074/jbc.M802946200.

Cherra, S. J., Kulich, S. M., Uechi, G., Balasubramani, M., Mountzouris, J., Day, B. W., et al. (2010). Regulation of the autophagy protein LC3 by phosphorylation. *J. Cell Biol.* 190, 533–539. doi:10.1083/jcb.201002108.

Chow, C. Y., Zhang, Y., Dowling, J. J., Jin, N., Adamska, M., Shiga, K., et al. (2007). Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. *Nature* 448, 68–72. doi:10.1038/nature05876.

Ciuffa, R., Lamark, T., Tarafder, A. K., Guesdon, A., Rybina, S., Hagen, W. J. H., et al. (2015). The Selective Autophagy Receptor p62 Forms a Flexible Filamentous Helical Scaffold. *Cell Rep.* 11, 748–758. doi:10.1016/j.celrep.2015.03.062.

Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., et al. (2016). p62-

and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci.* 113, E7490–E7499. doi:10.1073/pnas.1615455113.

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., and Mann, M. (2014). Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol. Cell. Proteomics* 13, 2513–2526. doi:10.1074/mcp.M113.031591.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi:10.1038/nbt.1511.

Crippa, V., Carra, S., Rusmini, P., Sau, D., Bolzoni, E., Bendotti, C., et al. (2010a). A role of small heat shock protein B8 (HspB8) in the autophagic removal of misfolded proteins responsible for neurodegenerative diseases. *Autophagy* 6, 958–960. doi:10.4161/auto.6.7.13042.

Crippa, V., D'Agostino, V. G., Cristofani, R., Rusmini, P., Cicardi, M. E., Messi, E., et al. (2016). Transcriptional induction of the heat shock protein B8 mediates the clearance of misfolded proteins responsible for motor neuron diseases. *Sci. Rep.* 6, 22827. doi:10.1038/srep22827.

Crippa, V., Sau, D., Rusmini, P., Boncoraglio, A., Onesto, E., Bolzoni, E., et al. (2010b). The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum. Mol. Genet.* 19, 3440–3456. doi:10.1093/hmg/ddq257.

Cuervo, a. M., Palmer, a., Rivett, a. J., and Knecht, E. (1995). Degradation of proteasomes by lysosomes in rat liver. *Eur. J. Biochem.* 227, 792–800. doi:10.1111/j.1432-1033.1995.tb20203.x.

d'Ydewalle, C., Krishnan, J., Chiheb, D. M., Van Damme, P., Irobi, J., Kozikowski, A. P., et al. (2011). HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. *Nat. Med.* 17, 968–974. doi:10.1038/nm.2396.

Dadakhujaev, S., Eun, J. J., Hae, S. N., Hah, Y. S., Chang, J. K., and Deok, R. K. (2009). Interplay between autophagy and apoptosis in TrkA-induced cell death. *Autophagy* 5, 103–105. doi:10.1016/j.yexcr.2008.08.013.

Darrow, M. C., Sergeeva, O. a., Isas, J. M., Galaz-Montoya, J. G., King§, J. a., Langen, R., et al. (2015). Structural mechanisms of mutant huntingtin aggregation suppression by the synthetic chaperonin-like CCT5 complex explained by cryoelectron tomography. *J. Biol. Chem.* 290, 17451–17461. doi:10.1074/jbc.M115.655373.

Datskevich, P. N., Mymrikov, E. V., and Gusev, N. B. (2012). Utilization of fluorescent chimeras for investigation of heterooligomeric complexes formed by human small heat shock proteins. *Biochimie* 94, 1794–1804. doi:10.1016/j.biochi.2012.04.012.

De Thonel, A., Le Mouël, A., and Mezger, V. (2012). Transcriptional regulation of small HSP - HSF1 and beyond. *Int. J. Biochem. Cell Biol.* 44, 1593–1612. doi:10.1016/j.biocel.2012.06.012.

Deng, H.-X., Klein, C. J., Yan, J., Shi, Y., Wu, Y., Fecto, F., et al. (2010). Scapuloperoneal spinal muscular atrophy and CMT2C are allelic disorders caused by alterations in TRPV4. *Nat. Genet.* 42, 165–9. doi:10.1038/ng.509.

Der Perng, M., Wen, S. F., van den IJssel, P., Prescott, A. R., and Quinlan, R. A. (2004). Desmin Aggregate Formation by R120G αB-Crystallin Is Caused by Altered Filament Interactions and Is Dependent upon Network Status in Cells. *Mol. Biol. Cell* 15, 2335–2346. doi:10.1091/mbc.E03-12-0893.

Deroyer, C., Renert, A. F., Merville, M. P., and Fillet, M. (2014). New role for EMD (emerin), a key inner nuclear membrane protein, as an enhancer of autophagosome formation in the C16-ceramide autophagy pathway. *Autophagy* 10, 1229–1240. doi:10.4161/auto.28777.

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, 651–657. doi:10.1038/nature05185.

Ding, Y., Gao, H., Zhao, L., Wang, X., and Zheng, M. (2015). Mitofusin 2-deficiency suppresses cell proliferation through disturbance of autophagy. *PLoS One* 10, 1–14. doi:10.1371/journal.pone.0121328.

Doshi, B. M., Hightower, L. E., and Lee, J. (2009). The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock. *Cell Stress Chaperones* 14, 445–457. doi:10.1007/s12192-008-0098-1.

Durieux, A. C., Vassilopoulos, S., Lainé, J., Fraysse, B., Briñas, L., Prudhon, B., et al.

(2012). A Centronuclear Myopathy - Dynamin 2 Mutation Impairs Autophagy in Mice. *Traffic* 13, 869–879. doi:10.1111/j.1600-0854.2012.01348.x.

Dyck, P. J., Litchy, W. J., Minnerath, S., Bird, T. D., Chance, P. F., Schaid, D. J., et al. (1994). Hereditary motor and sensory neuropathy with diaphragm and vocal cord paresis. *Ann. Neurol.* 35, 608–615. doi:10.1002/ana.410350515.

Echaniz-Laguna, A., Geuens, T., Petiot, P., Péréon, Y., Adriaenssens, E., Haidar, M., et al. (2017). Axonal Neuropathies due to Mutations in Small Heat Shock Proteins: Clinical, Genetic, and Functional Insights into Novel Mutations. *Hum. Mutat.* 38, 556–568. doi:10.1002/humu.23189.

Edvardson, S., Cinnamon, Y., Jalas, C., Shaag, A., Maayan, C., Axelrod, F. B., et al. (2012). Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. *Ann. Neurol.* 71, 569–572. doi:10.1002/ana.23524.

Eliceiri, K., Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ : 25 years of image analysis HISTORICAL commentary NIH Image to ImageJ : 25 years of image analysis. *Nat. Methods* 9, 671–675. doi:10.1038/nmeth.2089.

Erzberger, J. P., and Berger, J. M. (2006). Evolutionary Relationships and Structural Mechanisms of Aaa+ Proteins. *Annu. Rev. Biophys. Biomol. Struct.* 35, 93–114. doi:10.1146/annurev.biophys.35.040405.101933.

Evgrafov, O. V, Mersiyanova, I., Irobi, J., Van Den Bosch, L., Dierick, I., Leung, C. L., et al. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36, 602–606. doi:10.1038/ng1354.

Fan, G. C., Chu, G., and Kranias, E. G. (2005). Hsp20 and its cardioprotection. *Trends Cardiovasc. Med.* 15, 138–141. doi:10.1016/j.tcm.2005.05.004.

Fan, H., Chen, S., Sun, Y., Xu, S., and Wu, L. (2015). Seipin mutation at glycosylation sites activates autophagy in transfected cells via abnormal large lipid droplets generation. *Acta Pharmacol. Sin.* 36, 497–506. doi:10.1038/aps.2014.164.

Fang, B. a., Kovačević, Ž., Park, K. C., Kalinowski, D. S., Jansson, P. J., Lane, D. J. R., et al. (2014). Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy. *Biochim. Biophys. Acta - Rev. Cancer* 1845, 1–19. doi:10.1016/j.bbcan.2013.11.002.

Ferguson, C. J., Lenk, G. M., and Meisler, M. H. (2010). PtdIns(3,5)P2 and autophagy in mouse models of neurodegeneration. *Autophagy* 6, 170–171. doi:10.4161/auto.6.1.10626.

Ferraro, E., Giammarioli, A. M., Chiandotto, S., Spoletini, I., and Rosano, G. (2014). Exercise-Induced Skeletal Muscle Remodeling and Metabolic Adaptation: Redox Signaling and Role of Autophagy. *Antioxid. Redox Signal.* 21, 154–176. doi:10.1089/ars.2013.5773.

Ferrier, A., Boyer, J. G., and Kothary, R. (2013). *Cellular and Molecular Biology of Neuronal Dystonin*. Elsevier doi:10.1016/B978-0-12-405210-9.00003-5.

Ferrier, A., De Repentigny, Y., Lynch-Godrei, A., Gibeault, S., Eid, W., Kuo, D., et al. (2015). Disruption in the autophagic process underlies the sensory neuropathy in dystonia musculorum mice. *Autophagy* 11, 1025–1036. doi:10.1080/15548627.2015.1052207.

Fortun, J., Go, J. C., Li, J., Amici, S. a., Dunn, W. a., and Notterpek, L. (2006). Alterations in degradative pathways and protein aggregation in a neuropathy model based on PMP22 overexpression. *Neurobiol. Dis.* 22, 153–164. doi:10.1016/j.nbd.2005.10.010.

Frake, R. A., Ricketts, T., Menzies, F. M., and Rubinsztein, D. C. (2015). Autophagy and neurodegeneration. *J. Clin. Invest.* 125, 65–74. doi:10.1172/JCI73944.

Franco, M. L., Melero, C., Sarasola, E., Acebo, P., Luque, A., Calatayud-Baselga, I., et al. (2016). Mutations in TrkA causing congenital insensitivity to pain with anhidrosis (CIPA) induce misfolding, aggregation, and mutation-dependent neurodegeneration by dysfunction of the autophagic flux. *J. Biol. Chem.* 291, 21363–21374. doi:10.1074/jbc.M116.722587.

Gallolu Kankanamalage, S., Lee, A.-Y., Wichaidit, C., Lorente-Rodriguez, A., Shah, A. M., Stippec, S., et al. (2016). Multistep regulation of autophagy by WNK1. *Proc. Natl. Acad. Sci.* 113, 201617649. doi:10.1073/pnas.1617649113.

Gamerdinger, M., Hajieva, P., Kaya, a M., Wolfrum, U., Hartl, F. U., and Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J.* 28, 889–901. doi:10.1038/emboj.2009.29.

Gess, B., Auer-Grumbach, M., Schirmacher, A., Strom, T., Zitzelsberger, M., Rudnik-

Schöneborn, S., et al. (2014). Ovid: HSJ1-related hereditary neuropathies: Novel mutations and extended clinical spectrum. *Neurology* 83, 1726–32. doi:10.1212/WNL.0000000000000966.

Geuens, T., De Winter, V., Rajan, N., Achsel, T., Mateiu, L., Almeida-Souza, L., et al. (2017). Mutant HSPB1 causes loss of translational repression by binding to PCBP1, an RNA binding protein with a possible role in neurodegenerative disease. *Acta Neuropathol. Commun.* 5, 5. doi:10.1186/s40478-016-0407-3.

Glover, J. R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell* 94, 73–82. doi:10.1016/S0092-8674(00)81223-4.

Gomez-Suaga, P., Paillusson, S., Stoica, R., Noble, W., Hanger, D. P., and Miller, C. C. J. (2017). The ER-Mitochondria Tethering Complex VAPB-PTPIP51 Regulates Autophagy. *Curr. Biol.* 27, 371–385. doi:10.1016/j.cub.2016.12.038.

González-Jamett, A. M., Momboisse, F., Haro-Acuña, V., Bevilacqua, J. a., Caviedes, P., and Cárdenas, A. M. (2013). Dynamin-2 function and dysfunction along the secretory pathway. *Front. Endocrinol. (Lausanne).* 4, 1–9. doi:10.3389/fendo.2013.00126.

Gonzalez, M. a., Feely, S. M., Speziani, F., Strickland, A. V., Danzi, M., Bacon, C., et al. (2014). A novel mutation in VCP causes Charcot-Marie-Tooth Type 2 disease. *Brain* 137, 2–7. doi:10.1093/brain/awu224.

Guo, J., Qiu, W., Soh, S. L. Y., Wei, S., Radda, G. K., Ong, W.-Y., et al. (2013). Motor neuron degeneration in a mouse model of seipinopathy. *Cell Death Dis.* 4, e535. doi:10.1038/cddis.2013.64.

Guo, W., Naujock, M., Fumagalli, L., Vandoorne, T., Baatsen, P., Boon, R., et al. (2017). HDAC6 inhibition reverses axonal transport defects in motor neurons derived from FUS-ALS patients. *Nat. Commun.* 8, 1–14. doi:10.1038/s41467-017-00911-y.

Gutierrez, M. G., Munafó, D. B., Berón, W., and Colombo, M. I. (2004). Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J. Cell Sci.* 117, 2687–97. doi:10.1242/jcs.01114.

Haack, T. B. B., Iuso, A., Kremer, L. S. S., Hartig, M., Strom, T. M. M., Meitinger, T., et al. (2016). Absence of the Autophagy Adaptor SQSTM1/p62 Causes Childhood-Onset

Neurodegeneration with Ataxia, Dystonia, and Gaze Palsy. *Am. J. Hum. Genet.* 99, 735–743. doi:10.1016/j.ajhg.2016.06.026.

Haidar, M., and Timmerman, V. (2017). Autophagy as an Emerging Common Pathomechanism in Inherited Peripheral Neuropathies. *Front. Mol. Neurosci.* 10, 1–17. doi:10.3389/fnmol.2017.00143.

Hailey, D. W., Rambold, A. S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P. K., et al. (2010). Mitochondria Supply Membranes for Autophagosome Biogenesis during Starvation. *Cell* 141, 656–667. doi:10.1016/j.cell.2010.04.009.

Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., et al. (2013). Autophagosomes form at ER-mitochondria contact sites. *Nature* 495, 389–93. doi:10.1038/nature11910.

Han, B., Li, W., Sun, Y., Zhou, L., Xu, Y., and Zhao, X. (2014). A prolyl-hydroxylase inhibitor, ethyl-3,4-Dihydroxybenzoate, induces cell autophagy and apoptosis in esophageal squamous cell carcinoma cellsvia up-regulation of BNIP3 and N-myc downstream-regulated gene-1. *PLoS One* 9. doi:10.1371/journal.pone.0107204.

Hansen, K., Wagner, B., Hamel, W., Schweizer, M., Haag, F., Westphal, M., et al. (2007). Autophagic cell death induced by TrkA receptor activation in human glioblastoma cells. *J. Neurochem.* 103, 259–275. doi:10.1111/j.1471-4159.2007.04753.x.

Hara, K., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andrabi, K., Weng, Q. P., et al. (1997). Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* 272, 26457–26463. doi:10.1074/jbc.272.42.26457.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885–889. doi:10.1038/nature04724.

Harding, A. E., and P.K. Thomas (1980). Hereditary Distal Spinal Muscular Atrophy: A report on 34 cases and a review of the literature. *J. Neurol. Sci.* Volume 45, 337–348.

Harding, a E., and Thomas, P. K. (1980). Genetic aspects of hereditary motor and sensory neuropathy (types I and II). *J. Med. Genet.* 17, 329–336. doi:10.1136/jmg.17.5.329.

Haslbeck, M., Franzmann, T., Weinfurtner, D., and Buchner, J. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nat. Struct. Mol. Biol.* 12, 842–846.

doi:10.1038/nsmb993.

Haslbeck, M., and Vierling, E. (2015). A first line of stress defense: Small heat shock proteins and their function in protein homeostasis. *J. Mol. Biol.* 427, 1537–1548. doi:10.1016/j.jmb.2015.02.002.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. (2009). A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nat. Cell Biol.* 11, 1433–1437. doi:10.1038/ncb1991.

Hayes, V. H., Devlin, G., and Quinlan, R. A. (2008). Truncation of alpha-B-crystallin by the myopathy-causing Q151X mutation significantly destabilizes the protein leading to aggregate formation in transfected cells. *J. Biol. Chem.* 283, 10500–10512. doi:10.1074/jbc.M706453200.

He, C., Sumpter, R., and Levine, B. (2012). Exercise induces autophagy in peripheral tissues and in the brain. *Autophagy* 8, 1548–1551. doi:10.4161/auto.21327.

He, M., Ding, Y., Chu, C., Tang, J., Xiao, Q., and Luo, Z.-G. (2016). Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury. *Proc. Natl. Acad. Sci.* 113, 11324–11329. doi:10.1073/pnas.1611282113.

Heimer, G., Oz-Levi, D., Eyal, E., Edvardson, S., Nissenkorn, A., Ruzzo, E. K., et al. (2016). TECPR2 mutations cause a new subtype of familial dysautonomia like hereditary sensory autonomic neuropathy with intellectual disability. *Eur. J. Paediatr. Neurol.* 20, 69–79. doi:10.1016/j.ejpn.2015.10.003.

Holmgren, A. (2012). Molecular Biology of Small Heat Shock Proteins associated with Peripheral Neuropathies. *eLS. John Wiley Sons Ltd, Chichester*, 1–10. doi:10.1002/9780470015902.a0024294.

Holmgren, A., Bouhy, D., De Winter, V., Asselbergh, B., Timmermans, J. P., Irobi, J., et al. (2013). Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments. *Acta Neuropathol.* 126, 93–108. doi:10.1007/s00401-013-1133-6.

Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., et al. (2009). Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol. Biol. Cell* 20, 1981–1991. doi:10.1091/mbc.E08.

Hsiao, C. T., Tsai, P. C., Lin, C. C., Liu, Y. T., Huang, Y. H., Liao, Y. C., et al. (2016). Clinical and molecular characterization of BSCL2 mutations in a taiwanese cohort with hereditary neuropathy. *PLoS One* 11, 1–12. doi:10.1371/journal.pone.0147677.

Huber, N., Bieniossek, C., Wagner, K. M., Elsässer, H.-P., Suter, U., Berger, I., et al. (2016). Glutathione-conjugating and membrane-remodeling activity of GDAP1 relies on amphipathic C-terminal domain. *Sci. Rep.* 6, 36930. doi:10.1038/srep36930.

Huett, A., Heath, R. J., Begun, J., Sassi, S. O., Baxt, L. a., Vyas, J. M., et al. (2012). The LRR and RING domain protein LRSAM1 is an E3 ligase crucial for ubiquitin-dependent autophagy of intracellular salmonella typhimurium. *Cell Host Microbe* 12, 778–790. doi:10.1016/j.chom.2012.10.019.

Ikenaka, K., Kawai, K., Katsuno, M., Huang, Z., Jiang, Y. M., Iguchi, Y., et al. (2013). dnc-1/dynactin 1 Knockdown Disrupts Transport of Autophagosomes and Induces Motor Neuron Degeneration. *PLoS One* 8. doi:10.1371/journal.pone.0054511.

Imai, A. K., Hao, F., Fujita, N., Tsuji, Y., and Oe, Y. (2016). Atg9A Trafficking Through the Recycling Endosomes is Required for Autophagosome Formation.

Indo, Y., Tsuruta, M., Hayashida, Y., Karim, M., Ohta, K., Kawano, T., et al. (1996). Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat. Genet.* 13, 485–8. doi:10.1038/ng1096-146.

Irobi, J., Almeida-Souza, L., Asselbergh, B., de Winter, V., Goethals, S., Dierick, I., et al. (2010). Mutant HSPB8 causes motor neuron-specific neurite degeneration. *Hum. Mol. Genet.* 19, 3254–3265. doi:10.1093/hmg/ddq234.

Irobi, J., Impe, K. Van, Seeman, P., Jordanova, A., Dierick, I., Verpoorten, N., et al. (2004). Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. *Nat. Genet.* 36, 597–601. doi:10.1038/ng1328.

Itakura, E., and Mizushima, N. (2010). Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* 6, 764–776. doi:10.4161/auto.6.6.12709.

Itakura, E., and Mizushima, N. (2011). p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. *J. Cell Biol.* 192, 17–27. doi:10.1083/jcb.201009067.

Ito, D., Fujisawa, T., Iida, H., and Suzuki, N. (2008). Characterization of seipin/BSCL2, a protein associated with spastic paraplegia 17. *Neurobiol. Dis.* 31, 266–277. doi:10.1016/j.nbd.2008.05.004.

J. Cummings, C., and Y. Zoghbi, H. (2000). *Fourteen and counting: Unraveling trinucleotide repeat diseases*. doi:10.1093/hmg/9.6.909.

Jäger, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., et al. (2004). Role for Rab7 in maturation of late autophagic vacuoles. *J. Cell Sci.* 117, 4837–4848. doi:10.1242/jcs.01370.

Jang, S. Y., Shin, Y. K., Park, S. Y., Park, J. Y., Rha, S. H., Kim, J. K., et al. (2015). Autophagy is involved in the reduction of myelinating schwann cell cytoplasm during myelin maturation of the peripheral nerve. *PLoS One* 10, 1–14. doi:10.1371/journal.pone.0116624.

Jaya, N., Garcia, V., and Vierling, E. (2009). Substrate binding site flexibility of the small heat shock protein molecular chaperones. *Proc. Natl. Acad. Sci. U. S. A.* 106, 15604–15609. doi:10.1073/pnas.0902177106.

Jean, S., Cox, S., Nassari, S., and Kiger, A. a (2015). Starvation-induced MTMR 13 and RAB 21 activity regulates VAMP 8 to promote autophagosome – lysosome fusion. 16, 297–311.

Jiang, P., Nishimura, T., Sakamaki, Y., Itakura, E., Hatta, T., Natsume, T., et al. (2014). The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol. Biol. Cell* 25, 1327–1337. doi:10.1091/mbc.E13-08-0447.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R., et al. (2009). Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling. *Cell* 137, 721–735. doi:10.1016/j.cell.2009.03.015.

Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 7, 279–296. doi:10.4161/auto.7.3.14487.

Ju, J. S., Fuentealba, R. a., Miller, S. E., Jackson, E., Piwnica-Worms, D., Baloh, R. H., et al. (2009). Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *J. Cell Biol.* 187, 875–888. doi:10.1083/jcb.200908115.

Kabeya, Y. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in

autophagosome membranes after processing. *EMBO J.* 19, 5720–5728. doi:10.1093/emboj/19.21.5720.

Kakkar, V., Meister-Broekema, M., Minoia, M., Carra, S., and Kampinga, H. H. (2014). Barcoding heat shock proteins to human diseases: looking beyond the heat shock response. *Dis. Model. Mech.* 7, 421–34. doi:10.1242/dmm.014563.

Kalaydjieva, L., Gresham, D., Gooding, R., Heather, L., Baas, F., de Jonge, R., et al. (2000). N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. *Am. J. Hum. Genet.* 67, 47–58. doi:10.1086/302978.

Kalmar, B., Innes, A., Wanisch, K., Kolaszynska, A. K., Pandraud, A., Kelly, G., et al. (2017a). Mitochondrial deficits and abnormal mitochondrial retrograde axonal transport play a role in the pathogenesis of mutant Hsp27-induced Charcot Marie Tooth Disease. *Hum. Mol. Genet.* doi:10.1093/hmg/ddx216.

Kalmar, B., Innes, A., Wanisch, K., Kolaszynska, A. K., Pandraud, A., Kelly, G., et al. (2017b). Mitochondrial deficits and abnormal mitochondrial retrograde axonal transport play a role in the pathogenesis of mutant Hsp27-induced Charcot Marie Tooth Disease. *Hum. Mol. Genet.* 0, 1–14. doi:10.1093/hmg/ddx216.

Kamentsky, L., Jones, T. R., Fraser, A., Bray, M. A., Logan, D. J., Madden, K. L., et al. (2011). Improved structure, function and compatibility for cellprofiler: Modular high-throughput image analysis software. *Bioinformatics* 27, 1179–1180. doi:10.1093/bioinformatics/btr095.

Kampinga, H. H., and Garrido, C. (2012). HSPBs: Small proteins with big implications in human disease. *Int. J. Biochem. Cell Biol.* 44, 1706–1710. doi:10.1016/j.biocel.2012.06.005.

Kato, K., Goto, S., Inaguma, Y., Hasegawa, K., Morishita, R., and Asano, T. (1994). Purification and characterization of a 20-kDa protein that is highly homologous to ??B crystallin. *J. Biol. Chem.* 269, 15302–15309.

Katona, I., Zhang, X., Bai, Y., Shy, M. E., Guo, J., Yan, Q., et al. (2011). Distinct pathogenic processes between Fig4-deficient motor and sensory neurons. *Eur. J. Neurosci.* 33, 1401–1410. doi:10.1111/j.1460-9568.2011.07651.x.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al.

(2014a). The Functional Landscape of Hsp27 Reveals New Cellular Processes such as DNA Repair and Alternative Splicing and Proposes Novel Anticancer Targets. *Mol. Cell. Proteomics* 13, 3585–3601. doi:10.1074/mcp.M114.041228.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al. (2014b). The functional landscape of Hsp27 reveals new cellular processes such as DNA repair and alternative splicing and proposes novel anticancer targets. *Mol. Cell. Proteomics*, 3585–3601. doi:10.1074/mcp.M114.041228.

Katsuragi, Y., Ichimura, Y., and Komatsu, M. (2015). P62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J.* 282, 4672–4678. doi:10.1111/febs.13540.

Kennedy, D., Mnich, K., Oommen, D., Chakravarthy, R., Almeida-Souza, L., Krols, M., et al. (2017). HSPB1 facilitates ERK-mediated phosphorylation and degradation of BIM to attenuate endoplasmic reticulum stress-induced apoptosis. *Cell Death Dis.* 8, e3026. doi:10.1038/cddis.2017.408.

Khaminets, A., Heinrich, T., Mari, M., Huebner, A. K., Akutsu, M., Grumati, P., et al. (2015). Regulation of endoplasmic reticulum turnover by FAM134B-mediated selective autophagy. doi:10.1038/nature14498.

Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., and Ulrich Hartl, F. (2013). *Molecular Chaperone Functions in Protein Folding and Proteostasis*. doi:10.1146/annurev-biochem-060208-092442.

Kimonis, V. E., Kovach, M. J., Waggoner, B., Leal, S., Salam, a, Rimer, L., et al. (2000). Clinical and molecular studies in a unique family with autosomal dominant limb-girdle muscular dystrophy and Paget disease of bone. *Genet. Med.* 2, 232–241. doi:10.1097/00125817-200007000-00006.

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi:10.1080/15548627.2015.1100356.

Köchl, R., Hu, X. W., Chan, E. Y. W., and Tooze, S. a. (2006). Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. *Traffic* 7, 129–145. doi:10.1111/j.1600-0854.2005.00368.x.

Kolb, S. J., Snyder, P. J., Poi, E. J., Renard, E. A., Bartlett, A., Gu, S., et al. (2010). Mutant

small heat shock protein B3 causes motor neuropathy: Utility of a candidate gene approach. *Neurology* 74, 502–506. doi:10.1212/WNL.0b013e3181cef84a.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., et al. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441, 880–884. doi:10.1038/nature04723.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., et al. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* 169, 425–434. doi:10.1083/jcb.200412022.

Korolchuk, V. I., Mansilla, A., Menzies, F. M., and Rubinsztein, D. C. (2009). Autophagy Inhibition Compromises Degradation of Ubiquitin-Proteasome Pathway Substrates. *Mol. Cell* 33, 517–527. doi:10.1016/j.molcel.2009.01.021.

Köttgen, M., Buchholz, B., Garcia-Gonzalez, M. a., Kotsis, F., Fu, X., Doerken, M., et al. (2008). TRPP2 and TRPV4 form a polymodal sensory channel complex. *J. Cell Biol.* 182, 437–447. doi:10.1083/jcb.200805124.

Kurth, I., Pamminger, T., Hennings, J. C., Soehendra, D., Huebner, A. K., Rotthier, A., et al. (2009). Mutations in FAM134B, encoding a newly identified Golgi protein, cause severe sensory and autonomic neuropathy. *Nat. Genet.* 41, 1179–1181. doi:10.1038/ng.464.

Kwok, A. S., Phadwal, K., Turner, B. J., Oliver, P. L., Raw, A., Simon, A. K., et al. (2011). HspB8 mutation causing hereditary distal motor neuropathy impairs lysosomal delivery of autophagosomes. *J. Neurochem.* 119, 1155–1161. doi:10.1111/j.1471-4159.2011.07521.x.

Kyöstilä, K., Syrjä, P., Jagannathan, V., Chandrasekar, G., Jokinen, T. S., Seppälä, E. H., et al. (2015). A Missense Change in the ATG4D Gene Links Aberrant Autophagy to a Neurodegenerative Vacuolar Storage Disease. *PLoS Genet.* 11, 1–22. doi:10.1371/journal.pgen.1005169.

Lacerda, A. F. E., Hartjes, E., and Brunetti, C. R. (2014). LITAF mutations associated with Charcot-Marie-Tooth disease 1C show mislocalization from the late endosome/lysosome to the mitochondria. *PLoS One* 9, e103454. doi:10.1371/journal.pone.0103454.

Laganowsky, A., Benesch, J. L. P., Landau, M., Ding, L., Sawaya, M. R., Cascio, D., et al. (2010). Crystal structures of truncated alphaA and alphaB crystallins reveal structural

mechanisms of polydispersity important for eye lens function. *Protein Sci.* 19, 1031–1043. doi:10.1002/pro.380.

Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., et al. (2003). Interaction Codes within the Family of Mammalian Phox and Bem1p Domain-containing Proteins. *J. Biol. Chem.* 278, 34568–34581. doi:10.1074/jbc.M303221200.

Lamb, C. a, Nühlen, S., Judith, D., Frith, D., Snijders, A. P., Behrends, C., et al. (2016). TBC1D14 regulates autophagy via the TRAPP complex and ATG9 traffic. *EMBO J.* 35, 281–301. doi:10.15252/embj.201592695.

Lamb, C. a, Yoshimori, T., and Tooze, S. a (2013). The autophagosome: origins unknown, biogenesis complex. *Nat. Rev. Mol. Cell Biol.* 14, 759–74. doi:10.1038/nrm3696.

Lambert, H., Charette, S. J., André, F., Guimond, A., Landry, J., and Bernier, F. (1999). CELL BIOLOGY AND METABOLISM : HSP27 Multimerization Mediated by Interactions at the Amino Terminus HSP27 Multimerization Mediated by Phosphorylation-sensitive Intermolecular Interactions at the Amino Terminus *. *J. Biol. Chem.* 274, 9378–9385.

Larroquette, F., Seto, L., Gaub, P. L., Kamal, B., Wallis, D., Larivière, R., et al. (2015). Vapb/Amyotrophic lateral sclerosis 8 knock-in mice display slowly progressive motor behavior defects accompanying ER stress and autophagic response. *Hum. Mol. Genet.* 24, 6515–6529. doi:10.1093/hmg/ddv360.

Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* 16, 659–671. doi:10.1093/emboj/16.3.659.

Lee, J.-A. (2012). Neuronal Autophagy: A Housekeeper or a Fighter in Neuronal Cell Survival? *Exp. Neurobiol.* 21, 1. doi:10.5607/en.2012.21.1.1.

Lee, J.-Y., Koga, H., Kawaguchi, Y., Tang, W., Wong, E., Gao, Y.-S., et al. (2010). HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. *EMBO J.* 29, 969–980. doi:10.1038/emboj.2009.405.

Lee, S. M., Olzmann, J. a, Chin, L.-S., Li, L., Babst, M., Bendtsen, J. D., et al. (2011). Mutations associated with Charcot-Marie-Tooth disease cause SIMPLE protein mislocalization and degradation by the proteasome and aggresome-autophagy pathways.

J. Cell Sci. 124, 3319–31. doi:10.1242/jcs.087114.

Lenk, G. M., and Meisler, M. H. (2014). *Mouse models of PI(3,5)P2 deficiency with impaired lysosome function*. 1st ed. Elsevier Inc. doi:10.1016/B978-0-12-397926-1.00014-7.

Lev, S., Halevy, D. Ben, Peretti, D., and Dahan, N. (2008). The VAP protein family: from cellular functions to motor neuron disease. *Trends Cell Biol.* 18, 282–290. doi:10.1016/j.tcb.2008.03.006.

Lewis, S. E., Mannion, R. J., White, F. a, Coggeshall, R. E., Beggs, S., Costigan, M., et al. (1999). A role for HSP27 in sensory neuron survival. *J. Neurosci.* 19, 8945–8953.

Li, J., Kim, S. G., and Blenis, J. (2014). Rapamycin: One drug, many effects. *Cell Metab.* 19, 373–379. doi:10.1016/j.cmet.2014.01.001.

Li, J., Parker, B., Martyn, C., Natarajan, C., and Guo, J. (2012). The PMP22 Gene and Its Related Diseases. *Mol. Neurobiol.* 22, 1–26. doi:10.1007/s12035-012-8370-x.

Liang, C., Lee, J., Inn, K., Gack, M. U., Li, Q., Roberts, E. a, et al. (2008). Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat. Cell Biol.* 10, 776–87. doi:10.1038/ncb1740.

Lin, X., Li, S., Zhao, Y., Ma, X., Zhang, K., He, X., et al. (2013). Interaction domains of p62: a bridge between p62 and selective autophagy. *DNA Cell Biol.* 32, 220–7. doi:10.1089/dna.2012.1915.

Lipinski, M. M., Zheng, B., Lu, T., Yan, Z., Py, B. F., Ng, A., et al. (2010). Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Pnas* 107, 14164–14169. doi:10.1073/pnas.1009485107.

Liu, G.-S., Zhu, H., Cai, W.-F., Wang, X., Jiang, M., Essandoh, K., et al. (2017). Regulation of BECN1-Mediated Autophagy by HSPB6: Insights from A Human HSPB6 ^{S10F} Mutant. *Autophagy* 8627, 01–56. doi:10.1080/15548627.2017.1392420.

Liu, J., Xing, H., Chen, Y., Wang, L., Wang, D., Rao, Q., et al. (2012). PIG7, transactivated by AML1, promotes apoptosis and differentiation of leukemia cells with AML1-ETO fusion gene. *Leukemia* 26, 117–26. doi:10.1038/leu.2011.178.

Lo, K. Y., Kuzmin, a., Unger, S. M., Petersen, J. D., and Silverman, M. a. (2011). KIF1A is the primary anterograde motor protein required for the axonal transport of dense-core

vesicles in cultured hippocampal neurons. *Neurosci. Lett.* 491, 168–173. doi:10.1016/j.neulet.2011.01.018.

Longatti, A., Lamb, C. a., Razi, M., Yoshimura, S. I., Barr, F. a., and Tooze, S. a. (2012). TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J. Cell Biol.* 197, 659–675. doi:10.1083/jcb.201111079.

Lu, J. H., Tan, J. Q., Durairajan, S. S. K., Liu, L. F., Zhang, Z. H., Ma, L., et al. (2012). Isorhynchophylline, a natural alkaloid, promotes the degradation of ??-synuclein in neuronal cells via inducing autophagy. *Autophagy* 8, 98–108. doi:10.4161/auto.8.1.18313.

Lupski, J. R., Wise, C. a, Kuwano, a, Pentao, L., Parke, J. T., Glaze, D. G., et al. (1992). Gene dosage is a mechanism for Charcot-Marie-Tooth disease type 1A. *Nat Genet* 1, 29–33. doi:10.1038/ng0492-29.

Madorsky, I., Opalach, K., Waber, A., Verrier, J. D., Solmo, C., Foster, T., et al. (2009). Intermittent fasting alleviates the neuropathic phenotype in a mouse model of Charcot-Marie-Tooth disease. *Neurobiol. Dis.* 34, 146–154. doi:10.1016/j.nbd.2009.01.002.

Marchbank, K., Waters, S., Roberts, R. G., Solomon, E., and Whitehouse, C. A. (2012). MAP1B interaction with the FW domain of the autophagic receptor Nbr1 facilitates its association to the microtubule network. *Int. J. Cell Biol.* 2012. doi:10.1155/2012/208014.

Mari, M., Griffith, J., Rieter, E., Krishnappa, L., Klionsky, D. J., and Reggiori, F. (2010). An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *J. Cell Biol.* 190, 1005–1022. doi:10.1083/jcb.200912089.

Martinez-Vicente, M., and Cuervo, A. M. (2007). Autophagy and neurodegeneration: when the cleaning crew goes on strike. *Lancet Neurol.* 6, 352–361. doi:10.1016/S1474-4422(07)70076-5.

Matsumoto, T., Urushido, M., Ide, H., Ishihara, M., and Hamada-, K. (2015a). Small Heat Shock Protein Beta-1 (HSPB1) Is Upregulated and Regulates Autophagy and Apoptosis of Renal Tubular Cells in Acute Kidney Injury. 1, 1–22. doi:10.1371/journal.pone.0126229.

Matsumoto, T., Urushido, M., Ide, H., Ishihara, M., Hamada-Ode, K., Shimamura, Y., et al.

(2015b). Small Heat Shock Protein Beta-1 (HSPB1) Is Upregulated and Regulates Autophagy and Apoptosis of Renal Tubular Cells in Acute Kidney Injury. *PLoS One* 10, e0126229. doi:10.1371/journal.pone.0126229.

McDonald, E. T., Bortolus, M., Koteiche, H. A., and McHaourab, H. S. (2012). Sequence, structure, and dynamic determinants of Hsp27 (HspB1) equilibrium dissociation are encoded by the N-terminal domain. *Biochemistry* 51, 1257–1268. doi:10.1021/bi2017624.

McNeill, A., Magalhaes, J., Shen, C., Chau, K. Y., Hughes, D., Mehta, A., et al. (2014). Ambroxol improves lysosomal biochemistry in glucocerebrosidase mutation-linked Parkinson disease cells. *Brain* 137, 1481–1495. doi:10.1093/brain/awu020.

Menzies, F. M., Fleming, A., Caricasole, A., Bento, C. F., Andrews, S. P., Ashkenazi, A., et al. (2017). Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. 2. doi:10.1016/j.neuron.2017.01.022.

Mersiyanova, I. V, Perepelov, a V, Polyakov, a V, Sitnikov, V. F., Dadali, E. L., Oparin, R. B., et al. (2000). A new variant of Charcot-Marie-Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene. *Am. J. Hum. Genet.* 67, 37–46. doi:10.1086/302962.

Mijaljica, D., Prescott, M., and Devenish, R. J. (2011). Microautophagy in mammalian cells: Revisiting a 40-year-old conundrum. *Autophagy* 7, 673–682. doi:10.4161/auto.7.7.14733.

Miller, F. D., and Kaplan, D. R. (2001). Neurotrophin signalling pathways regulating neuronal apoptosis. *Cell. Mol. Life Sci.* 58, 1045–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11529497.

Mizushima, N., and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. *Autophagy* 3, 542–545. doi:10.4161/auto.4600.

Morelli, F. F., Verbeek, D. S., Bertacchini, J., Vinet, J., Mediani, L., Marmiroli, S., et al. (2017). Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function. *Cell Rep.* 20, 2100–2115. doi:10.1016/j.celrep.2017.08.018.

Moriguchi, T., Urushiyama, S., Hisamoto, N., Iemura, S. I., Uchida, S., Natsume, T., et al.

(2005). WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J. Biol. Chem.* 280, 42685–42693. doi:10.1074/jbc.M510042200.

Moscat, J., and Diaz-Meco, M. T. (2009). p62 at the Crossroads of Autophagy, Apoptosis, and Cancer. *Cell* 137, 1001–1004. doi:10.1016/j.cell.2009.05.023.

Murrow, L., Malhotra, R., and Debnath, J. (2015). ATG12–ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. *Nat. Cell Biol.* 17, 1–10. doi:10.1038/ncb3112.

Mymrikov, E. V., Seit-Nebi, a. S., and Gusev, N. B. (2011). Large Potentials of Small Heat Shock Proteins. *Physiol. Rev.* 91, 1123–1159. doi:10.1152/physrev.00023.2010.

Nakamura, K., Uhlik, M., and Johnson, N. (2006). PB1 domain-dependent signaling complex is required for extracellular signal-regulated kinase 5 activation. *Mol. Cell. Biol.* 26, 2065–2079. doi:10.1128/MCB.26.6.2065.

Nalbandian, A., Llewellyn, K. J., Badadani, M., Yin, H. Z., Nguyen, C., Katheria, V., et al. (2013). A progressive translational mouse model of human valosin-containing protein disease: The VCPR155H/+ mouse. *Muscle and Nerve* 47, 260–270. doi:10.1002/mus.23522.

Ng, A. C. Y., Eisenberg, J. M., Heath, R. J. W., Huett, A., Robinson, C. M., Nau, G. J., et al. (2011). Human leucine-rich repeat proteins: a genome-wide bioinformatic categorization and functional analysis in innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl, 4631–8. doi:10.1073/pnas.1000093107.

Nicholson, G. A., Valentijn, L. J., Cherryson, A. K., Kennerson, M. L., Bragg, T. L., DeKroon, R. M., et al. (1994). A frame shift mutation in the PMP22 gene in hereditary neuropathy with liability to pressure palsies. *Nat. Genet.* 6, 263–6. doi:10.1038/ng0394-263.

Nicolaou, P., Knöll, R., Haghighi, K., Fan, G. C., Dorn, G. W., Hasenfuß, G., et al. (2008). Human mutation in the anti-apoptotic heat shock protein 20 abrogates its cardioprotective effects. *J. Biol. Chem.* 283, 33465–33471. doi:10.1074/jbc.M802307200.

Niemann, A., Ruegg, M., La Padula, V., Schenone, A., and Suter, U. (2005). Ganglioside-

induced differentiation associated protein 1 is a regulator of the mitochondrial network: New implications for Charcot-Marie-Tooth disease. *J. Cell Biol.* 170, 1067–1078. doi:10.1083/jcb.200507087.

Nishimura, A. L., Mitne-Neto, M., Silva, H. C. a, Richieri-Costa, A., Middleton, S., Cascio, D., et al. (2004). A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am. J. Hum. Genet.* 75, 822–31. doi:10.1086/425287.

Notterpek, L., Shooter, E. M., and Snipes, G. J. (1997). Upregulation of the endosomal-lysosomal pathway in the trembler-J neuropathy. *J. Neurosci.* 17, 4190–4200.

Novoselov, S. S., Mustill, W. J., Gray, A. L., Dick, J. R., Kanuga, N., Kalmar, B., et al. (2013). Molecular Chaperone Mediated Late-Stage Neuroprotection in the SOD1G93A Mouse Model of Amyotrophic Lateral Sclerosis. *PLoS One* 8. doi:10.1371/journal.pone.0073944.

Ohta, K., Mizuno, A., Ueda, M., Li, S., Suzuki, Y., Hida, Y., et al. (2010). Autophagy impairment stimulates PS1 expression and ??-secretase activity. *Autophagy* 6, 345–352. doi:10.4161/auto.6.3.11228.

Outeiro, T. F., Klucken, J., Strathearn, K. E., Liu, F., Nguyen, P., Rochet, J. C., et al. (2006). Small heat shock proteins protect against α-synuclein-induced toxicity and aggregation. *Biochem. Biophys. Res. Commun.* 351, 631–638. doi:10.1016/j.bbrc.2006.10.085.

Oz-Levi, D., Ben-Zeev, B., Ruzzo, E. K., Hitomi, Y., Gelman, A., Pelak, K., et al. (2012). Mutation in TECPR2 reveals a role for autophagy in hereditary spastic paraparesis. *Am. J. Hum. Genet.* 91, 1065–1072. doi:10.1016/j.ajhg.2012.09.015.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., et al. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. *J. Biol. Chem.* 282, 24131–24145. doi:10.1074/jbc.M702824200.

Papadopoulos, C., Kirchner, P., Bug, M., Grum, D., Koerver, L., Schulze, N., et al. (2016). VCP/p97 cooperates with YOD1, UBXD1 and PLAA to drive clearance of ruptured lysosomes by autophagy. *EMBO J.* 36, 1–16. doi:10.15252/embj.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., et

al. (2003). HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol. Cell. Biol.* 23, 5790–5802. doi:10.1128/MCB.23.16.5790.

Pareyson, D., Saveri, P., and Piscosquito, G. (2014). Charcot-Marie-Tooth Disease and Related Hereditary Neuropathies : From Gene Function to Associated Phenotypes. 1009–1033.

Parzych, K. R., and Klionsky, D. J. (2014). An overview of autophagy: morphology, mechanism, and regulation. *Antioxid. Redox Signal.* 20, 460–73. doi:10.1089/ars.2013.5371.

Patel, S., Vierling, E., and Tama, F. (2014). Replica exchange molecular dynamics simulations provide insight into substrate recognition by small heat shock proteins. *Biophys. J.* 106, 2644–2655. doi:10.1016/j.bpj.2014.04.048.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Xiao, H. L., Mizushima, N., et al. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939. doi:10.1016/j.cell.2005.07.002.

Pavel, M., Imarisio, S., Menzies, F. M., Jimenez-Sanchez, M., Siddiqi, F. H., Wu, X., et al. (2016). CCT complex restricts neuropathogenic protein aggregation via autophagy. *Nat. Commun.* 7, 13821. doi:10.1038/ncomms13821.

Perrot, R., Berges, R., Bocquet, A., and Eyer, J. (2008). Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration. *Mol. Neurobiol.* 38, 27–65. doi:10.1007/s12035-008-8033-0.

Petiot, A., Ogier-denis, E., Edward, F. C., Meijer, A. J., Chem, J. B., Blommaart, E. F. C., et al. (2000). Distinct Classes of Phosphatidylinositol 3'-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in Distinct Classes of Phosphatidylinositol 3 ˜-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in HT-29 Cells. *J. Biol. Chem.* 275, 992–998. doi:10.1074/jbc.275.2.992.

Previtali, S. C., Zerega, B., Sherman, D. L., Brophy, P. J., Dina, G., King, R. H. M., et al. (2003). Myotubularin-related 2 protein phosphatase and neurofilament light chain protein, both mutated in CMT neuropathies, interact in peripheral nerve. *Hum. Mol. Genet.* 12, 1713–1723. doi:10.1093/hmg/ddg179.

Prior, R., Van Helleputte, L., Benoy, V., and Den Bosch, L. Van (2017). Defective axonal

transport: A common pathological mechanism in inherited and acquired peripheral neuropathies. *Neurobiol. Dis.* doi:10.1016/j.nbd.2017.02.009.

Proikas-Cezanne, T., Ruckerbauer, S., Stierhof, Y. D., Berg, C., and Nordheim, A. (2007). Human WIPI-1 puncta-formation: A novel assay to assess mammalian autophagy. *FEBS Lett.* 581, 3396–3404. doi:10.1016/j.febslet.2007.06.040.

Puls, I., Jonnakuty, C., LaMonte, B. H., Holzbaur, E. L. F., Tokito, M., Mann, E., et al. (2003). Mutant dynactin in motor neuron disease. *Nat. Genet.* 33, 455–456. doi:10.1038/ng1123.

Qian, J., Ren, X., Wang, X., Zhang, P., Jones, W. K., Molkentin, J. D., et al. (2009). Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circ. Res.* 105, 1223–1231. doi:10.1161/CIRCRESAHA.109.200378.

Quinlan, R. A., Brenner, M., Goldman, J. E., and Messing, A. (2007). GFAP and its role in Alexander disease. *Exp. Cell Res.* 313, 2077–2087. doi:10.1016/j.yexcr.2007.04.004.

Rahvar, M., Nikseresht, M., Shafiee, S. M., Naghibalhossaini, F., Rasti, M., Panjehshahin, M. R., et al. (2011). Effect of oral resveratrol on the BDNF gene expression in the hippocampus of the rat brain. *Neurochem. Res.* 36, 761–765. doi:10.1007/s11064-010-0396-8.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi:10.1038/nprot.2013.143.

Rangaraju, S., Verrier, J. D., Madorsky, I., Nicks, J., Dunn, W. a, and Notterpek, L. (2010). Rapamycin activates autophagy and improves myelination in explant cultures from neuropathic mice. *J. Neurosci.* 30, 11388–11397. doi:10.1523/JNEUROSCI.1356-10.2010.

Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., and Rubinsztein, D. C. (2010). Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nat. Cell Biol.* 12, 747–757. doi:10.1038/ncb2078.

Reggiori, F., and Tooze, S. a. (2012). Autophagy regulation through Atg9 traffic. *J. Cell Biol.* 198, 151–153. doi:10.1083/jcb.201206119.

Rekas, A., Adda, C. G., Andrew Aquilina, J., Barnham, K. J., Sunde, M., Galatis, D., et al. (2004). Interaction of the molecular chaperone αB-crystallin with α-Synuclein: Effects on amyloid fibril formation and chaperone activity. *J. Mol. Biol.* 340, 1167–1183. doi:10.1016/j.jmb.2004.05.054.

Rivière, J. B., Verlaan, D. J., Shekarabi, M., Lafrenière, R. G., Bénard, M., Der Kaloustian, V. M., et al. (2004). A mutation in the HSN2 gene causes sensory neuropathy type II in a lebanese family. *Ann. Neurol.* 56, 572–575. doi:10.1002/ana.20237.

Rivire, J. B., Ramalingam, S., Lavastre, V., Shekarabi, M., Holbert, S., Lafontaine, J., et al. (2011). KIF1A, an axonal transporter of synaptic vesicles, is mutated in hereditary sensory and autonomic neuropathy type 2. *Am. J. Hum. Genet.* 89, 219–301. doi:10.1016/j.ajhg.2011.06.013.

Rizzo, F., Ronchi, D., Salani, S., Nizzardo, M., Fortunato, F., Bordoni, A., et al. (2016). Selective mitochondrial depletion, apoptosis resistance, and increased mitophagy in human Charcot-Marie-Tooth 2A motor neurons. *Hum. Mol. Genet.* 25, 4266–4281.

Roberts-Clarke, D., Fornusek, C., Saigal, N., Halaki, M., Burns, J., Nicholson, G., et al. (2016). Relationship between physical performance and quality of life in Charcot-Marie-Tooth disease: a pilot study. *J. Peripher. Nerv. Syst.* doi:10.1111/jns.12191.

Roberts, R. C., Peden, A. a., Buss, F., Bright, N. a., Latouche, M., Reilly, M. M., et al. (2010). Mistargeting of SH3TC2 away from the recycling endosome causes Charcot-Marie-Tooth disease type 4C. *Hum. Mol. Genet.* 19, 1009–1018. doi:10.1093/hmg/ddp565.

Robinson, F. L., and Dixon, J. E. (2005). The phosphoinositide-3-phosphatase MTMR2 associates with MTMR13, a membrane-associated pseudophosphatase also mutated in type 4B Charcot-Marie-Tooth disease. *J. Biol. Chem.* 280, 31699–31707. doi:10.1074/jbc.M505159200.

Rose, C., Menzies, F. M., Renna, M., Acevedo-Arozena, A., Corrochano, S., Sadiq, O., et al. (2010). Rilmenidine attenuates toxicity of polyglutamine expansions in a mouse model of Huntington's disease. *Hum. Mol. Genet.* 19, 2144–2153. doi:10.1093/hmg/ddq093.

Rose, J. M., Novoselov, S. S., Robinson, P. a., and Cheetham, M. E. (2011). Molecular chaperone-mediated rescue of mitophagy by a Parkin RING1 domain mutant. *Hum. Mol.*

Genet. 20, 16–27. doi:10.1093/hmg/ddq428.

Rosenzweig, R., Moradi, S., Zarrine-Afsar, A., Glover, J. R., and Kay, L. E. (2013). Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. *Science (80-.).* 339, 1080–1083. doi:10.1126/science.1233066.

Rossor, a. M., Kalmar, B., Greensmith, L., and Reilly, M. M. (2012). The distal hereditary motor neuropathies. *J. Neurol. Neurosurg. Psychiatry* 83, 6–14. doi:10.1136/jnnp-2011-300952.

Rotthier, A., Baets, J., Timmerman, V., and Janssens, K. (2012). Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat. Rev. Neurol.* 8, 73–85. doi:10.1038/nrneurol.2011.227.

Russo, M., Laurá, M., Polke, J. M., Davis, M. B., Blake, J., Brandner, S., et al. (2011). Variable phenotypes are associated with PMP22 missense mutations. *Neuromuscul. Disord.* 21, 106–114. doi:10.1016/j.nmd.2010.11.011.

Sahni, S., Bae, D. H., Lane, D. J. R., Kovacevic, Z., Kalinowski, D. S., Jansson, P. J., et al. (2014). The metastasis suppressor, N-myc Downstream-regulated gene 1 (NDRG1), inhibits stress-induced autophagy in cancer cells. *J. Biol. Chem.* 289, 9692–9709. doi:10.1074/jbc.M113.529511.

Saio, T., Yokochi, M., and Inagaki, F. (2009). The NMR structure of the p62 PB1 domain, a key protein in autophagy and NF-κB signaling pathway. *J. Biomol. NMR* 45, 335–341. doi:10.1007/s10858-009-9370-7.

Saitsu, H., Nishimura, T., Muramatsu, K., Kodera, H., Kumada, S., Sugai, K., et al. (2013). De novo mutations in the autophagy gene WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood. *Nat. Genet.* 45, 445–449. doi:10.1038/ng.2562.

Salmon, P., and Trono, D. (2006). Production and Titration of Lentivial Vectors. *Curr. Protoc. Neurosci.* 4, 1–24. doi:10.1002/0471142301.ns0421s37.

Sanchez, E., Darvish, H., Mesias, R., Taghavi, S., Firouzabadi, S. G., Walker, R. H., et al. (2016). Identification of a Large DNAJB2 Deletion in a Family with Spinal Muscular Atrophy and Parkinsonism. *Hum. Mutat.* 37, 1180–1189. doi:10.1002/humu.23055.

Santel, a, and Fuller, M. T. (2001). Control of mitochondrial morphology by a human

mitofusin. *J. Cell Sci.* 114, 867–874. doi:mitochondrial fusion fzo human gtpase dynamin related protein mitofusin outer-membrane protein dynamin-related gtpase saccharomyces-cerevisiae coiled coils fusion yeast reticulum fzo1p maintenance fission.

Sanz, L. (2000). The atypical PKC-interacting protein p62 channels NF-kappa B activation by the IL-1-TRAF6 pathway. *EMBO J.* 19, 1576–1586. doi:10.1093/emboj/19.7.1576.

Sarkar, S., Davies, J. E., Huang, Z., Tunnacliffe, A., and Rubinsztein, D. C. (2007). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and ??-synuclein. *J. Biol. Chem.* 282, 5641–5652. doi:10.1074/jbc.M609532200.

Sasaki, T., Gotow, T., Shiozaki, M., Sakaue, F., Saito, T., Julien, J. P., et al. (2006). Aggregate formation and phosphorylation of neurofilament-L Pro22 Charcot-Marie-Tooth disease mutants. *Hum. Mol. Genet.* 15, 943–952. doi:10.1093/hmg/ddl011.

Schrepfer, E., and Scorrano, L. (2016). Mitofusins, from Mitochondria to Metabolism. *Mol. Cell* 61, 683–694. doi:10.1016/j.molcel.2016.02.022.

Schroer, T. a. (2004). Dynactin. *Annu. Rev. Cell Dev. Biol.* 20, 759–779. doi:10.1146/annurev.cellbio.20.012103.094623.

Schulze, R. J., and McNiven, M. a. (2014). A well-oiled machine DNM2/dynamin 2 helps keep hepatocyte lipophagy running smoothly. *Autophagy* 10, 388–389. doi:10.4161/auto.27486.

Sebastián, D., Sorianello, E., Segalés, J., Irazoki, A., Ruiz-Bonilla, V., Sala, D., et al. (2016). Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway. *EMBO J.*, e201593084. doi:10.15252/embj.201593084.

Selcen, D., and Engel, a G. (2003). Myofibrillar myopathy caused by novel dominant negative alpha B- crystallin mutations. *Ann Neurol* 54, 804–810. doi:10.1002/ana.10767.

Senderek, J., Bergmann, C., Ramaekers, V. T., Nelis, E., Bernert, G., Makowski, A., et al. (2003a). Mutations in the ganglioside-induced differentiation-associated protein-1 (GDAP1) gene in intermediate type autosomal recessive Charcot-Marie-Tooth neuropathy. *Brain* 126, 642–649. doi:10.1093/brain/awg068.

Senderek, J., Bergmann, C., Stendel, C., Kirfel, J., Verpoorten, N., De Jonghe, P., et al. (2003b). Mutations in a gene encoding a novel SH3/TPR domain protein cause

autosomal recessive Charcot-Marie-Tooth type 4C neuropathy. *Am. J. Hum. Genet.* 73, 1106–19. doi:10.1086/379525.

Shaw, C. E. (2010). Capturing VCP: Another Molecular Piece in the ALS Jigsaw Puzzle. *Neuron* 68, 812–814. doi:10.1016/j.neuron.2010.11.040.

Shintani, T., and Klionsky, D. J. (2004). Autophagy in Health and Disease : A Double-edged sword. 306, 990–996.

Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J.* 18, 5234–5241. doi:10.1093/emboj/18.19.5234.

Sivera, R., Espinõs, C., Vílchez, J. J., Mas, F., Martínez-Rubio, D., Chumillas, M. J., et al. (2010). Phenotypical features of the p.R120W mutation in the GDAP1 gene causing autosomal dominant Charcot-Marie-Tooth disease. *J. Peripher. Nerv. Syst.* 15, 334–344. doi:10.1111/j.1529-8027.2010.00286.x.

Spencer, B., Potkar, R., Trejo, M., Rockenstein, E., Patrick, C., Gindi, R., et al. (2009). Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J. Neurosci.* 29, 13578–88. doi:10.1523/JNEUROSCI.4390-09.2009.

Spillantini, M. G., Schmidt, M. L., Lee, V. M.-Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). alpha-Synuclein in Lewy bodies. *Nature* 388, 839–840. doi:10.1038/42166.

Stadel, D., Millarte, V., Tillmann, K. D., Huber, J., Tamin-Yecheskel, B. C., Akutsu, M., et al. (2015). TECPR2 Cooperates with LC3C to Regulate COPII-Dependent ER Export. *Mol. Cell* 60, 89–104. doi:10.1016/j.molcel.2015.09.010.

Stavoe, A. K. H., Hill, S. E., Hall, D. H., and Colón-Ramos, D. A. (2016). KIF1A/UNC-104 Transports ATG-9 to Regulate Neurodevelopment and Autophagy at Synapses. *Dev. Cell* 38, 171–85. doi:10.1016/j.devcel.2016.06.012.

Stendel, C., Roos, A., Kleine, H., Arnaud, E., ??z??elik, M., Sidiropoulos, P. N. M., et al. (2010). SH3TC2, a protein mutant in Charcot-Marie-Tooth neuropathy, links peripheral nerve myelination to endosomal recycling. *Brain* 133, 2462–2474. doi:10.1093/brain/awq168.

Stengel, F., Baldwin, A. J., Painter, A. J., Jaya, N., Basha, E., Kay, L. E., et al. (2010).

Quaternary dynamics and plasticity underlie small heat shock protein chaperone function. *Proc. Natl. Acad. Sci.* 107, 2007–2012. doi:10.1073/pnas.0910126107.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* 10, 513–25. doi:10.1038/nrm2728.

Stephan, J. S., Yeh, Y.-Y., Ramachandran, V., Deminoff, S. J., and Herman, P. K. (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17049–17054. doi:10.1073/pnas.0903316106.

Stojkovic, T. (2016). Hereditary neuropathies: An update. *Rev. Neurol. (Paris).* 172, 775–778. doi:10.1016/j.neurol.2016.06.007.

Sugiyama, Y., Suzuki, A., Akutsu, R., Hirose, T., Waye, M. M. Y., Tsui, S. K. W., et al. (2000). CELL BIOLOGY AND METABOLISM : Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of Myogenic Differentiation Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of MKBP / HSPB2 and HSPB3 during Myogenic D. 275, 1095–1104.

Sun, P., Zhang, S., Qin, X., Chang, X., Cui, X., Li, H., et al. (2017). Foot-and-mouth disease virus capsid protein VP2 activates the cellular EIF2S1-ATF4 pathway and induces autophagy via HSPB1. *Autophagy* 8627, 00–00. doi:10.1080/15548627.2017.1405187.

Sun, X., Zhou, Z., Fink, D. J., and Mata, M. (2013). HspB1 silences translation of PDZ-RhoGEF by enhancing miR-20a and miR-128 expression to promote neurite extension. *Mol. Cell. Neurosci.* 57, 111–119. doi:10.1016/j.mcn.2013.10.006.

Takahashi, Y., Tsotakos, N., Liu, Y., Young, M. M., Serfass, J., Tang, Z., et al. (2016). The Bif-1-Dynamin 2 membrane fission machinery regulates Atg9-containing vesicle generation at the Rab11-positive reservoirs. *Oncotarget* 7. doi:10.18632/oncotarget.8028.

Tang, B. S., Zhao, G. H., Luo, W., Xia, K., Cai, F., Pan, Q., et al. (2005). Small heat-shock protein 22 mutated in autosomal dominant Charcot-Marie-Tooth disease type 2L. *Hum. Genet.* 116, 222–224. doi:10.1007/s00439-004-1218-3.

Tang, D., Kang, R., Livesey, K. M., Kroemer, G., Billiar, T. R., Van Houten, B., et al. (2011). High-mobility group box 1 is essential for mitochondrial quality control. *Cell*

Metab. 13, 701–711. doi:10.1016/j.cmet.2011.04.008.

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., et al. (1999). Apg7p/Cvt2p: A Novel Protein-activating Enzyme Essential for Autophagy. *Mol. Biol. Cell* 10, 1367–1379. doi:10.1091/mbc.10.5.1367.

Taylor, R. P., and Benjamin, I. J. (2005). Small heat shock proteins: A new classification scheme in mammals. *J. Mol. Cell. Cardiol.* 38, 433–444. doi:10.1016/j.yjmcc.2004.12.014.

Teyssou, E., Takeda, T., Lebon, V., Boillée, S., Doukouré, B., Bataillon, G., et al. (2013). Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: Genetics and neuropathology. *Acta Neuropathol.* 125, 511–522. doi:10.1007/s00401-013-1090-0.

Thomas, P. D. P. K. (2005). *Peripheral Neuropathy*. 4th ed. , ed. P. Dyck Saunders Available at: https://www.elsevier.com/books/peripheral-neuropathy/dyck/978-0-7216-9491-7.

Timmerman, V., Nelis, E., Van Hul, W., Nieuwenhuijsen, B. W., Chen, K. L., Wang, S., et al. (1992). The peripheral myelin protein gene PMP-22 is contained within the Charcot-Marie-Tooth disease type 1A duplication. *Nat. Genet.* 1, 171–5. doi:10.1038/ng0692-171.

Timmerman, V., Strickland, A. V., and Züchner, S. (2014). Genetics of Charcot-Marie-Tooth (CMT) disease within the frame of the human genome project success. *Genes (Basel).* 5, 13–32. doi:10.3390/genes5010013.

Towers, C. G., and Thorburn, A. (2016). Therapeutic Targeting of Autophagy. *EBioMedicine* 14, 15–23. doi:10.1016/j.ebiom.2016.10.034.

Treweek, T. M., Ecroyd, H., Williams, D. M., Meehan, S., Carver, J. A., and Walker, M. J. (2007). Site-directed mutations in the C-terminal extension of human ??B-crystallin affect chaperone function and block amyloid fibril formation. *PLoS One* 2. doi:10.1371/journal.pone.0001046.

V.A. Street, C.L. Bennett, J.D. Goldy, A.J. Shirk, K.A. Kleopa, B.L Tempel, et al. (2003). Mutation of a putative protein degradation gene LITAF/SIMPLE in Charcot-Marie-Tooth disease 1C. *Neurology* 60, 22–26. doi:10.1212/WNL.60.1.22.

Vaccari, I., Carbone, A., Previtali, S. C., Mironova, Y. A., Alberizzi, V., Noseda, R., et al. (2015). Loss of Fig4 in both Schwann cells and motor neurons contributes to CMT4J

neuropathy. *Hum. Mol. Genet.* 24, 383–396. doi:10.1093/hmg/ddu451.

Vallat, J. M., Goizet, C., Tazir, M., Couratier, P., Magy, L., and Mathis, S. (2016). Classifications of neurogenetic diseases: An increasingly complex problem. *Rev. Neurol. (Paris).* 172, 339–349. doi:10.1016/j.neurol.2016.04.005.

Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998). The small heat-shock protein IbpB from Escherichia coli stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem.* 273, 11032–11037. doi:10.1074/jbc.273.18.11032.

Verhoeven, K., De Jonghe, P., Coen, K., Verpoorten, N., Auer-Grumbach, M., Kwon, J. M., et al. (2003). Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. *Am J Hum Genet* 72, 722–727. doi:10.1086/367847.

Vicart, P., Caron, a, Guicheney, P., Li, Z., Prévost, M. C., Faure, a, et al. (1998). A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat. Genet.* 20, 92–5. doi:10.1038/1765.

Vingtdeux, V., Giliberto, L., Zhao, H., Chandakkar, P., Wu, Q., Simon, J. E., et al. (2010). AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-β peptide metabolism. *J. Biol. Chem.* 285, 9100–9113. doi:10.1074/jbc.M109.060061.

Vita, G., La Foresta, S., Russo, M., Vita, G. L., Messina, S., Lunetta, C., et al. (2016). Sport activity in Charcot-Marie-Tooth disease: A case study of a Paralympic swimmer. *Neuromuscul. Disord.* 26, 614–618. doi:10.1016/j.nmd.2016.06.002.

Vos, M. J., Hageman, J., Carra, S., and Kampinga, H. H. (2008). Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. *Biochemistry* 47, 7001–7011. doi:10.1021/bi800639z.

Vos, M. J., Zijlstra, M. P., Carra, S., Sibon, O. C. M., and Kampinga, H. H. (2011). Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy* 7, 101–103. doi:10.4161/auto.7.1.13935.

Vos, M. J., Zijlstra, M. P., Kanon, B., van Waarde-Verhagen, M. a W. H., Brunt, E. R. P., Oosterveld-Hut, H. M. J., et al. (2010). HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones. *Hum. Mol. Genet.* 19,

4677–4693. doi:10.1093/hmg/ddq398.

Wagstaff, M. J. D., Smith, J., De, J. S., Coffin, R. S., David, S., and Belleroche, J. S. De (1999). NUCLEIC ACIDS , PROTEIN SYNTHESIS , AND MOLECULAR GENETICS : Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus-based Vector Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus. 274, 5061–5069. doi:10.1074/jbc.274.8.5061.

Wang, Z., Hu, J., Li, G., Qu, L., He, Q., Lou, Y., et al. (2014). PHF23 (plant homeodomain finger protein 23) negatively regulates cell autophagy by promoting ubiquitination and degradation of E3 ligase LRSAM1. *Autophagy* 10, 2158–2170. doi:10.4161/auto.36439.

Watanabe, Y., Tsujimura, A., Taguchi, K., and Tanaka, M. (2017). HSF1 stress response pathway regulates autophagy receptor SQSTM1/p62-associated proteostasis. *Autophagy* 13, 133–148. doi:10.1080/15548627.2016.1248018.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., et al. (2005). Thermotolerance Requires Refolding of Aggregated Proteins by Substrate Translocation through the Central Pore of ClpB. *Trends Biochem. Sci.* 30, i. doi:10.1016/S0968-0004(05)00043-5.

Weis, J., Claeys, K. G., Roos, A., Azzedine, H., Katona, I., Schr??der, J. M., et al. (2016). Towards a functional pathology of hereditary neuropathies. *Acta Neuropathol.*, 1–23. doi:10.1007/s00401-016-1645-y.

Westhoff, B., Chapple, J. P., Van Der Spuy, J., H??hfeld, J., and Cheetham, M. E. (2005). HSJ1 is a neuronal shuttling factor for the sorting of chaperone clients to the proteasome. *Curr. Biol.* 15, 1058–1064. doi:10.1016/j.cub.2005.04.058.

Weterman, M. a J., Sorrentino, V., Kasher, P. R., Jakobs, M. E., van Engelen, B. G. M., Fluiter, K., et al. (2012). A frameshift mutation in LRSAM1 is responsible for a dominant hereditary polyneuropathy. *Hum. Mol. Genet.* 21, 358–370. doi:10.1093/hmg/ddr471.

Wiesner, D., Sinniger, J., Henriques, A., Dieterlé, S., Müller, H. P., Rasche, V., et al. (2015). Low dietary protein content alleviates motor symptoms in mice with mutant dynactin/dynein-mediated neurodegeneration. *Hum. Mol. Genet.* 24, 2228–2240.

doi:10.1093/hmg/ddu741.

Wilhelmus, M. M. M., Boelens, W. C., Otte-Höller, I., Kamps, B., Kusters, B., Maat-Schieman, M. L. C., et al. (2006a). Small heat shock protein HspB8: Its distribution in Alzheimer's disease brains and its inhibition of amyloid-β protein aggregation and cerebrovascular amyloid-β toxicity. *Acta Neuropathol.* 111, 139–149. doi:10.1007/s00401-005-0030-z.

Wilhelmus, M. M. M., Otte-Höller, I., Wesseling, P., De Waal, R. M. W., Boelens, W. C., and Verbeek, M. M. (2006b). Specific association of small heat shock proteins with the pathological hallmarks of Alzheimer's disease brains. *Neuropathol. Appl. Neurobiol.* 32, 119–130. doi:10.1111/j.1365-2990.2006.00689.x.

Windpassinger, C., Auer-Grumbach, M., Irobi, J., Patel, H., Petek, E., Hörl, G., et al. (2004). Heterozygous missense mutations in BSCL2 are associated with distal hereditary motor neuropathy and Silver syndrome. *Nat. Genet.* 36, 271–276. doi:10.1038/ng1313.

Wong, P.-M., Feng, Y., Wang, J., Shi, R., and Jiang, X. (2015). Regulation of autophagy by coordinated action of mTORC1 and protein phosphatase 2A. *Nat. Commun.* 6, 8048. doi:10.1038/ncomms9048.

Wurzer, B., Zaffagnini, G., Fracchiolla, D., Turco, E., Abert, C., Romanov, J., et al. (2015). Oligomerization of p62 allows for selection of ubiquitinated cargo and isolation membrane during selective autophagy. *Elife* 4, 1–28. doi:10.7554/eLife.08941.

Wyttenbach, A. (2002). Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* 11, 1137–1151. doi:10.1093/hmg/11.9.1137.

Xiaoren Tang, Daniel Metzger, Susan Leeman, and S. A. (2007). Correction for Tang et al., LPS-induced TNF- factor (LITAF)-deficient mice express reduced LPS-induced cytokine: Evidence for LITAF-dependent LPS signaling pathways. *Proc. Natl. Acad. Sci.* 104, 3015–3015. doi:10.1073/pnas.0700697104.

Yang, Z., and Klionsky, D. J. (2010a). Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* 12, 814–822. doi:10.1038/ncb0910-814.

Yang, Z., and Klionsky, D. J. (2010b). Mammalian autophagy: Core molecular machinery and signaling regulation. *Curr. Opin. Cell Biol.* 22, 124–131.

doi:10.1016/j.ceb.2009.11.014.

Zhan, L., Yang, Y., Ma, T. T., Huang, C., Meng, X. M., Zhang, L., et al. (2015). Transient receptor potential vanilloid 4 inhibits rat HSC-T6 apoptosis through induction of autophagy. *Mol. Cell. Biochem.* 402, 9–22. doi:10.1007/s11010-014-2298-6.

Zhang, F., Kumano, M., Beraldi, E., Fazli, L., Du, C., Moore, S., et al. (2014). Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. *Nat. Commun.* 5, 1–13. doi:10.1038/ncomms6775.

Zhang, H., Rajasekaran, N. S., Orosz, A., Xiao, X., Rechsteiner, M., and Benjamin, I. J. (2010). Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin-proteasome pathways. *J. Mol. Cell. Cardiol.* 49, 918–930. doi:10.1016/j.yjmcc.2010.09.004.

Zhang, X., Chen, S., Huang, K., and Le, W. (2013). Why should autophagic flux be assessed? *Acta Pharmacol. Sin.* 34, 595–9. doi:10.1038/aps.2012.184.

Zhou, J., Yang, Z., Tsuji, T., Gong, J., Xie, J., Chen, C., et al. (2011). LITAF and TNFSF15, two downstream targets of AMPK, exert inhibitory effects on tumor growth. *Oncogene* 30, 1892–1900. doi:10.1038/onc.2010.575.

Zobel, A. T. C., Loranger, A., Marceau, N., Thériault, J. R., Lambert, H., and Landry, J. (2003). Distinct chaperone mechanisms can delay the formation of aggresomes by the myopathy-causing R120G αB-crystallin mutant. *Hum. Mol. Genet.* 12, 1609–1620. doi:10.1093/hmg/ddg173.

Züchner, S., Mersiyanova, I. V, Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E. L., et al. (2004). Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. *Nat. Genet.* 36, 449–451. doi:10.1038/ng1341.

Züchner, S., Noureddine, M., Kennerson, M., Verhoeven, K., Claeys, K., De Jonghe, P., et al. (2005). Mutations in the pleckstrin homology domain of dynamin 2 cause dominant intermediate Charcot-Marie-Tooth disease. *Nat. Genet.* 37, 289–294. doi:10.1212/WNL.64.10.1826-a.

Żwirowski, S., Kłosowska, A., Obuchowski, I., Nillegoda, N. B., Piróg, A., Ziętkiewicz, S., et al. (2017). Hsp70 displaces small heat shock proteins from aggregates to initiate protein refolding. *EMBO J.* 36, 783–796. doi:10.15252/embj.201593378.

CHAPTER 2

Small heat shock proteins: Their role in proteostasis and neurodegeneration

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ABSTRACT

The small heat shock proteins (sHSPs) are a family of stress-induced molecular chaperones. They play an important role in the prevention of protein aggregation and maintenance of protein homeostasis (proteostasis). Mammalian sHSPs, HSPBs, are a family of ten members of proteins sharing a highly conserved alpha-crystallin domain necessary for the modulation of their functional and structural properties. Classically, HSPBs act as the first line of defence against aggregation prone proteins by binding to these misfolded substrates and allowing ATP-dependent chaperones to refold them. The role of HSPBs in preventing protein aggregation is not limited to their chaperone activity. Several members of this protein family can activate degradative pathways such as autophagy and the proteasome. The different HSPB proteins have different anti-aggregation and pro-degradation functions. They can have distinct substrates for their molecular chaperone function, and they can activate different degradative pathways. Due to their role in proteostasis, HSPBs are also highly implicated in neurodegenerative and neuromuscular disorders. The levels and activity of HSPBs are associated with many protein deposit disorders of both the central and peripheral nervous system. In addition, mutations in genes coding for HSPBs, lead to a variety of neuropathic and myopathic diseases. In this chapter, I will briefly describe the HSPB protein family. Then I will further elaborate on what is known about their anti-aggregation and pro-degradation functions. In the last part, I will provide an overview of their links to neurodegenerative and neuromuscular disorders due to either protein deposits or to mutations in the *HSPB* genes.

1. The small heat shock proteins:

The small heat shock proteins are a family of molecular chaperones with a relatively low molecular weight (ranging from 15 to 45 kDa). In mammals, this family, also called the HSPB proteins, consists of 10 members (HSPB1-HSPB10). HSPBs have been divided to two classes depending on their tissue distribution. Class I includes ubiquitously expressed HSPBs (HSPB1, HSPB5, HSPB6, HSPB8); while class II includes HSPBs with tissue-restricted pattern of expression (HSPB2, HSPB3, HSPB4, HSPB7, HSPB9, HSPB10) (Taylor and Benjamin, 2005) (Table 1).

In addition to their small molecular weight and their induction by stress, HSPBs share characteristic features including: (i) the presence of a highly conserved alpha-crystallin domain (ACD), and (ii) the tendency to form large oligomers and dynamic quaternary structures (Haslbeck et al., 2005).

The ACD, a region of ~ 90 residues, consists of six to eight β-strands organized in two β-sheets and is flanked by C- and N-terminal extensions which vary in length and structure between different HSPBs. The ACD plays an important role in regulating the structural and functional properties of HSPBs. Through the ACD the HSPBs are capable of forming dynamic interactions of variable sizes and natures and of binding to protein substrates. The flexible N- and C-terminal loops stabilize these interactions (Laganowsky et al., 2010; Lambert et al., 1999). The N-terminus contains multiple phosphorylation sites that can modulate the formation of dimers by HSPBs (McDonald et al., 2012). The HSPB dimers are the building units of larger quaternary structures (oligomers) (Vos et al., 2008). These oligomers are subject to a dynamic association/dissociation process believed to be essential to the function of HSPB proteins (Datskevich et al., 2012).

HSPBs are biochemically defined as molecular chaperones and they play a role in protein quality control. HSPB proteins bind to misfolded, aggregation-prone proteins and in conjunction with ATP-dependent chaperones (e.g. HSP70) prevent their aggregation. The fate of HSPB-bound clients can be either refolding by the ATP-dependent chaperones or degradation by the proteasome or autophagy.

Below, I summarize the anti-aggregation properties of HSPBs. I have divided these properties into two categories: 1) The classical molecular chaperone function and 2) the ability to stimulate degradative pathways such as autophagy and the proteasome.

Name	**Expression**	**Degradative Pathway**	**Target disease aggregates**	**Associated disease(s)**
HSPB1	Ubiquitous	Proteasome Autophagy	ALS: SOD1	CMT2F, dHMN II, Cancer, ischemia and reperfusion
HSPB2 (MKBP)	Heart and skeletal muscle	N.A.	N.A.	Myotonic dystrophy
HSPB3	Heart, Brain, Skeletal and smooth muscle	N.A.	N.A.	dHMN II
HSPB4 (αA-crystallin)	Eye lens, Skeletal muscle	N.A.	N.A.	Cataract
HSPB5 (αB-crystallin)	Ubiquitous	N.A.	α-synuclein Amyloid β SOD1	Cataract, desmin-related myopathy, cardiomyopathy, ischemia and reperfusion
HSPB6	Ubiquitous	Autophagy	PolyQ aggregates (Huntingtin)	Cardiomyopathy, ischemia and reperfusion
HSPB7	Heart and skeletal muscle	Autophagy	PolyQ aggregates (Huntingtin)	Muscular dystrophy
HSPB8	Ubiquitous	Autophagy	PolyQ aggregates, SOD1, TDP43	CMT2L, dHMNII, cancer
HSPB9 (CT51)	Testis	Proteasome	PolQ aggregates	Cancer
HSPB10 (ODF27)	Testis	N.A.	N.A.	N.A.

Table 1. An overview of the 10 different HSPB proteins, their expression pattern and known associations with anti-aggregation and disease. N.A. = Not Applicable

2.1. Anti-aggregation properties of HSPBs: Molecular Chaperone activity:

As a result of diverse stress conditions (e.g. temperature, oxidative stress), the formation of misfolded, aggregation-prone proteins increases. Aggregating proteins not only lose their activity, but can also promote the aggregation of other proteins (Kim et al., 2013). HSPBs perform their task as molecular chaperones by binding to and stabilizing the early unfolding intermediates of these proteins. Since HSPBs need to bind to their substrates while they are unfolding, not before nor after; they are among the first to respond to cell stress (Haslbeck and Vierling, 2015). The binding of HSPBs to their substrates is ATP-independent and leads to the formation of assemblies containing both the HSPBs and the substrates (Lee et al., 1997) (Fig1). The substrate proteins are retrieved from these assemblies by ATP-dependent chaperones, namely HSP70 and HSP100 systems reducing the need for resynthesis of essential stress-recovery proteins (Veinger et al., 1998). HSP70 and HSP100 cooperate to perform their disaggregation and refolding actions (Glover and Lindquist, 1998). First HSP70 binds to the aggregates, then it recruits HSP100 hexamers (Rosenzweig et al., 2013). The activated HSP100 then extracts the captured polypeptides by running them through its central channel (Weibezahn et al., 2005) (Fig1). The initial binding of HSPBs to substrate proteins prevents the formation of large aggregates and provides a highly accessible surface for the HSP70-HSP100 machinery. Recently, it has been shown that HSPB-substrate assemblies comprise two substructures: a stable core of HSPB-bound substrates and a dynamic outer shell. The outer shell consists exclusively of HSPBs which are later displaced by HSP70 in the refolding process (Żwirowski et al., 2017) (Fig1).

The stability of the interactions between HSPBs and their substrate is determined by the identity of the substrate, the degree to which it is unfolded and the specific properties of the HSPB (Stengel et al., 2010). How HSPBs recognize denaturing protein substrates remains unknown. Some studies used proteomic approaches to check which cytosolic proteins are associated with HSPBs under heat shock conditions (Basha et al., 2004). The identified proteins reflected a great promiscuity despite an apparent preference for translation-related proteins (Basha et al., 2004). One observation points out that HSPBs are less effective in preventing the aggregation of larger proteins, indicating a correlation with the mass ratio rather than the molar ratio, and a charge- and/or hydrophobicity-oriented binding to substrates (Basha et al., 2012). It remains also poorly understood which regions of substrate proteins do HSPBs bind to and whether they share common recognition motifs. Early studies using hydrophobic dyes suggested that substrates are bound to the N-terminal extension of HSPBs (Lee et al., 1997).

This claim is supported by studies using cross-linking and mass spectrometry analysis (Cheng et al., 2008; Jaya et al., 2009). On the other hand, other studies using directed mutations point to the involvement of the ACD and the C-terminal extension (Treweek et al., 2007). The ACD is however less accessible than the flanking termini due to the hydrophobic groove between its β4 and β8 strands, and few of the interactions of the ACD with substrates have been confirmed in cross-linking experiments (Jaya et al., 2009; Patel et al., 2014). Put together, binding to substrates seems mainly attained by the non-conserved sequences outside the ACD, which may justify the variability in substrate specificity among different HSPBs.

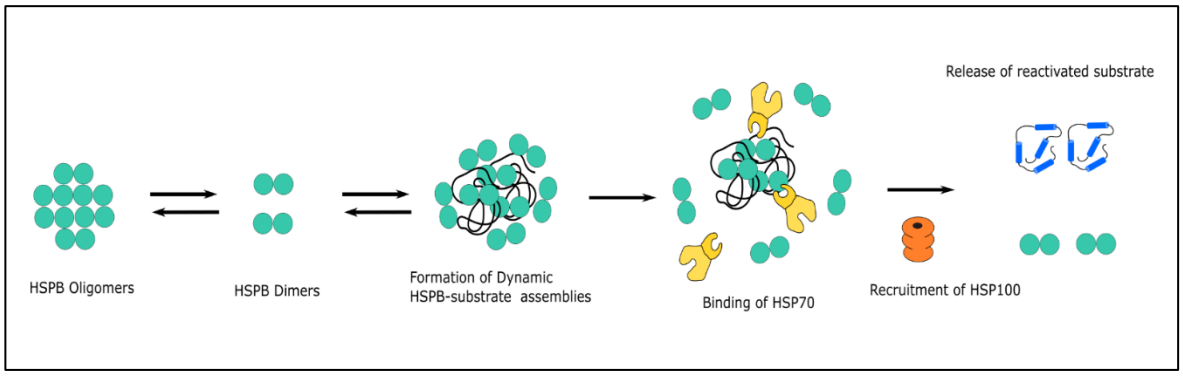

Fig.1: The molecular chaperone activity of HSPBs

HSPBs exist in dynamic structures of large oligomers and the active dimers. The dimers bind to aggregate-prone substrates, forming dynamic assemblies that are accessible to the ATP-dependent chaperones HSP70 and its co-chaperones, and HSP90. These assemblies consist of a core formed of HSPBs and substrates and an outer HSPB-only shell. Binding of HSP70 displaces the HSPBs on the outer shell and recruits HSP100 hexamers. HSP100 then extracts the reactivated protein substrates through its channel and releases the bound HSPBs.

2.2. Anti-aggregation properties of HSPBs: stimulating degradative pathways:

A study by Vos et. al in 2010 compared the anti-aggregation efficiency of the different HSPBs, some of them showed strong aggregate clearance capacity yet low efficiency in facilitating refolding of client proteins (Vos et al., 2010). These data propose that different HSPB proteins have not only different client specificity but may have different impact on client processing. Indeed some of the HSPB members seem to have the potential to stimulate or enhance proteasomal and/or autophagic degradation of misfolded proteins. Interestingly, different HSPBs trigger degradative pathways in distinct ways (Fig 2, Table 1). This feature is in line with the ability of HSPBs to interact with different proteins and modulate different cellular functions (Carra et al., 2013). Below I summarize the HSPBs with known function in facilitating the proteasome and/or autophagy degradation pathways.

2.2.1. HSPB1:

HSPB1 has been reported to mediate the proteasomal degradation of HSPB5, another member of the small heat shock proteins family. It was shown that HSPB1 expression increases the polyubiquitination of aggregation-prone HSPB5, and directs it for proteasomal degradation (Zhang et al., 2010). While the exact nature of the pro-proteasomal action of HSPB1 is not clear, HSPB1 has been reported as an ubiquitin-binding protein and a binding partner to the 26S proteasome subunit (Parcellier et al., 2003) (Fig 2). This binding property of HSPB1 is necessary for the proteasomal degradation of phosphorylated IκBα where HSPB1 also mediates its ubiquitination and proteasomal recognition (Parcellier et al., 2003). On the other hand, a few studies hinted at the possible function of HSPB1 in stimulating autophagy. First, the wide repertoire of HSPB1-interacting partners, and its ability to mediate diverse cellular pathways, particularly those related to cell survival, hints at a possible function in autophagy (Katsogiannou et al., 2014b). Furthermore, the participation of HSPB1 in clearing out a wide array of aggregating proteins (An et al., 2009; Vos et al., 2010; Wyttenbach, 2002) points to a function in activating a more bulk-degradation pathway such as autophagy. More recently, HSPB1 overexpression has been shown to increase the autophagic flux in kidney cells (Matsumoto et al., 2015b). However, a concrete connection and an exact mechanism of action of HSPB1 in autophagy has never been revealed before.

2.2.2. HSPB6:

Earlier studies have shown that HSPB6 can modulate autophagy. It was shown that phosphorylation of HSPB6 activates autophagy in ischemic injury to protect from cell death. Mouse models expressing mutant forms of HSPB6 targeting the serine 16 phosphorylation site show decreased autophagic flux and high sensitivity of the hearts to ischemia/reperfusion injury (Qian et al., 2009). More recently, a clearer view of the role of HSPB6 in autophagy has been revealed using models of cardiomyopathy caused by mutations in *HSPB6*. It was shown that HSPB6 interacts with the autophagy receptor BECN1/Beclin 1 (Liu et al., 2017) (Fig 2). BECN1 serves as a checkpoint between autophagy and apoptosis. Binding of BECN1 to autophagy-related proteins such as ATG14 leads to upregulation of autophagy, while binding to the apoptosis regulator BCL2 activates apoptosis (Pattingre et al., 2005). Recent data suggest that HSPB6 binding to BECN1 suppresses the binding of BECN1 to BCL2 thus stimulating autophagy and downregulating the apoptosis pathway (Liu et al., 2017).

2.2.3. HSPB7:

HSPB7 is one of the most potent HSPB proteins in suppressing polyglutamine protein aggregation. The ability of HSPB7 to suppress polyglutamine aggregation has been tested both in vitro, in cell lines, and in vivo in Drosophila models (Vos et al., 2010). The counter-aggregation activity of HSPB7 does not seem dependent on a chaperone-like refolding activity neither on proteasomal degradation. However, HSPB7 fails to clear out polyglutamine aggregates in cells where ATG5, a crucial gene for the stimulation of autophagy is knocked out (Vos et al., 2010). Though the exact link between HSPB7 and autophagy remains unclear, this indicates, that HSPB7 needs a functional autophagy machinery for the clearing of aggregates. Interestingly, HSPB7 expression levels do not affect the autophagy flux. The more likely mechanism is that HSPB7 primes early aggregates for degradation by autophagy (Vos et al., 2011).

2.2.4. HSPB8:

Several studies show that the anti-aggregation activity of HSPB8 is mediated by autophagy (Carra et al., 2008a, 2008b; Crippa et al., 2010b). In this process, HSPB8 forms a stable CASA (chaperone assisted selective autophagy) complex with the co-chaperone BAG3 (Bcl-2-associated athanogene 3) and HSC70 (Heat shock cognate protein 70) (Carra et al., 2008b) (Fig 2). The association with BAG3 is crucial for the pro-degradative function of HSPB8 as BAG3 is required for the stimulation of autophagy and its knock-down prevents HSPB8 from exerting

its protective function (Carra et al., 2008a). The current view suggests that HSPB8 recognizes the degradation targets and delivers them to the autophagosomes for degradation via BAG3. In addition to stimulating autophagy, HSPB8 plays a role in translational shut-down during proteotoxic stress. HSPB8 together with BAG3 perform this task by inducing the phosphorylation of eIF2 alpha (eukaryotic initiation factor 2 alpha) (Carra et al., 2009a). The exact manner of which HSPB8-BAG3 complex phosphorylates eIF2 alpha, and the link between the stimulation of autophagy and repression of translation remains poorly understood.

2.2.5- HSPB9:

HSPB9 is expressed exclusively in the testis, rendering it a less attractive candidate to study in more broad spectra. Nevertheless, it has been reported that expressing HSPB9 in cell models can aid the degradation of nucleating polyglutamine aggregates by the proteasome machinery (Carra et al., 2013). How HSPB9 links its targets to the proteasome is still to be elucidated.

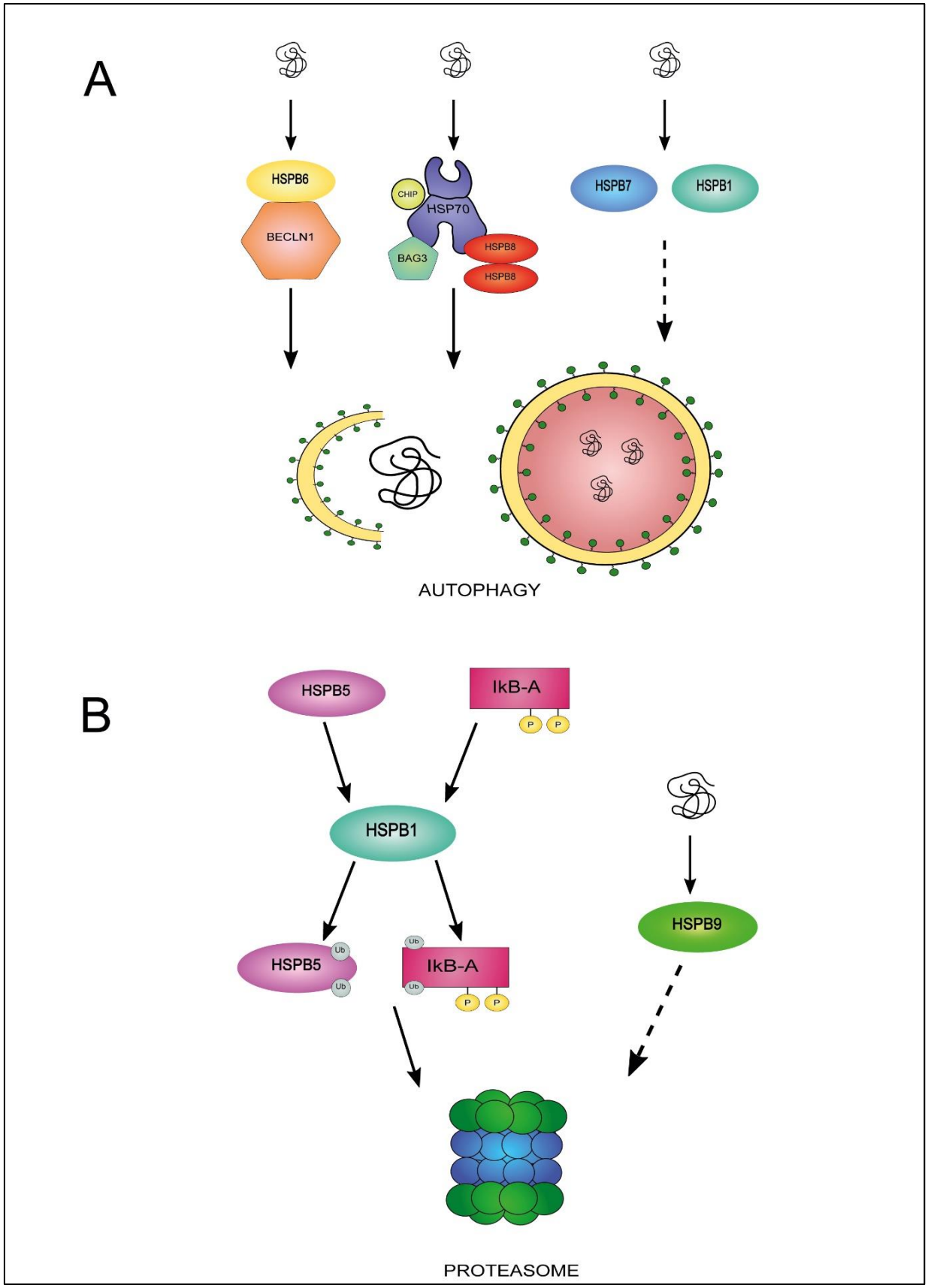
A
HSPB6
BECLN1
CHIP
HSP70
BAG3
HSPB8
HSPB8
HSPB7
HSPB1
AUTOPHAGY
B
HSPB5
IkB-A
P
P
HSPB1
HSPB5
Ub
Ub
IkB-A
Ub
P
P
HSPB9
PROTEASOME

Fig.2: Roles of HSPBs in stimulating degradative pathways

A- Different HSPBs have been reported to promote the autophagic clearance of aggregates. HSPB6 initiates autophagy by interacting with the autophagy receptor BCLN1. HSPB8 on the other hand, acts in a Chaperone Assisted Selective Autophagy (CASA) complex containing HSP70, CHIP, and BAG3 to direct aggregates for degradation by autophagy. HSPB7, does not activate the autophagy pathway but rather primes its substrates to be cleared out by autophagy in a yet unknown mechanism. On the other hand, a role for HSPB1 in autophagy has also been suggested even though the exact mechanism of this role is not fully understood. **B-** Some members of the HSPB family also stimulate the proteasome degradation. HSPB9 can direct protein aggregates to the proteasome when expressed in different cell models. HSPB1 has been shown to enhance the poly-ubiquitination of HSPB5 and of IkB-A, thus labelling them for degradation by the proteasome.

3. HSPBs in neurodegenerative and neuromuscular diseases:

HSPBs have an integral association with neurodegenerative and neuromuscular diseases. This association stems from two main observations: i) several neurodegenerative diseases are marked with protein deposits and aggregations and a heightened expression of HSPBs. ii) Mutations in HSPBs can lead to neuropathies, myopathies and neuromuscular disorders. Below I expand more on these two topics and what is known about them so far.

3.1. HSPBs and protein deposit diseases:

Protein deposit diseases are characterized by the accumulation of insoluble protein aggregates leading to cell degeneration and are usually aggravated with aging (Bucciantini, 2002). These diseases can be classified depending on the nature of protein deposits. Polyglutamine protein aggregates are seen in Huntington (huntingtin), spinocerebellar ataxia type-3 (Ataxin-3), and spinobulbar muscular atrophy, SBMA (mutant androgen receptor). Non-polyglutamine proteins are hallmark deposits in Alzheimer's disease (mutant amyloid beta), Alexander disease (GFAP), and amyotrophic lateral sclerosis, ALS (SOD1, TDP43) (J. Cummings and Y. Zoghbi, 2000). The anti-aggregation properties of the HSPB family members make them a compelling role players in fighting mutant aggregate proteins.

HSPBs have been shown to associate with the pathological hallmarks in Alzheimer's disease brains. HSPB1, HSPB2, HSPB6 and HSPB8 were shown to be extracellularly expressed in classic senile plaques (SP), with HSPB6 also being expressed in brain lesions of diffuse senile plaques (Wilhelmus et al., 2006a, 2006b). In addition, HSPB2 and HSPB8 were shown to be expressed in brain lesions of cerebral amyloid angiopathy (CAA) while HSPB1 and HSPB5 are highly detected in astrocytes associated with both CAA and SP lesions (Wilhelmus et al., 2006b). Interestingly, HSPBs have also been detected in alpha-synucleinopathy cases of dementia with Lewi bodies (DLB) and Parkinson's disease (Outeiro et al., 2006; Spillantini et al., 1997). These observations suggest a correlation between HSPBs and neurodegenerative diseases marked with protein aggregation. Indeed, several studies using overexpression of some HSPB proteins showed that they can decrease the aggregation of mutant proteins and/or increase their clearance (Bailey et al., 2002; Bilen and Bonini, 2007; Crippa et al., 2010a; Vos et al., 2010). Interestingly, the ability of HSPB proteins to inhibit aggregation seems to be different between the different members of HSPBs and the different type of aggregates (Table 1). HSPB6, HSPB7, HSPB8, and HSPB9 show strong inhibition of mutated polyglutamine

protein aggregation (Vos et al., 2010). HSPB7, the most potent of the four, could inhibit polyglutamine protein aggregates with both long and short stretches, while HSPB6, HSPB8, and HSPB9 are mainly efficient against short polyglutamine containing proteins. The manner by which these proteins suppress polyglutamine aggregation is also different. HSPB7 seems to keep aggregation prone proteins in a state competent for autophagic degradation (see above). HSPB8 and HSPB6 each activate the autophagic degradation machinery in a different way (Carra et al., 2008b; Liu et al., 2017). HSPB9 on the other hand is an activator of proteasomal degradation (Carra et al., 2013). HSPB1 and HSPB5, though ineffective against polyglutamine protein aggregation are capable of inhibiting other types of aggregates. HSPB5 shows potent inhibition of α-synuclein and GFAP (Quinlan et al., 2007; Rekas et al., 2004). HSPB1, on the other hand, can suppress the aggregation of mutant SOD1 (An et al., 2009). Intriguingly, even though overexpression of HSPB1 fails to inhibit aggregation in Huntington's disease cell models, it still exerts cell-protective effects there and lead to reduction in cell death (Wyttenbach, 2002). The ability of different HSPBs to inhibit the accumulation of aggregates in multiple neurodegenerative disorders has proposed their expression as an interesting therapeutic target in these diseases (Kampinga and Garrido, 2012).

3.2. Neuropathic and myopathic diseases caused by mutations in HSPBs:

3.2.1. HSPB1:

Mutations in *HSPB1* are associated with distal hereditary motor neuropathies (dHMN) and the axonal form of Charcot-Marie-Tooth disease (CMT2) (Evgrafov et al., 2004; Irobi et al., 2004). The majority of mutations in *HSPB1* leading to inherited neuropathies occur in the ACD, but mutations in the N- or C-terminus also exist (Adriaenssens et al., 2017) (Fig 3). There is of yet no clear relationship between the position of the mutation and the type of peripheral neuropathy. Interestingly, several mutations are located in key regions of the protein that might influence its ability to form dimers and oligomers (Almeida-Souza et al., 2010). Still exactly how mutations in *HSPB1* lead to neuropathic phenotypes remains unclear. Studies on the C-terminal P182L mutant show that it can be intrinsically unstable and form aggregates that sequester neurofilament middle chain subunit (NF-M) and dynactin in primary cortical neurons (Ackerley et al., 2006). Another way mutations in *HSPB1* contribute to disease might be the altered binding properties of mutant proteins to specific clients. R127W and S135F mutations

display increased chaperone activity and overstabilisation of microtubules (Almeida-Souza et al., 2010, 2011). The C-terminal mutant P182L was also shown to affect the translational repression function of the RNA-binding protein Poly(rC)-binding protein 1 (PCBP1) (Geuens et al., 2017). Other studies suggested that mutations in *HSPB1* can affect mitochondrial transport and phosphorylation of neurofilaments (Holmgren et al., 2013; Kalmar et al., 2017a). Recently, a frameshift mutation in *HSPB1* was found in an ALS patient. Functional analysis of this mutation showed that the mutant protein sequesters the wildtype protein leading to an impairment of the chaperone function of HSPB1 (Capponi et al., 2016). While several mutations in *HSPB1* have been studied as toxic gain of function mutations, loss of HSPB1 can also be harmful in neuronal context. Indeed, knock-down of HSPB1 in rat adult sensory neurons induces apoptotic cell death both in vivo and in vitro, a condition which can be rescued by ectopic HSPB1 delivery (Lewis et al., 1999; Wagstaff et al., 1999). Taken together, the exact functional causative of neuropathy caused by *HSPB1* mutations remains obscure, and a mechanism that is common to the different mutations is not yet clear.

3.2.2. HSPB3:

One N-terminal mutation (R7S) leading to motor neuropathy has been reported in *HSPB3* (Fig 3) (Kolb et al., 2010). Recently, a frame-shift mutation leading to a stop codon (A33AfsX50) and a point mutation inside the ACD (R116P) both causing myopathy have been reported (Morelli et al., 2017). HSPB3 is known to form a stable complex with HSPB2, and plays a vital role in muscle differentiation (Sugiyama et al., 2000). HSPB2 undergoes physiological phase separation leading to lamin A (LMNA) and chromatin rearrangements which are essential for myoblast differentiation (Morelli et al., 2017). HSPB3 exerts an inhibitory control on the HSPB2 phase separation and prevents its aberrant compartmentalization. Functional analysis of the myopathy-causing mutations has shown that they both disrupt the HSPB2-HSPB3 complex, leading to abnormal HSPB2 phase separation and ultimately to impairment in nuclear homeostasis and transcription (Morelli et al., 2017). Therefore, the deregulated HSPB2 function due to *HSPB3* mutations can contribute to the myopathy condition seen in patients with *HSPB3* mutations.

3.2.3. HSPB8:

Mutations in *HSPB8* have been reported to cause distal hereditary motor neuropathy type II (dHMNII) and axonal CMT (CMT2L) (Fig 3) (Irobi et al., 2004). Similarly to some of the mutations in HSPB1, mutations in HSPB8 occur in the β6/β7 strands of the ACD which are reportedly crucial for the formation of homo/heterodimers (Mymrikov et al., 2011). Overexpression of mutant HSPB8 has been shown to lead to the formation of perinuclear aggregates in COS cells (Irobi et al., 2004) and to neurite degeneration in primary motor neurons (Irobi et al., 2010). The ability of HSPB8 to form an autophagy-stimulating complex with BAG3 and Hsc70 (see above) is affected by neuropathy-causing mutations in *HSPB8*. A study by Carra, et al., in 2010 showed that the K141E and K141N mutations in *HSPB8* lead to a weaker binding to BAG3 and an impaired stimulation of autophagy-dependent clearance of aggregates (Carra et al., 2010). Another study has shown that the lysosomal delivery of autophagosomes is impaired in NSC34 cells and in peripheral blood mononuclear cells of dHMNII patients carrying the K141E mutation (Kwok et al., 2011). This observation is in line with the observation that disrupted autophagy is often seen in motor neuron diseases, muscular disorders and inherited neuropathies (Haidar and Timmerman, 2017; Shintani and Klionsky, 2004).

3.2.4- HSPB5:

Mutations in *HSPB5* can lead to myofibrillar myopathy (MFM) characterized with slow progression of proximal and distal muscle weakness (Fig 3) (Selcen and Engel, 2003; Vicart et al., 1998). Expression of the R120G mutation in cells leads to the formation of desmin-containing (Der Perng et al., 2004). Whether HSPB5 loses its function of properly chaperoning desmin, or whether the instability of the R120G mutant leads to the aggregation is unclear, but the fact that overexpression of HSPB1 or HSPB8 but not HSPB5 inhibit this aggregation, indicates that it might be caused by toxicity of HSPB5 mutants (Zobel et al., 2003). In fact, other mutations affecting the C-terminus of HSPB5 yield intrinsically unstable and self-aggregating proteins (Hayes et al., 2008). For the Q151X mutant, this has been seen together with increased substrate-binding affinity and poor chaperone activity (Hayes et al., 2008). Regardless of the exact mechanism by which mutations in *HSPB5* lead to a myopathic phenotype, protein aggregation and disrupted protein quality control seems to be at the basis of this pathomechanism.

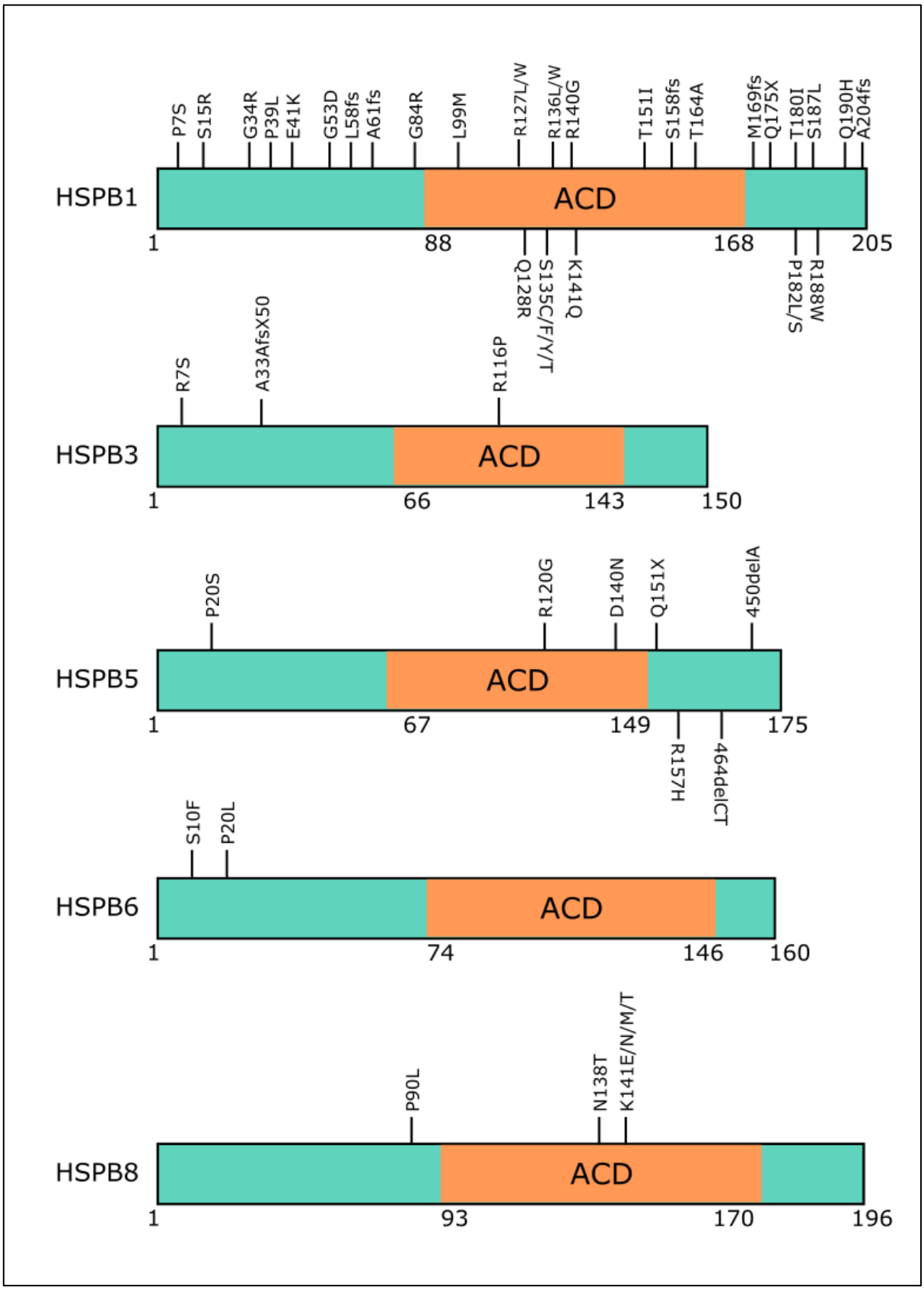

Fig.3: Overview of all mutations reported in several HSPB proteins to cause neurodegenerative, neuropathic and/or myopathic diseases

3.2.5. HSPB6:

HSPB6 is ubiquitously expressed but mostly abundant in cardiac, smooth, and skeletal muscles (Kato et al., 1994). HSPB6 was found to be upregulated in failing human hearts and upon exercise training or ischemic injury hinting at a role in maintaining cardiac health (Fan et al., 2005). A mutation in *HSPB6* has been reported to abrogate the cardioprotective effects of HSPB6 (Nicolaou et al., 2008). Very recently, a new mutation in *HSPB6* (S10F) was identified in dilated cardiomyopathy patients (Fig 3) (Liu et al., 2017). The change of the serine to a phenylalanine was shown to reduce the interaction of HSPB6 with the autophagy mediator Beclin-1, leading to the degradation of Beclin-1 by the proteasome (Liu et al., 2017). Beclin-1 is important for decision making between autophagy and apoptosis (Pattingre et al., 2005). The reduced interaction with HSPB6 and the degradation of Beclin-1 leads to the inhibition of autophagy and activation of apoptosis and cell death pathways leading to cardiomyopathy (Liu et al., 2017). Expression of wild-type HSPB6 stimulates autophagy and produces cardio-protective effects in cardiomyopathic mice. This confirms that HSPB6, through its involvement in maintaining autophagy exerts a cell protective function, and that messing this particular function leads to disease manifestation.

4. Concluding remarks:

The role of HSPBs in preventing protein aggregation stretches beyond their traditional chaperone activity to the activation of degradative pathways such as autophagy and the proteasome. The different HSPB proteins can have different anti-aggregation and pro-degradation functions. These differences are represented by the distinct substrates they have for their molecular chaperone function, and the different ways they can activate autophagy and/or the proteasome. Due to their role in proteostasis, HSPBs are also highly implicated in neurodegenerative and neuromuscular disorders and mutations in *HSPB* genes lead to a variety of neuropathic and myopathic conditions. Understanding the role of HSPBs in proteostasis is key to understanding their disease implications.

REFERENCES

Ackerley, S., James, P. A., Kalli, A., French, S., Davies, K. E., and Talbot, K. (2006). A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. *Hum. Mol. Genet.* 15, 347–354. doi:10.1093/hmg/ddi452.

Adriaenssens, E., Geuens, T., Baets, J., Echaniz-Laguna, A., and Timmerman, V. (2017). Novel insights in the disease biology of mutant small heat shock proteins in neuromuscular diseases. *Brain* 140, 2541–2549. doi:10.1093/brain/awx187.

Almeida-Souza, L., Asselbergh, B., d'Ydewalle, C., Moonens, K., Goethals, S., de Winter, V., et al. (2011). Small Heat-Shock Protein HSPB1 Mutants Stabilize Microtubules in Charcot-Marie-Tooth Neuropathy. *J. Neurosci.* 31, 15320–15328. doi:10.1523/JNEUROSCI.3266-11.2011.

Almeida-Souza, L., Goethals, S., De Winter, V., Dierick, I., Gallardo, R., Van Durme, J., et al. (2010). Increased monomerization of mutant HSPB1 leads to protein hyperactivity in Charcot-Marie-Tooth neuropathy. *J. Biol. Chem.* 285, 12778–12786. doi:10.1074/jbc.M109.082644.

An, J. J., Lee, Y. P., Kim, D. W., Sohn, E. J., Jeong, H. J., Kang, H. W., et al. (2009). Transduced HSP27 protein protects neuronal cell death by enhancing FALS-associated SOD1 mutant activity. *BMB Rep.* 42, 136–141. doi:10.5483/BMBRep.2009.42.3.136.

Bailey, C. K., Andriola, I. F. M., Kampinga, H. H., and Merry, D. E. (2002). Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* 11, 515–523. doi:10.1093/hmg/11.5.515.

Basha, E., Lee, G. J., Breci, L. A., Hausrath, A. C., Buan, N. R., Giese, K. C., et al. (2004). The Identity of Proteins Associated with a Small Heat Shock Protein during Heat Stress *in Vivo* Indicates That These Chaperones Protect a Wide Range of Cellular Functions. *J. Biol. Chem.* 279, 7566–7575. doi:10.1074/jbc.M310684200.

Basha, E., O'Neill, H., and Vierling, E. (2012). Small heat shock proteins and alpha-crystallins: Dynamic proteins with flexible functions. *Trends Biochem. Sci.* 37, 106–117. doi:10.1016/j.tibs.2011.11.005.

Bilen, J., and Bonini, N. M. (2007). Genome-wide screen for modifiers of ataxin-3 neurodegeneration in Drosophila. *PLoS Genet.* 3, 1950–1964. doi:10.1371/journal.pgen.0030177.

Bucciantini, M. (2002). Inherent cytotoxicity of aggregates implies a common origin for protein misfolding diseases. *Nature* 416, 507–511. Available at: http://dx.doi.org/10.1038/416507a.

Capponi, S., Geuens, T., Geroldi, A., Origone, P., Verdiani, S., Cichero, E., et al. (2016). Molecular Chaperones in the Pathogenesis of Amyotrophic Lateral Sclerosis: The Role of HSPB1. *Hum. Mutat.* 37, 1202–1208. doi:10.1002/humu.23062.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., et al. (2010). Identification of the Drosophila ortholog of HSPB8: Implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* 285, 37811–37822. doi:10.1074/jbc.M110.127498.

Carra, S., Brunsting, J. F., Lambert, H., Landry, J., and Kampinga, H. H. (2009). HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2?? phosphorylation. *J. Biol. Chem.* 284, 5523–5532. doi:10.1074/jbc.M807440200.

Carra, S., Rusmini, P., Crippa, V., Giorgetti, E., Boncoraglio, A., Cristofani, R., et al. (2013). Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368, 20110409. doi:10.1098/rstb.2011.0409.

Carra, S., Seguin, S. J., Lambert, H., and Landry, J. (2008a). HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J. Biol. Chem.* 283, 1437–1444. doi:10.1074/jbc.M706304200.

Carra, S., Seguin, S. J., and Landry, J. (2008b). HspB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 4, 237–239. doi:5407 [pii].

Cheng, G., Basha, E., Wysocki, V. H., and Vierling, E. (2008). Insights into small heat shock protein and substrate structure during chaperone action derived from hydrogen/deuterium exchange and mass spectrometry. *J. Biol. Chem.* 283, 26634–26642. doi:10.1074/jbc.M802946200.

Crippa, V., Carra, S., Rusmini, P., Sau, D., Bolzoni, E., Bendotti, C., et al. (2010a). A role of small heat shock protein B8 (HspB8) in the autophagic removal of misfolded proteins responsible for neurodegenerative diseases. *Autophagy* 6, 958–960. doi:10.4161/auto.6.7.13042.

Crippa, V., Sau, D., Rusmini, P., Boncoraglio, A., Onesto, E., Bolzoni, E., et al. (2010b). The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum. Mol. Genet.* 19, 3440–3456. doi:10.1093/hmg/ddq257.

Datskevich, P. N., Mymrikov, E. V., and Gusev, N. B. (2012). Utilization of fluorescent chimeras for investigation of heterooligomeric complexes formed by human small heat shock proteins. *Biochimie* 94, 1794–1804. doi:10.1016/j.biochi.2012.04.012.

Der Perng, M., Wen, S. F., van den IJssel, P., Prescott, A. R., and Quinlan, R. A. (2004). Desmin Aggregate Formation by R120G αB-Crystallin Is Caused by Altered Filament Interactions and Is Dependent upon Network Status in Cells. *Mol. Biol. Cell* 15, 2335–2346. doi:10.1091/mbc.E03-12-0893.

Evgrafov, O. V, Mersiyanova, I., Irobi, J., Van Den Bosch, L., Dierick, I., Leung, C. L., et al. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36, 602–606. doi:10.1038/ng1354.

Fan, G. C., Chu, G., and Kranias, E. G. (2005). Hsp20 and its cardioprotection. *Trends Cardiovasc. Med.* 15, 138–141. doi:10.1016/j.tcm.2005.05.004.

Geuens, T., De Winter, V., Rajan, N., Achsel, T., Mateiu, L., Almeida-Souza, L., et al. (2017). Mutant HSPB1 causes loss of translational repression by binding to PCBP1, an RNA binding protein with a possible role in neurodegenerative disease. *Acta Neuropathol. Commun.* 5, 5. doi:10.1186/s40478-016-0407-3.

Glover, J. R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell* 94, 73–82. doi:10.1016/S0092-8674(00)81223-4.

Haidar, M., and Timmerman, V. (2017). Autophagy as an Emerging Common Pathomechanism in Inherited Peripheral Neuropathies. *Front. Mol. Neurosci.* 10, 1–17. doi:10.3389/fnmol.2017.00143.

Haslbeck, M., Franzmann, T., Weinfurtner, D., and Buchner, J. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nat. Struct. Mol. Biol.* 12, 842–846. doi:10.1038/nsmb993.

Haslbeck, M., and Vierling, E. (2015). A first line of stress defense: Small heat shock proteins and their function in protein homeostasis. *J. Mol. Biol.* 427, 1537–1548. doi:10.1016/j.jmb.2015.02.002.

Hayes, V. H., Devlin, G., and Quinlan, R. A. (2008). Truncation of alpha-B-crystallin by the myopathy-causing Q151X mutation significantly destabilizes the protein leading to aggregate formation in transfected cells. *J. Biol. Chem.* 283, 10500–10512. doi:10.1074/jbc.M706453200.

Holmgren, A., Bouhy, D., De Winter, V., Asselbergh, B., Timmermans, J. P., Irobi, J., et al. (2013). Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments. *Acta Neuropathol.* 126, 93–108. doi:10.1007/s00401-013-1133-6.

Irobi, J., Almeida-Souza, L., Asselbergh, B., de Winter, V., Goethals, S., Dierick, I., et al. (2010). Mutant HSPB8 causes motor neuron-specific neurite degeneration. *Hum. Mol. Genet.* 19, 3254–3265. doi:10.1093/hmg/ddq234.

Irobi, J., Impe, K. Van, Seeman, P., Jordanova, A., Dierick, I., Verpoorten, N., et al. (2004). Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. *Nat. Genet.* 36, 597–601. doi:10.1038/ng1328.

J. Cummings, C., and Y. Zoghbi, H. (2000). *Fourteen and counting: Unraveling trinucleotide repeat diseases*. doi:10.1093/hmg/9.6.909.

Jaya, N., Garcia, V., and Vierling, E. (2009). Substrate binding site flexibility of the small heat shock protein molecular chaperones. *Proc. Natl. Acad. Sci. U. S. A.* 106, 15604–15609. doi:10.1073/pnas.0902177106.

Kalmar, B., Innes, A., Wanisch, K., Kolaszynska, A. K., Pandraud, A., Kelly, G., et al. (2017). Mitochondrial deficits and abnormal mitochondrial retrograde axonal transport play a role in the pathogenesis of mutant Hsp27-induced Charcot Marie Tooth Disease. *Hum. Mol. Genet.* doi:10.1093/hmg/ddx216.

Kampinga, H. H., and Garrido, C. (2012). HSPBs: Small proteins with big implications in human disease. *Int. J. Biochem. Cell Biol.* 44, 1706–1710. doi:10.1016/j.biocel.2012.06.005.

Kato, K., Goto, S., Inaguma, Y., Hasegawa, K., Morishita, R., and Asano, T. (1994). Purification and characterization of a 20-kDa protein that is highly homologous to ??B crystallin. *J. Biol. Chem.* 269, 15302–15309.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al. (2014). The functional landscape of Hsp27 reveals new cellular processes such as DNA repair and alternative splicing and proposes novel anticancer targets. *Mol. Cell. Proteomics*, 3585–3601. doi:10.1074/mcp.M114.041228.

Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., and Ulrich Hartl, F. (2013). *Molecular*

Chaperone Functions in Protein Folding and Proteostasis. doi:10.1146/annurev-biochem-060208-092442.

Kolb, S. J., Snyder, P. J., Poi, E. J., Renard, E. A., Bartlett, A., Gu, S., et al. (2010). Mutant small heat shock protein B3 causes motor neuropathy: Utility of a candidate gene approach. *Neurology* 74, 502–506. doi:10.1212/WNL.0b013e3181cef84a.

Kwok, A. S., Phadwal, K., Turner, B. J., Oliver, P. L., Raw, A., Simon, A. K., et al. (2011). HspB8 mutation causing hereditary distal motor neuropathy impairs lysosomal delivery of autophagosomes. *J. Neurochem.* 119, 1155–1161. doi:10.1111/j.1471-4159.2011.07521.x.

Laganowsky, A., Benesch, J. L. P., Landau, M., Ding, L., Sawaya, M. R., Cascio, D., et al. (2010). Crystal structures of truncated alphaA and alphaB crystallins reveal structural mechanisms of polydispersity important for eye lens function. *Protein Sci.* 19, 1031–1043. doi:10.1002/pro.380.

Lambert, H., Charette, S. J., André, F., Guimond, A., Landry, J., and Bernier, F. (1999). CELL BIOLOGY AND METABOLISM : HSP27 Multimerization Mediated by Interactions at the Amino Terminus HSP27 Multimerization Mediated by Phosphorylation-sensitive Intermolecular Interactions at the Amino Terminus *. *J. Biol. Chem.* 274, 9378–9385.

Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* 16, 659–671. doi:10.1093/emboj/16.3.659.

Lewis, S. E., Mannion, R. J., White, F. a, Coggeshall, R. E., Beggs, S., Costigan, M., et al. (1999). A role for HSP27 in sensory neuron survival. *J. Neurosci.* 19, 8945–8953.

Liu, G.-S., Zhu, H., Cai, W.-F., Wang, X., Jiang, M., Essandoh, K., et al. (2017). Regulation of BECN1-Mediated Autophagy by HSPB6: Insights from A Human HSPB6 ^{S10F} Mutant. *Autophagy* 8627, 01–56. doi:10.1080/15548627.2017.1392420.

Matsumoto, T., Urushido, M., Ide, H., Ishihara, M., Hamada-Ode, K., Shimamura, Y., et al. (2015). Small Heat Shock Protein Beta-1 (HSPB1) Is Upregulated and Regulates Autophagy and Apoptosis of Renal Tubular Cells in Acute Kidney Injury. *PLoS One* 10, e0126229. doi:10.1371/journal.pone.0126229.

McDonald, E. T., Bortolus, M., Koteiche, H. A., and McHaourab, H. S. (2012). Sequence, structure, and dynamic determinants of Hsp27 (HspB1) equilibrium dissociation are encoded by the N-terminal domain. *Biochemistry* 51, 1257–1268. doi:10.1021/bi2017624.

Morelli, F. F., Verbeek, D. S., Bertacchini, J., Vinet, J., Mediani, L., Marmiroli, S., et al. (2017). Aberrant Compartment Formation by HSPB2 Mislocalizes Lamin A and Compromises Nuclear Integrity and Function. *Cell Rep.* 20, 2100–2115. doi:10.1016/j.celrep.2017.08.018.

Mymrikov, E. V., Seit-Nebi, a. S., and Gusev, N. B. (2011). Large Potentials of Small Heat Shock Proteins. *Physiol. Rev.* 91, 1123–1159. doi:10.1152/physrev.00023.2010.

Nicolaou, P., Knöll, R., Haghighi, K., Fan, G. C., Dorn, G. W., Hasenfuß, G., et al. (2008). Human mutation in the anti-apoptotic heat shock protein 20 abrogates its cardioprotective effects. *J. Biol. Chem.* 283, 33465–33471. doi:10.1074/jbc.M802307200.

Outeiro, T. F., Klucken, J., Strathearn, K. E., Liu, F., Nguyen, P., Rochet, J. C., et al. (2006). Small heat shock proteins protect against α-synuclein-induced toxicity and aggregation.

Biochem. Biophys. Res. Commun. 351, 631–638. doi:10.1016/j.bbrc.2006.10.085.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., et al. (2003). HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol. Cell. Biol.* 23, 5790–5802. doi:10.1128/MCB.23.16.5790.

Patel, S., Vierling, E., and Tama, F. (2014). Replica exchange molecular dynamics simulations provide insight into substrate recognition by small heat shock proteins. *Biophys. J.* 106, 2644–2655. doi:10.1016/j.bpj.2014.04.048.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Xiao, H. L., Mizushima, N., et al. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939. doi:10.1016/j.cell.2005.07.002.

Qian, J., Ren, X., Wang, X., Zhang, P., Jones, W. K., Molkentin, J. D., et al. (2009). Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circ. Res.* 105, 1223–1231. doi:10.1161/CIRCRESAHA.109.200378.

Quinlan, R. A., Brenner, M., Goldman, J. E., and Messing, A. (2007). GFAP and its role in Alexander disease. *Exp. Cell Res.* 313, 2077–2087. doi:10.1016/j.yexcr.2007.04.004.

Rekas, A., Adda, C. G., Andrew Aquilina, J., Barnham, K. J., Sunde, M., Galatis, D., et al. (2004). Interaction of the molecular chaperone αB-crystallin with α-Synuclein: Effects on amyloid fibril formation and chaperone activity. *J. Mol. Biol.* 340, 1167–1183. doi:10.1016/j.jmb.2004.05.054.

Rosenzweig, R., Moradi, S., Zarrine-Afsar, A., Glover, J. R., and Kay, L. E. (2013). Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. *Science (80-.).* 339, 1080–1083. doi:10.1126/science.1233066.

Selcen, D., and Engel, a G. (2003). Myofibrillar myopathy caused by novel dominant negative alpha B- crystallin mutations. *Ann Neurol* 54, 804–810. doi:10.1002/ana.10767.

Shintani, T., and Klionsky, D. J. (2004). Autophagy in Health and Disease : A Double-edged sword. 306, 990–996.

Spillantini, M. G., Schmidt, M. L., Lee, V. M.-Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). alpha-Synuclein in Lewy bodies. *Nature* 388, 839–840. doi:10.1038/42166.

Stengel, F., Baldwin, A. J., Painter, A. J., Jaya, N., Basha, E., Kay, L. E., et al. (2010). Quaternary dynamics and plasticity underlie small heat shock protein chaperone function. *Proc. Natl. Acad. Sci.* 107, 2007–2012. doi:10.1073/pnas.0910126107.

Sugiyama, Y., Suzuki, A., Akutsu, R., Hirose, T., Waye, M. M. Y., Tsui, S. K. W., et al. (2000). CELL BIOLOGY AND METABOLISM : Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of Myogenic Differentiation Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of MKBP / HSPB2 and HSPB3 during Myogenic D. 275, 1095–1104.

Taylor, R. P., and Benjamin, I. J. (2005). Small heat shock proteins: A new classification scheme in mammals. *J. Mol. Cell. Cardiol.* 38, 433–444. doi:10.1016/j.yjmcc.2004.12.014.

Treweek, T. M., Ecroyd, H., Williams, D. M., Meehan, S., Carver, J. A., and Walker, M. J. (2007). Site-directed mutations in the C-terminal extension of human ??B-crystallin affect

chaperone function and block amyloid fibril formation. *PLoS One* 2. doi:10.1371/journal.pone.0001046.

Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998). The small heat-shock protein IbpB from Escherichia coli stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem.* 273, 11032–11037. doi:10.1074/jbc.273.18.11032.

Vicart, P., Caron, a, Guicheney, P., Li, Z., Prévost, M. C., Faure, a, et al. (1998). A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat. Genet.* 20, 92–5. doi:10.1038/1765.

Vos, M. J., Hageman, J., Carra, S., and Kampinga, H. H. (2008). Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. *Biochemistry* 47, 7001–7011. doi:10.1021/bi800639z.

Vos, M. J., Zijlstra, M. P., Carra, S., Sibon, O. C. M., and Kampinga, H. H. (2011). Small heat shock proteins, protein degradation and protein aggregation diseases. *Autophagy* 7, 101–103. doi:10.4161/auto.7.1.13935.

Vos, M. J., Zijlstra, M. P., Kanon, B., van Waarde-Verhagen, M. a W. H., Brunt, E. R. P., Oosterveld-Hut, H. M. J., et al. (2010). HSPB7 is the most potent polyQ aggregation suppressor within the HSPB family of molecular chaperones. *Hum. Mol. Genet.* 19, 4677–4693. doi:10.1093/hmg/ddq398.

Wagstaff, M. J. D., Smith, J., De, J. S., Coffin, R. S., David, S., and Belleroche, J. S. De (1999). NUCLEIC ACIDS , PROTEIN SYNTHESIS , AND MOLECULAR GENETICS : Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus-based Vector Protection of Neuronal Cells from Apoptosis by Hsp27 Delivered with a Herpes Simplex Virus. 274, 5061–5069. doi:10.1074/jbc.274.8.5061.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., et al. (2005). Thermotolerance Requires Refolding of Aggregated Proteins by Substrate Translocation through the Central Pore of ClpB. *Trends Biochem. Sci.* 30, i. doi:10.1016/S0968-0004(05)00043-5.

Wilhelmus, M. M. M., Boelens, W. C., Otte-Höller, I., Kamps, B., Kusters, B., Maat-Schieman, M. L. C., et al. (2006a). Small heat shock protein HspB8: Its distribution in Alzheimer's disease brains and its inhibition of amyloid-β protein aggregation and cerebrovascular amyloid-β toxicity. *Acta Neuropathol.* 111, 139–149. doi:10.1007/s00401-005-0030-z.

Wilhelmus, M. M. M., Otte-Höller, I., Wesseling, P., De Waal, R. M. W., Boelens, W. C., and Verbeek, M. M. (2006b). Specific association of small heat shock proteins with the pathological hallmarks of Alzheimer's disease brains. *Neuropathol. Appl. Neurobiol.* 32, 119–130. doi:10.1111/j.1365-2990.2006.00689.x.

Wyttenbach, A. (2002). Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* 11, 1137–1151. doi:10.1093/hmg/11.9.1137.

Zhang, H., Rajasekaran, N. S., Orosz, A., Xiao, X., Rechsteiner, M., and Benjamin, I. J. (2010). Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin-proteasome pathways. *J. Mol. Cell. Cardiol.* 49, 918–930. doi:10.1016/j.yjmcc.2010.09.004.

Zobel, A. T. C., Loranger, A., Marceau, N., Thériault, J. R., Lambert, H., and Landry, J. (2003). Distinct chaperone mechanisms can delay the formation of aggresomes by the myopathy-causing R120G αB-crystallin mutant. *Hum. Mol. Genet.* 12, 1609–1620. doi:10.1093/hmg/ddg173.

Żwirowski, S., Kłosowska, A., Obuchowski, I., Nillegoda, N. B., Piróg, A., Ziętkiewicz, S., et al. (2017). Hsp70 displaces small heat shock proteins from aggregates to initiate protein refolding. *EMBO J.* 36, 783–796. doi:10.15252/embj.201593378.

CHAPTER 3

HSPB1 is required for Autophagy: Insights from CMT-causing mutations

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ABSTRACT

The heat shock protein B1 (HSPB1) is a ubiquitously expressed molecular chaperone with a function in counteracting protein misfolding. A wider role for HSPB1 in proteostasis has been proposed, and recently some studies that it can play a role in autophagy, a bulk protein degradation pathway. Mutations in *HSPB1* cause axonal Charcot-Marie-Tooth neuropathies (CMT2) and distal hereditary motor neuropathies (dHMN). Several studies have contributed to understanding the pathogenicity of such mutations, and through it revealed basic functions of HSPB1. In this study we show that mutations in *HSPB1* lead to the impairment of autophagy. We also see that knocking-out *HSPB1* also impairs the autophagy process while expression of HSPB1 rescues these deficits, indicating that it is necessary for the autophagy pathway. We show that while autophagy is disrupted by mutant HSPB1, the proteasome pathway is not affected suggesting an autophagy-specific effect of these mutations. Interestingly, we confirm the autophagy deficits in motor neurons differentiated from patient derived iPSC-lines, indicating that the impairment of autophagy might be one of the pathomechanisms by which mutations in *HSPB1* lead to peripheral neuropathy.

Introduction:

The heat shock protein B1 (HSPB1) protein is a ubiquitously expressed molecular chaperone belonging to the small heat shock protein family. HSPB1 contains an evolutionary conserved α-crystallin domain (ACD) which is important for its interaction and oligomerization with other small heat shock proteins (Holmgren, 2012). Under acute stress conditions, such as heat shock or oxidative stress, HSPB1 is upregulated and protects the cell from aggregates and stress-unfolded proteins (Kakkar et al., 2014). HSPB1 performs its chaperone function by binding to non-native proteins, aggregates or misfolded proteins and mediating their refolding by ATP-dependent chaperones or degradation by the proteasome molecular machinery (Haslbeck and Vierling, 2015). In addition to its chaperone activity, HSPB1 is involved in several essential cellular functions, such as apoptosis and redox balance, as well as the regulation of cytoskeletal dynamics (Mymrikov et al., 2011).

Mutations in *HSPB1* are associated with Charcot-Marie-Tooth axonal neuropathies (CMT2), distal hereditary motor neuropathy (dHMN) (Echaniz-Laguna et al., 2017; Evgrafov et al., 2004), and amyotrophic lateral sclerosis (ALS) (Capponi et al., 2016). The majority of disease-causing mutations in *HPSB1* occur in the ACD, but some mutations do exist in the N- or C-termini (Adriaenssens et al., 2017). Functional analysis of the different reported mutations show that the location of the mutation can affect different functions of the protein (Almeida-Souza et al., 2011). Few studies have reported a common effect by mutations in and outside of the ACD (Holmgren et al., 2013).

Recently, some studies suggested that HSPB1 might play a role in autophagy and that overexpression of HSPB1 upregulates macroautophagy in renal tubular cells (Matsumoto et al., 2015a; Sun et al., 2017). Macroautophagy, hereafter referred to as autophagy, is a homeostatic cellular process by which protein aggregates and cellular organelles are targeted, degraded, and recycled. Autophagy proceeds through a series of steps including: induction, membrane nucleation and elongation, to finally form a double-membraned autophagosome. The autophagosome then fuses with the lysosomes to ensue further degradation of the autophagic contents (Yang and Klionsky, 2010a). In long-lived cells like neurons, autophagy is crucial as it helps to remove dysfunctional organelles and cellular waste that with ageing (Boland and Nixon, 2006). Interestingly, mouse models where key autophagy genes are

knocked-out display features of peripheral neuropathy evident with poor motor behavior (Komatsu et al., 2005). This suggests that disruption of the autophagy process could contribute to the pathomechanisms of CMT neuropathies and of other neurodegenerative diseases. Indeed, recently several genes associated with inherited neuropathy have been linked with autophagy disruption (Haidar and Timmerman, 2017).

In this study we investigated the role of HSPB1 in autophagy and how CMT-causing mutations affect this role. Our study reveals that HSPB1 plays a role in autophagy which is disrupted by CMT-causing mutations in and outside of the ACD. This was further confirmed both in cells stably expressing the wild-type and mutant forms of HSPB1, and in patient-derived motor neurons. Knock-out of HSPB1 also decreased autophagy levels upon starvation. Importantly, re-expression of HSPB1 rescued these deficits, indicating that HSPB1 is itself necessary for starvation-induced autophagy. We also show that mutations in HSPB1 do not affect the proteasome or cause a compensatory increase in the proteasomal activity suggesting an autophagy-specific effect of these mutations on proteostasis.

Materials and Methods:

Creation of constructs and stables cell lines:

Constructs used to generate stable HeLa and SH-SY5Y cell lines were designed using the Gateway recombination system (Life Technologies). The open reading frames of HSPB1 and EGFP were amplified by PCR using specific primers flanked by attB recombination sites to allow the insertion of the PCR product in a pDONR221 vector. The HSPB1 mutations used in this study (HSPB1-R127W, HSPB1-S135F, and HSPB1-P182L) were generated by site-directed mutagenesis. Sequence validated pDONRs were transferred by recombination to a pLenti6/V5 destination vector (Life Technologies) to generate constructs where the ORF is fused to a V5-tag. All the finally obtained plasmids were validated by Sanger sequencing. Stable cell lines were generated by lentiviral transduction of HeLa cell lines or the neuroblastoma cell line SH-SY5Y according to the method described previously (Salmon and Trono, 2006). The expression levels and growth rates of the generated cell lines were monitored and shown to be similar (data not shown).

Cell culture material and conditions:

Unless otherwise mentioned, all the cell culture media and supplements were purchased from Life Technologies. The HeLa cell line was purchased from ATCC and grown at 37ºC and 5% CO2 in MEM, supplemented with 10% fetal calf serum (FCS), and 1% Glutamine and Penicillin-Streptomycin. The human neuroblastoma cell line SH-SY5Y was purchased from ATCC and cultured at 37ºC and 5% CO_2 in DMEM supplemented with 10% FCS, non-essential amino acids, glutamine and Penicillin-Streptomycin .

Transient transfection

HeLa cell lines were transfected using polyethylenimine (PEI) according to an in-house optimized protocol. Briefly, cells were seeded out in a 24-well plate at $7x10^4$ cells per well 24 hrs. before transfection. On the day of transfection, 500 ng plasmid DNA was diluted in 36 µl Opti-MEM (Life Technologies) and in parallel, 2.5 µl PEI (1 ug/ul) was diluted in 36 µl Opti-MEM. The diluted PEI was then added to the DNA and mixed by vortexing for 10 seconds. Afterwards, the PEI-DNA mix was incubated for 10 minutes at room temperature, and then added drop-wise onto the cells.

Autophagy induction and treatment

Autophagy was induced by serum starvation by culturing cells in culture medium deprived of FCS for the indicated amount of time. Where necessary, the lysosomal inhibitor Bafilomycin A1 (Enzo Life Sciences) was used at 10nM concentration to block the lysosomal degradation. For the motor neurons, the culture medium was replaced with medium lacking any supplements or growth factors for 3 hours.

Western blotting

Cells were lysed in lysis buffer (0.5% Nonidet P-40, 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 4mM Sodium orthovanadate, 20mM Glycerol-2-Phosphate, 10 mM Sodium Fluoride, 1 mM Sodium Pyrophosphate, together with complete protease and Phospho-STOP inhibitor mixtures - Roche Applied Science) for 30 min on ice and cleared by centrifugation for 10min at 14.000RPM. After protein concentration was determined, by using the Pierce BCA protein assay kit (ThermoFisher Scientific), cell lysates were boiled for 5 min at 95°C in reducing Laemmli buffer (Life Technologies) supplemented with 100mM 1,4-Dithiothreitol (DTT). Proteins were separated on NuPAGE gels (Life Technologies) or on 18% gels made in-house (for detection of LC3 isoforms) and transferred to a nitro-cellulose-membrane (Hybond^{TM}-P, GE Healthcare). Blocking of the membrane was performed using 5% milk powder diluted in PBS, supplemented with 0.1% Tween 20. Afterwards, membranes were incubated with a primary antibody over night at 4°C and one hour with a secondary horseradish peroxidase-conjugated antibody. Blots were developed by using the Enhanced Chemiluminescence ECL Plus^{TM} detection system (Thermoscientific) and imaged with an ImageQuant imager (GE Healthcare). Band intensities were determined by quantifying the mean pixel grey values using the ImageJ software (Schneider, et al, 2012). Mean pixel grey values were measured in a rectangular region of interest.

Immunocytochemistry and immunofluorescence microscopy

Immunostainings were performed according to a standardized protocol. Briefly, cells were seeded out on glass coverslips the day before fixation. Fixation was performed by incubating the cells with 4% paraformaldehyde or Ice Cold Methanol for 20 min. Afterwards cells were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS). Blocking was performed with 5% BSA diluted in PBS for 1 hour, primary and secondary antibodies were incubated for 1 hour diluted in 1% BSA in PBS supplemented with 0.1% Tween 20. Nuclear staining was done by using Hoechst33342 (Life Technologies), afterwards cells were mounted

with fluorescent mounting medium (Dako). Images were taken on a LSM700 confocal fluorescence microscope using a 40x/1.40 plan-apochromatic objective. For assays using automated image acquisition, cells were seeded (15.000 cells per well) in Ibidi µ-Plate IbiTreat 96-well plates and fixed 24h later in 4% paraformaldehyde. Three replicate wells were used per genotype and immunocytochemical staining was performed as described above. Images were acquired with an automated Nikon Eclipse TiE microscope, equipped with DS-Qi2 camera, PFS3, 40x Plan Fluor 0.75 NA objective (DIC M N2), Spectra X epifluorescence excitation source, motorized stage (Prior) and using the JOBS module of Nikon Elements software (Nikon Instruments Europe B.V.). Per well, 30 randomly positioned multichannel image sets (1608 x 1608 pixels, 4 channels, 0.183 µm pixels) were acquired using the PS3 hardware autofocus. Image analysis and quantification was done automatically using CellProfiler software (Kamentsky et al., 2011). An image analysis pipeline was designed in CellProfiler to allow for the segmentation and quantification of individual cells, punctate structures inside the cells, and their relative intensities. Statistical analysis was then performed in R and GraphPad Prism.

Antibodies and plasmids used

The following anti-bodies were used in this study: anti-HSPB1 (Enzo Life Sciences), anti-V5 (Life Technologies), anti-LC3 (Sigma Aldrich), anti-GFP (Abcam), anti-phospho ULK1 ser757 (Cell Signaling), anti-4E-BP1 (Cell Signaling), Anti-beta-Actin (Sigma Aldrich), anti-BAG1 (Santa Cruz), anti-BAG3 (ImTec Diagnostics), anti-ChAT (Bio-Connect), anti-Islet-1 (Abcam). The pEGFPC2-DFCP1 plasmid was a kind gift from the group of Prof. Nicholas Ktistakis (Babraham Institute, Cambridge, UK) . The shRNAs against HSPB1 were purchased from Dharmacon and the following clones were used: shRNA752: TRCN0000008752 and shRNA466: Clone ID: TRCN0000011466

Generation of HSPB1 CRISPR knock-out cell lines

Knock-out cell lines for HSPB1 were generated according to the protocol of Ran, et al, 2013 (Ran et al., 2013). In brief, two guide RNAs (gRNAs) targeting the first nucleotides after the start codon, each selected based on its specificity score and number of off-targets (online tool MIT, http://crispr.mit.edu). The gRNAs were ordered as phosphorylated primers from IDT (www.idtdna.com) and cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene). gRNA efficiency was verified by quantifying the reduction of HSPB1 protein expression in HEK293T cells after transfecting the gRNAs with polyethylenimine and performing a western

blot after 72 h. Next, HeLa cells were transfected in 6-well dishes with the PX459 plasmids using Lipofectamine LTX Plus (Life Technologies) and puromycin was added (1ug/ml) for 72 h. Surviving cells were then serially diluted in a 96-well plate in order to isolate single colonies. After expansion of the single colonies, complete knock-out of HSPB1 was assessed by western blotting and the presence of premature stop codons was verified by Sanger sequencing.

Differentiation of motor neurons from iPSC lines

Induced pluripotential stem cells (iPSCs) derived from fibroblasts of a patient with HSPB1-P182L mutation and a healthy control individual were provided from the VIB Stem Cell Center, Leuven, Belgium. iPSCs were cultured on matrigel matrix (VWR biotechnologies) and in E8 flex medium and supplement (Life Technologies) and penicillin-streptomycin (Life Technologies). The differentiation of the iPSCs to motor neurons was done as published by Guo, et al. (Guo et al., 2017). In brief, on Day 0, the E8 flex medium is removed and iPSC are detached using collagenase (Stem Cell Technologies). The cells are then spinned down and incubated in a new neuronal medium consisting of neurobasal medium (Life Technologies), DMEM/F12 medium (Life Technologies) (both media in a 1:1 ratio) in addition to Penicillin-Streptomycin (Life Technologies), N2 supplement (Life Technologies), B27 supplement (Life Technologies), beta-mercaptoethanol (Life Technologies), ascorbic acid (Sigma Aldrich), ROCK inhibitor (For embryoid bodies formation, ThermoFisher Scientific), SB (dual SMAD, inhibitor, Bio-Techne), LDN (dual SMAD inhibitor, Selleck Chemicals), and CHIR (wnt antagonist, Bio-Techne). The following days the medium was sequentially refreshed or replaced with media containing compounds necessary for motor neuron differentiation. Compounds used include: Retinoic Acid (Sigma Aldrich), SAG (Bio-Techne, the growth factors BDNF and GDNF (Immunosource), DAPT (Bio-Techne), Laminin (Sigma Aldrich), ROCK inhibitor, Cell One (CO) supplement (ThermoFisher Scientific), and CNTF (Sigma Aldrich). The differentiated motor neurons were treated and collected on Day 22. Medical ethical approval to perform our studies using patient derived material was obtained from our local medical ethical committees.

Proteasome activity assay

The proteasome activity was measured using a fluorometric proteasome 20S assay kit and according to the supplied protocol (Sigma Aldrich). In brief, cells were seeded in 96-well plates at equal density (80,000 cells/well/90µl medium) and cultured overnight in growth medium. Then 100 µl of Proteasome Assay Loading Solution was added, and the 96-well plate was

incubated overnight protected from light at room temperature. Afterwards, the fluorescence intensity was measured using a Synergy HT spectrophotometer at excitation = 490 nm and emission = 525 nm. Blank wells with medium but without cells were used and background fluorescence was corrected by subtracting the blank fluorescence from the fluorescence in the test wells. Comparison between the corrected fluorescence intensities demonstrated relative proteasome 20S activity.

Results:

HSPB1 mutations impair the autophagy pathway

In order to assess the effect of HSPB1 mutations on the autophagy pathway, we performed western blotting analysis to examine the levels of the autophagy marker LC3II in HeLa cells stably expressing HSPB1 WT, or one of the mutants: R127W, S135F, and P182L. Upon the induction of autophagy, the microtubule-associated protein light chain 3 (LC3) is usually converted from the LC3I form to the cleaved and lipidated LC3II form which correlates with the number of autophagosomes (Mizushima and Yoshimori, 2007). Cells were treated with serum withdrawal and bafilomycin A1 (10 nM) for 3 hours, or left untreated, then subjected to western blotting for detection of LC3II levels. Serum withdrawal is known to induce autophagy, while bafilomycin A1 blocks the fusion of autophagosomes with the lysosomes by blocking Na+/H+ lysosomal pumps and rendering the lumen less acidic (Klionsky et al., 2016). This allows us to compare the "autophagic load" between the different cell lines (Zhang et al., 2013). Mutant cell lines showed a significantly lower amount of LC3II compared to the WT after treatment, indicating a disruption in the levels of autophagy in these cells (Fig. 1 C). We also performed immunofluorescence labelling of autophagosomes in the same cell lines by staining for LC3. The LC3 positive puncta were then quantified by automated counting using the CellProfiler software (Kamentsky et al., 2011) and compared between the different cell lines. The mutant cell lines showed a lower number of LC3 positive puncta compared to the WT and control lines (Fig. 1 A and B). These results were also confirmed in the neuroblastoma SH-SY5Y cells stably expressing the different HSPB1 variants (Fig. S1).

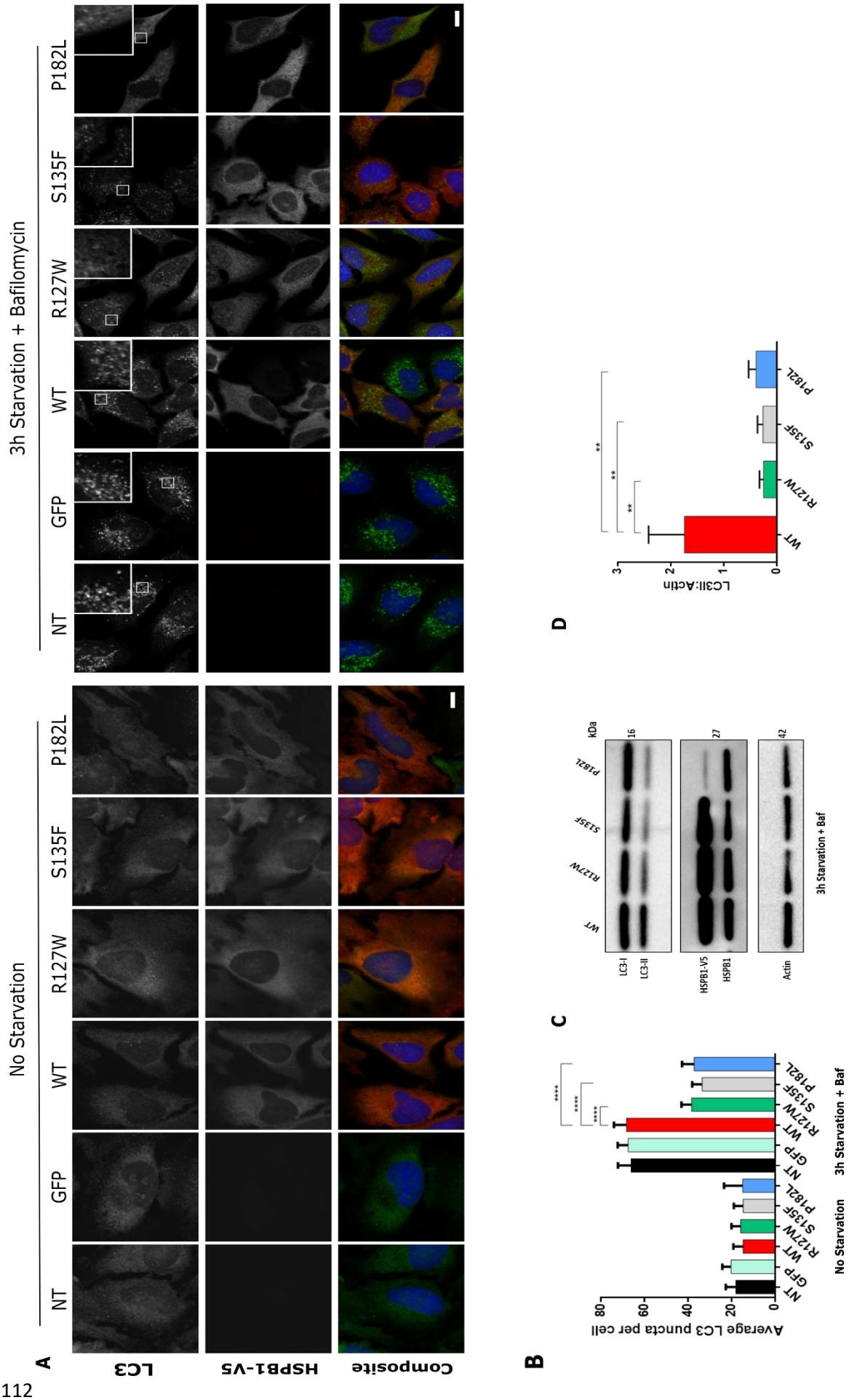

A
No Starvation
3h Starvation + Bafilomycin
NT
GFP
WT
R127W
S135F
P182L
LC3
HSPB1-V5
Composite
B
Average LC3 puncta per cell
No Starvation
3h Starvation + Baf
C
kDa
WT
R127W
S135F
P182L
LC3-I
LC3-II
HSPB1-V5
HSPB1
Actin
3h Starvation + Baf
D
LC3II:Actin

Figure 1. HSPB1 mutations impair the autophagy pathway.

HeLa cells expressing HSPB1 WT or the mutants were treated or not with serum starvation and bafilomycin A1 for 3 hours. (**A**) Immunofluorescence staining of HeLa cells transduced with V5-tagged HSPB1-WT, the mutant forms (R127W, S135F, P182L), or GFP. NT cells are not transduced. LC3 (Green) HSPB1 (Red), and the DAPI nucleus stain (Blue). Scale Bar = 10 µm. (**B**) Quantification of LC3 positive puncta detected by immunofluorescence. Data originates from four independent experiments (50+ cells per experiment). (**C**)Western blotting analysis of LC3II (**D**) The level of LC3II calculated from 3 independent western blotting experiments and normalised to actin (loading control). Note that the lower levels of exogenous HSPB1 in the P182L cell lines are due to the tendency of this mutant to precipitate into the cell pellet rather than due to lower expression (as validated in the immunofluorescence pictures). One-way ANOVA, with Tukey's correction range test. Error bars= Mean with 95% CI (**B**) S.D (**D**), ** = $p<0.01$ **** = $p<0.0001$

HSPB1 mutations do not disturb the initiation steps of autophagy

Autophagy is under the inhibitory control of the nutrient sensor: mechanistic target of rapamycin (mTORC1). During nutrient starvation, mTORC1 is inhibited, leading to the activation of the autophagy initiating complexes (Wong et al., 2015). We tested whether HSPB1 mutants fail to induce autophagy upon starvation due to a constantly active mTORC1. To this end, we assessed the phosphorylation levels of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). The inhibition of mTORC1 lowers the phosphorylation levels of 4E-BP1 and causes a shift from a hyper- to a hypo-phosphorylation pattern (Hara et al., 1997). We saw that 4E-BP1 displayed a hypo-phosphorylated pattern upon serum starvation in both the mutants and the controls (Fig 2 A). This indicates that starvation leads to inhibition of mTORC1 in the cells expressing mutant HSPB1. To test whether the autophagy initiation complex is affected by mutant HSPB1, we studied the phosphorylation levels of the autophagy initiator unc-51 like kinase 1 (ULK1). AMP-activated protein kinase (AMPK) is activated upon starvation and in turn it phosphorylates ULK1 at multiple sites including Ser317, Ser555, and Ser777. Conversely, mTORC1 phosphorylates ULK1 at Ser757 and disrupts the interaction between ULK1 and AMPK. The decrease in phosphorylation levels of ULK1 at Ser757 signals the activation of the autophagy initiation complex (Hosokawa et al., 2009). We observed no difference in the Ser757 phosphorylation levels between HSPB1 mutant, WT and control cell lines with or without starvation treatment, showing that the initiation complex is not affected by mutations in *HSPB1* (Fig 2 A).

Several studies have shown that the nucleation of autophagic isolation membranes (phagophores) happens at the endoplasmic reticulum (ER)-derived omegasomes. While the omegasomes and the ER are not the sole contributors of material for the forming membranes, the building blocks for the forming autophagosomes seem to be recruited to the omegasome sites where the nucleation of autophagic membranes starts (Axe et al., 2008). Upon starvation, DFCP1, a FYVE domain-containing protein, translocates to the omegasomes rendering it a good marker to study omegasome formation (Axe et al., 2008). To test whether the effect of HSPB1 mutants on autophagy affects omegasome formation, we transfected the stably-expressing HeLa cell lines with GFP-DFCP1 and then subjected them to serum starvation for 1.5 hrs as recommended by the study of Itakura and Mizushima (Itakura and Mizushima, 2010). The number of DFCP1 positive puncta was then counted and compared between the different

cell lines. No difference in DFCP1-positive omegasomes was observed between the different cell lines further confirming that the autophagy deficits must be located after the initiation signaling steps and before the completion of autophagy (Fig 2 B).

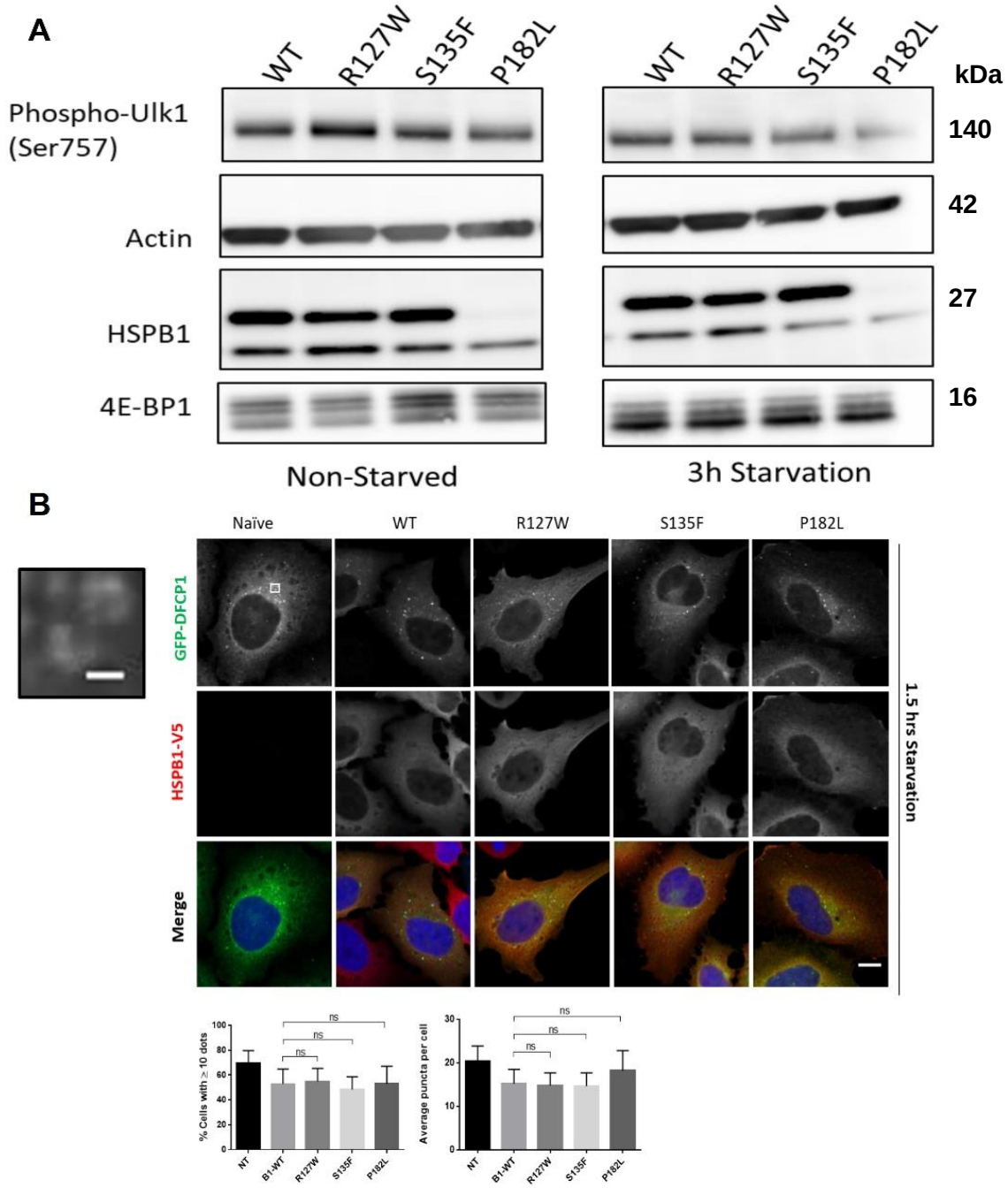
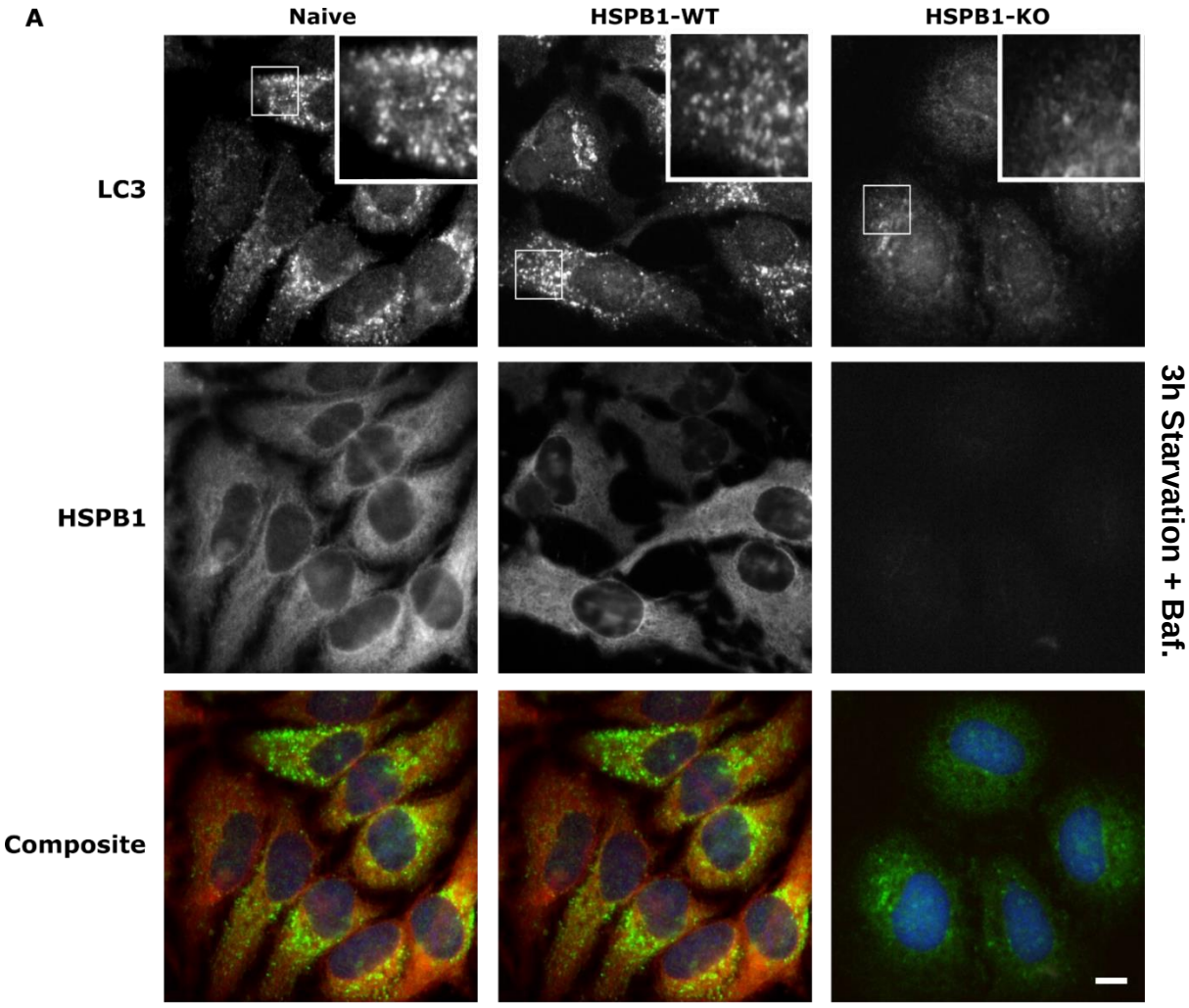


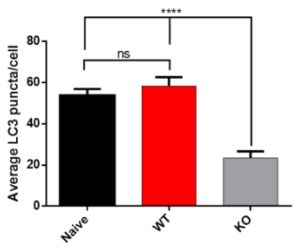
Fig 2. HSPB1 mutations do not disturb the initiation steps of autophagy.

(**A**) Western blot analysis of HeLa cells expressing HSPB1 WT or the mutant forms (R127W, S135F, P182L) treated or not with 3hrs serum starvation. Phosphorylation patterns of ULK1 Ser757, and 4E-BP1 are visualized. Actin was used as a loading control. (**B**) HeLa cells expressing or not HSPB1 WT or the mutants were transfected with GFP-DFCP1. DFCP1 (Green), HSPB1 (Red), and the DAPI nucleus stain (Blue) (Scale bar = 10 μm). positive omega-shaped omegasomes are formed (zoomed square, Scale bar = 1 μm). Statistical analysis was performed using GraphPad Prism and compared the percentage of cells with 10 or more DFCP1-positive puncta (Left graph) and the average number of puncta per cell (Right graph) One-way ANOVA, with Tukey's correction range test. N>2000, Error bars= Mean with 95% CI, **** = $p<0.001$.

CRISPR Knock-out of HSPB1 disrupts autophagy and expression of WT HSPB1 rescues the phenotype

Given the prominent effect of *HSPB1* mutations on the levels of autophagy, and given that overexpression of HSPB1 was shown to increase the rate of autophagy in kidney cells (Matsumoto et al., 2015a), we decided to test if HSPB1 is required for autophagy. Using the CRISPR technology, we knocked-out HSPB1 in HeLa cells and then used automated immunofluorescence microscopy to quantify the number of LC3 positive puncta (autophagosomes) upon autophagy induction. We indeed saw that cells lacking HSPB1 displayed a drastically lower number of autophagosomes compared to the control cell lines (Fig 3 A). We also confirmed our results using western blotting analysis of LC3II levels (Fig 3 B). More interestingly, when we re-expressed HSPB1-WT in the CRISPR knock-out cell lines, the LC3II levels returned to normal (Fig 3B). This illustrates that HSPB1 is needed for starvation induced autophagy and that the expression of HSPB1 rescues the lower autophagy levels in the knock-out cell lines.


A
Naive
HSPB1-WT
HSPB1-KO
LC3
HSPB1
Composite
3h Starvation + Baf.


B
Average LC3 puncta/cell
80
60
40
20
0
ns
****
Naive
WT
KO

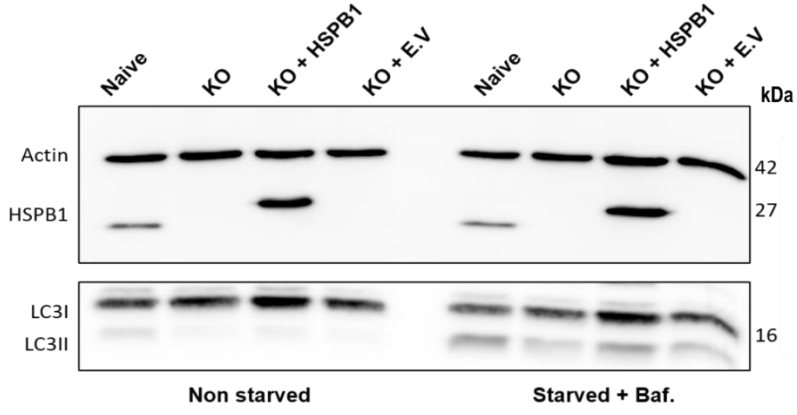

C
Naive
KO
KO + HSPB1
KO + E.V
Naive
KO
KO + HSPB1
KO + E.V
kDa
Actin
HSPB1
42
27
LC3I
LC3II
16
Non starved
Starved + Baf.

Fig 3. Knock-out of HSPB1 impairs autophagy and expression of WT HSPB1 rescues the phenotype.

(**A**) Immunofluorescence microscopy analysis of Naïve, HSPB1-WT expressing, and HSPB1 knock-out (KO) HeLa cell lines treated with serum starvation and 10 nM Bafilomycin A1. LC3 (Green), HSPB1 (Red) and the nuclear stain DAPI (Blue), Scale bar = 20 μm. (**B**) The average number of LC3 puncta per cell from more than 500 cells from 3 independent experiments. One-way ANOVA, with Tukey's correction range test. Error bars= Mean with 95% CI, **** = $p<0.001$ (**C**) Western blot analysis of naïve HeLa cells, HSPB1 KO cells, and KO cells transfected with V5-Tagged HSPB1 or an empty vector (E.V). The levels of LC3II were detected, together with the expression levels of HSPB1. Actin was used as a loading control.

HSPB1 mutants do not disrupt the proteasome or cause a compensatory shift between the autophagy and proteasome pathways

HSPB1 has been shown to interact with the proteasome and to direct some protein substrates to the proteasome for degradation (Parcellier et al., 2003). In addition, a compensatory mechanism is reported to exist between autophagy and proteasomal degradation, mediated by the change in the expression ratio of the BAG (BCL2 Associated Athanogene) family proteins BAG3 and BAG1. An increase in this ratio towards BAG3 can signal a preference towards autophagy and while an increase towards BAG1 marks the proteasomal activation (Gamerdinger et al., 2009). Since BAG proteins have been reported to interact with sHSPs we looked whether the lower levels of autophagy in HSPB1 mutant cell lines lead to a shift in the BAG3-BAG1 ratio and hence an increased activation of proteasomal degradation. Western blotting analysis showed no difference in the BAG3-BAG1 ratio between mutant and WT HeLa cell lines under serum starvation (Fig 4). Next we measured the proteasomal activity in these cells using a fluorogenic indicator substrate (LLVY-R110), which upon cleavage by the proteasome produces a green fluorescent product (R110) which can be measured fluorometrically. The proteasomal activity was not altered in the mutant cell lines compared to the WT or the controls (Fig 4). This indicates that the role for HSPB1 in autophagy is

specifically disrupted by CMT-causing mutations and that the proteasome is not activated in the mutant lines to compensate for the autophagy loss.

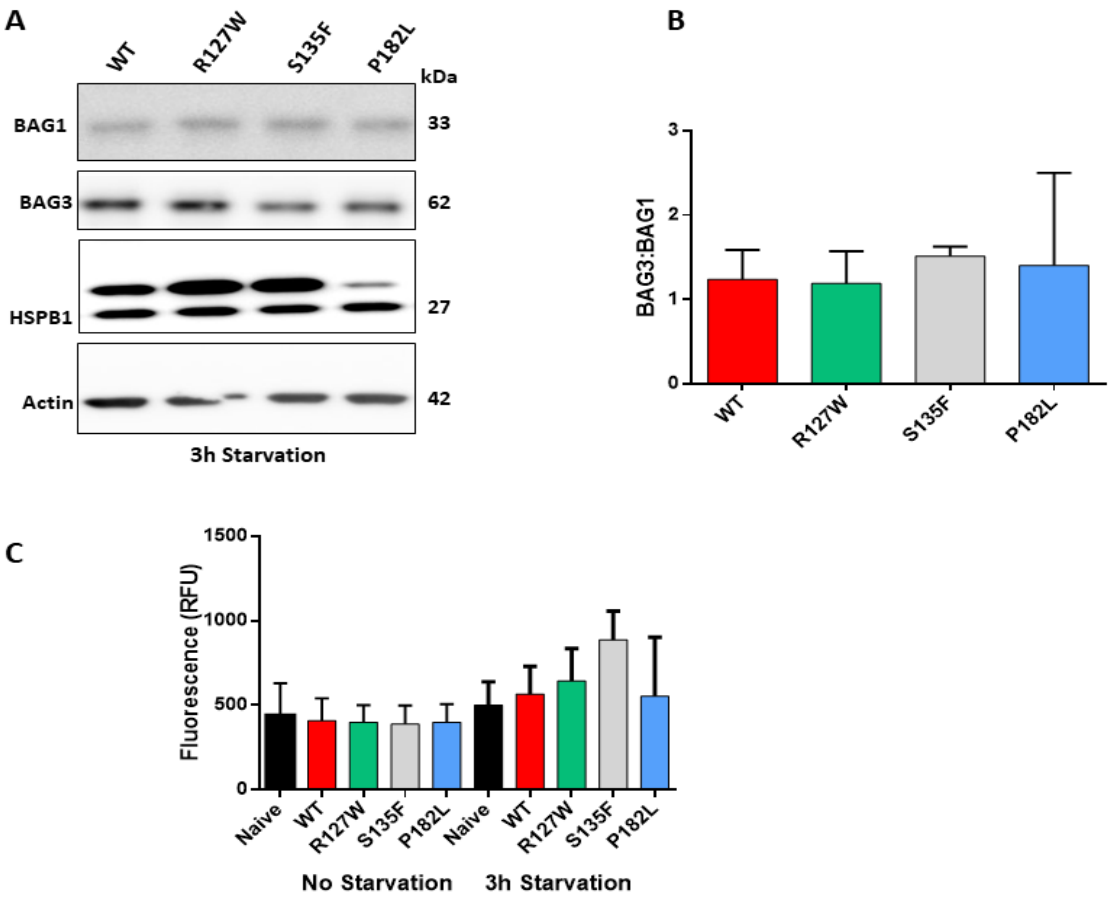


FIG 4. HSPB1 mutants do not disrupt the proteasome or cause a compensatory shift in the BAG3-BAG1 ratio

(**A**) Western blot analysis of BAG1 and BAG3 ratios in HeLa cells expressing HSPB1 WT or the mutant forms (R127W, S135F, P182L). (**B**) The BAG3:BAG1 ratios from 3 independent experiments were compared using One-way ANOVA with Tukey's correction test. Error bars = S.D. B- Fluorometric analysis from 4 independent experiments of Chymotrypsin-like activity of the 20S proteasome in HeLa cell lines before and after starvation treatment. RFU = relative fluorescence units. One-way ANOVA with Tukey's correction test. Error bars = S.D.

Autophagy is disrupted in motor neurons differentiated from patient-derived iPSC

Mutations in *HSPB1* lead to the peripheral neuropathies (CMT2F and dHMNII), meaning that the target of the deficits caused by these mutations are motor neurons. Furthermore, HSPB1 might play different roles in different cell lines (Carra et al., 2013). After establishing that neuropathy-causing mutations in *HSPB1* lead to disruption of autophagy, we decided to validate these results in a motor neuronal model. We used iPSC derived from fibroblasts of a patient carrying the P182L mutation in *HSPB1* and from a healthy control individual and we differentiated these cell lines to motor neurons. The success of the differentiation was validated by the morphology of the neurons and the expression of motor neuron markers: choline acyltransferase (ChAT) and the insulin gene enhancer protein (Islet 1) (Fig 5 A and B). The motor neuron cultures where then treated with nutrient free medium and the lysosomal inhibitor bafilomycin A1 or left untreated. The levels of LC3II were then detected western blotting. We saw that the P182L mutant motor neurons displayed lower levels of LC3II compared to those derived from the healthy control (Fig 5 B, C). We also examined the formation of autophagic puncta by immunofluorescencece (Fig. 5D). Quantification of LC3 puncta from different neurite-dense areas confirmed the lower autophagy levels in the P182L mutant lines upon starvation and bafilomycin A1 treatment (Fig. 5E).

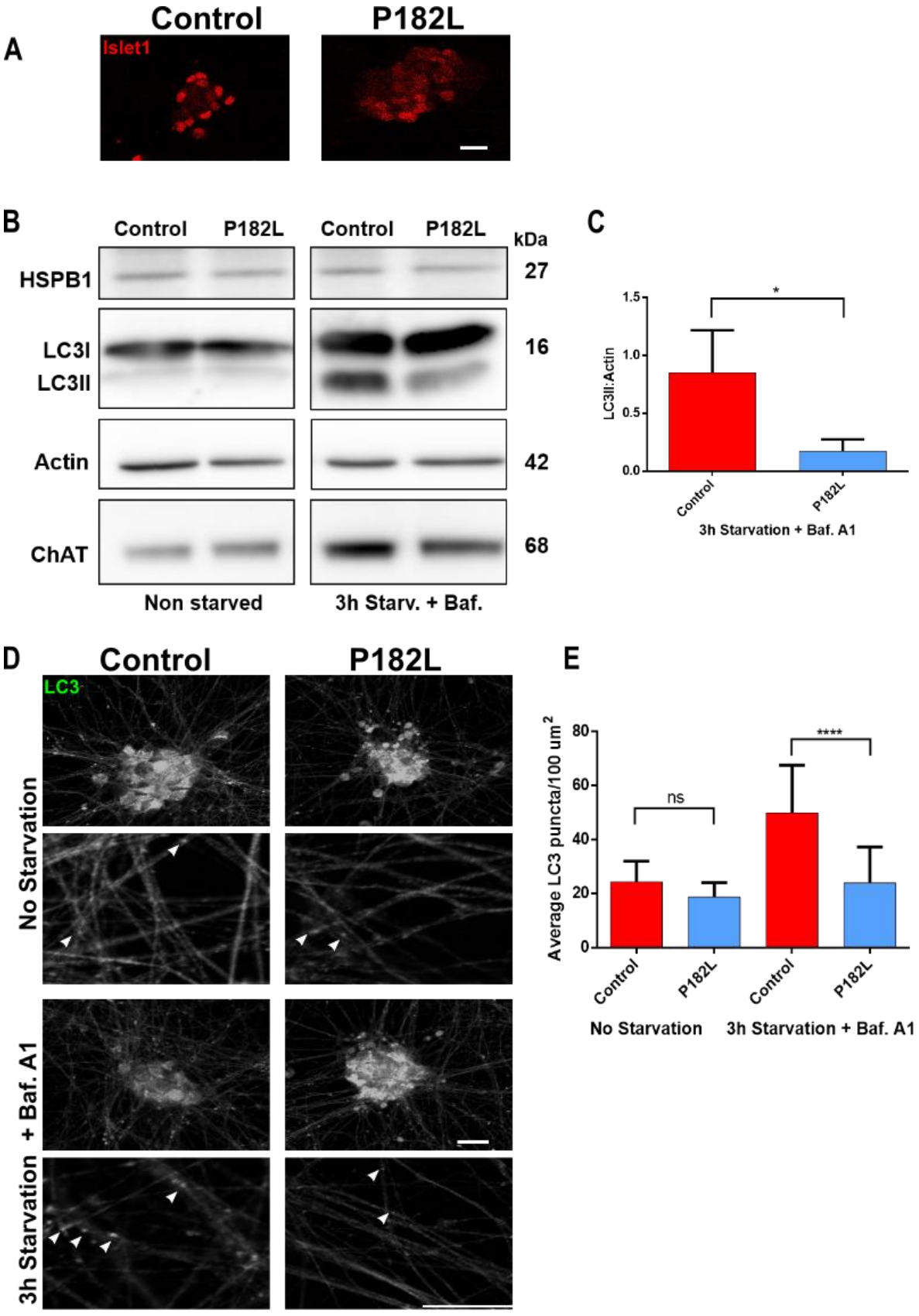

A
Control
P182L
Islet1
B
Control
P182L
Control
P182L
kDa
HSPB1
27
LC3I
LC3II
16
Actin
42
ChAT
68
Non starved
3h Starv. + Baf.
C
LC3II:Actin
1.5
1.0
0.5
0.0
*
Control
P182L
3h Starvation + Baf. A1
D
Control
P182L
LC3
No Starvation
+ Baf. A1
3h Starvation
E
Average LC3 puncta/100 um2
80
60
40
20
0
ns
****
Control
P182L
Control
P182L
No Starvation
3h Starvation + Baf. A1

Fig 5. Motor neurons differentiated from patient derived iPSC show decreased autophagy levels

(**A**) Immunofluorescence analysis of motor neurons differentiated from iPSCs derived from patient fibroblasts carrying the P182L mutation and a healthy control. The motor neurons were stained with the neuronal marker Islet-1 (Red), Scale Bar = 20 μm. (**B**) Western blotting analysis of motor neurons treated and untreated with 3 hrs starvation and bafilomycin A1. (**C**) Quantification of the ratio of LC3II (autophagy marker) over Actin (Loading control) from 3 independent western blotting experiments. Two-tailed t-test, with Tukey's correction range test. Error bars= S.D, * = $p<0.05$. (**D**) Immunofluorescence staining of the autophagy marker LC3 (Green) in motor neurons at 20X and 63X (Scale bar = 20 μm). White arrows indicate LC3-positive puncta (**E**) Quantification of LC3 puncta per 100 μm2 neurite area. One-way ANOVA, with Tukey's correction range test. Error bars= S.D, ns = non significant, **** = $p<0.0001$

Discussion:

In this study, we present evidence that HSPB1 is required for autophagy and that neuropathy-causing mutations in it can disrupt the autophagy pathway. Autophagy has been recently proposed as a common pathomechanism in inherited neuropathies (Haidar and Timmerman, 2017). Defects in autophagy found in neuropathy-causing mutations can answer a question that has puzzled scientists for a while: how can mutations in ubiquitously expressed genes lead to neuronal-specific symptoms? The fact that neurons are more vulnerable to disturbance in autophagy (Lee, 2012; Martinez-Vicente and Cuervo, 2007) means that they are more likely to suffer from the defects in this protein degradation pathway. Another important aspect of neuropathic and neurodegenerative disorders is the fact that they get worse with aging which directly correlates with the accumulation of dead organelles and aggregating proteins due to failure of the pro-degradation processes such as autophagy (Menzies et al., 2017). Importantly, we show that mutations inside and outside of the ACD disrupt the autophagy process which further highlights the important role of HSPB1 in autophagy. While a role for HSPB1 in stimulating the proteasomal degradation of some client proteins has been reported (Parcellier et al., 2003; Zhang et al., 2010) we found that only autophagy is disrupted by mutations in *HSPB1* and by knocking-out *HSPB1*. This might reflect that HSPB1 is more important for

autophagic degradation or that its function in proteasomal degradation is limited to certain client proteins. Our findings are in line with studies showing that other HSPB proteins might be required for autophagy. HSPB8 and HSPB6 have both been shown to play a role in stimulating the autophagic degradation of protein substrates and organelles (Carra et al., 2009b; Fan et al., 2005). Interestingly, not unlike our findings, mutations in *HSPB6* and *HSPB8* causing cardiomyopathy and neuropathy respectively, disturb their ability of stimulating autophagy (Carra et al., 2010; Liu et al., 2017). The specific role of HSPB1 in autophagy is not clear yet, and it might be context-specific as other studies have shown HSPB1 to play a role in autophagy in infection conditions and in kidney injury (Matsumoto et al., 2015a). Preliminary data suggests its role to be related to stress-induced autophagy as treatment with trehalose (an mTORC1 independent activator of autophagy) (Sarkar et al., 2007) does not show the same deficits as serum-starvation treatment in cells expressing mutant HSPB1 (data not shown). Our study do not show that the initiation step of autophagy is affected by *HSPB1* mutations. HSPB1 might be required for the steps after the initiation signals have been induced, and the nucleation of autophagosomes is about to begin rather than the induction of autophagy itself. HSPB1 has the ability to interact with other proteins and mediate several functions and pathways in the cell (Katsogiannou et al., 2014a). Therefore, HSPB1 might be necessary for mediating the formation of autophagosomes through interaction with other protein(s) necessary for nucleating these membranes. A more elaborate study on the role of HSPB1 in autophagy is needed to fully understand it.

Acknowledgements:

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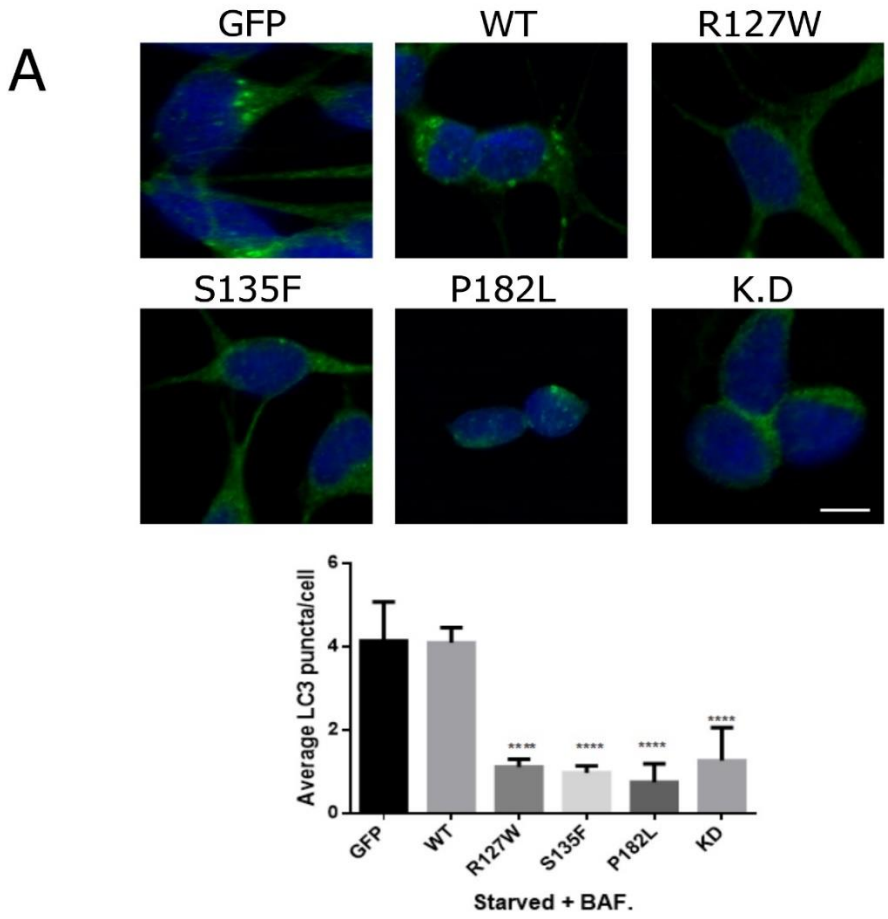

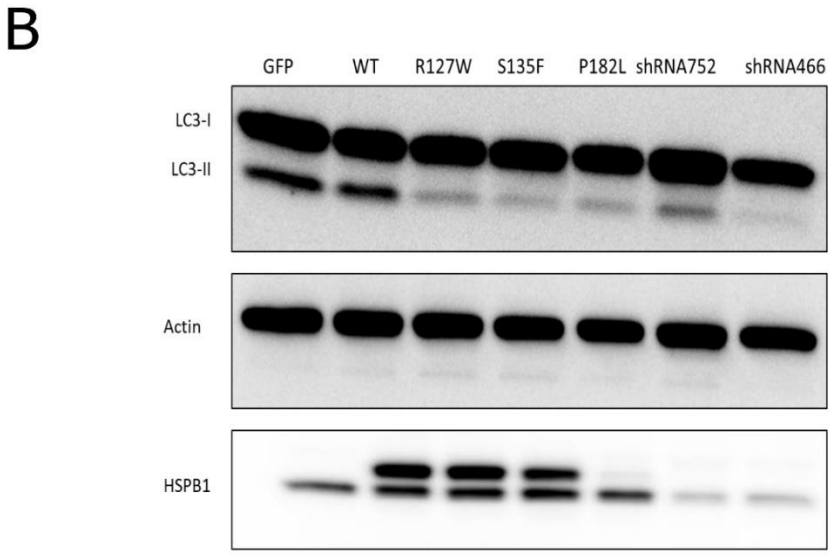

Fig S1: HSPB1 mutations and knock-down disrupt autophagy in neuroblastoma cell lines.

(**A**) Immunofluorescence analysis and quantification of LC3 puncta (Green) in SH-SY5Y neuroblastoma cell lines expressing HSPB1 WT, mutants (R127W, S135F, P182L), or GFP and cells with shRNA knock-down of HSPB1 after starvation treatment with bafilomycin A1. Scale Bar = 10 µm, N= 150 cells. One-way ANOVA with Tukey's correction test, ****= $p<0.0001$, Error bars = Mean with 95% Confidence Interval. (**B**) Western blot analysis of LC3II levels in the different SH-SY5Y cell lines after starvation and bafilomycin A1 treatment.

REFERENCES

Adriaenssens, E., Geuens, T., Baets, J., Echaniz-Laguna, A., and Timmerman, V. (2017). Novel insights in the disease biology of mutant small heat shock proteins in neuromuscular diseases. *Brain* 140, 2541–2549. doi:10.1093/brain/awx187.

Almeida-Souza, L., Asselbergh, B., d'Ydewalle, C., Moonens, K., Goethals, S., de Winter, V., et al. (2011). Small Heat-Shock Protein HSPB1 Mutants Stabilize Microtubules in Charcot-Marie-Tooth Neuropathy. *J. Neurosci.* 31, 15320–15328. doi:10.1523/JNEUROSCI.3266-11.2011.

Axe, E. L., Walker, S. a., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., et al. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J. Cell Biol.* 182, 685–701. doi:10.1083/jcb.200803137.

Boland, B., and Nixon, R. a. (2006). Neuronal macroautophagy: From development to degeneration. *Mol. Aspects Med.* 27, 503–519. doi:10.1016/j.mam.2006.08.009.

Capponi, S., Geuens, T., Geroldi, A., Origone, P., Verdiani, S., Cichero, E., et al. (2016). Molecular Chaperones in the Pathogenesis of Amyotrophic Lateral Sclerosis: The Role of HSPB1. *Hum. Mutat.* 37, 1202–1208. doi:10.1002/humu.23062.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., et al. (2010). Identification of the Drosophila ortholog of HSPB8: Implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* 285, 37811–37822. doi:10.1074/jbc.M110.127498.

Carra, S., Brunsting, J. F., Lambert, H., Landry, J., and Kampinga, H. H. (2009). HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2α phosphorylation. *J. Biol. Chem.* 284, 5523–5532. doi:10.1074/jbc.M807440200.

Carra, S., Rusmini, P., Crippa, V., Giorgetti, E., Boncoraglio, A., Cristofani, R., et al. (2013). Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368, 20110409. doi:10.1098/rstb.2011.0409.

Echaniz-Laguna, A., Geuens, T., Petiot, P., Péréon, Y., Adriaenssens, E., Haidar, M., et al. (2017). Axonal Neuropathies due to Mutations in Small Heat Shock Proteins: Clinical, Genetic, and Functional Insights into Novel Mutations. *Hum. Mutat.* 38, 556–568. doi:10.1002/humu.23189.

Evgrafov, O. V, Mersiyanova, I., Irobi, J., Van Den Bosch, L., Dierick, I., Leung, C. L., et al. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36, 602–606. doi:10.1038/ng1354.

Fan, G. C., Chu, G., and Kranias, E. G. (2005). Hsp20 and its cardioprotection. *Trends Cardiovasc. Med.* 15, 138–141. doi:10.1016/j.tcm.2005.05.004.

Gamerdinger, M., Hajieva, P., Kaya, a M., Wolfrum, U., Hartl, F. U., and Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J.* 28, 889–901. doi:10.1038/emboj.2009.29.

Guo, W., Naujock, M., Fumagalli, L., Vandoorne, T., Baatsen, P., Boon, R., et al. (2017). HDAC6 inhibition reverses axonal transport defects in motor neurons derived from FUS-ALS

patients. *Nat. Commun.* 8, 1–14. doi:10.1038/s41467-017-00911-y.

Haidar, M., and Timmerman, V. (2017). Autophagy as an Emerging Common Pathomechanism in Inherited Peripheral Neuropathies. *Front. Mol. Neurosci.* 10, 1–17. doi:10.3389/fnmol.2017.00143.

Hara, K., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andrabi, K., Weng, Q. P., et al. (1997). Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* 272, 26457–26463. doi:10.1074/jbc.272.42.26457.

Haslbeck, M., and Vierling, E. (2015). A first line of stress defense: Small heat shock proteins and their function in protein homeostasis. *J. Mol. Biol.* 427, 1537–1548. doi:10.1016/j.jmb.2015.02.002.

Holmgren, A. (2012). Molecular Biology of Small Heat Shock Proteins associated with Peripheral Neuropathies. *eLS. John Wiley Sons Ltd, Chichester*, 1–10. doi:10.1002/9780470015902.a0024294.

Holmgren, A., Bouhy, D., De Winter, V., Asselbergh, B., Timmermans, J. P., Irobi, J., et al. (2013). Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments. *Acta Neuropathol.* 126, 93–108. doi:10.1007/s00401-013-1133-6.

Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., et al. (2009). Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol. Biol. Cell* 20, 1981–1991. doi:10.1091/mbc.E08.

Itakura, E., and Mizushima, N. (2010). Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* 6, 764–776. doi:10.4161/auto.6.6.12709.

Kakkar, V., Meister-Broekema, M., Minoia, M., Carra, S., and Kampinga, H. H. (2014). Barcoding heat shock proteins to human diseases: looking beyond the heat shock response. *Dis. Model. Mech.* 7, 421–34. doi:10.1242/dmm.014563.

Kamentsky, L., Jones, T. R., Fraser, A., Bray, M. A., Logan, D. J., Madden, K. L., et al. (2011). Improved structure, function and compatibility for cellprofiler: Modular high-throughput image analysis software. *Bioinformatics* 27, 1179–1180. doi:10.1093/bioinformatics/btr095.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al. (2014). The Functional Landscape of Hsp27 Reveals New Cellular Processes such as DNA Repair and Alternative Splicing and Proposes Novel Anticancer Targets. *Mol. Cell. Proteomics* 13, 3585–3601. doi:10.1074/mcp.M114.041228.

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi:10.1080/15548627.2015.1100356.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., et al. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* 169, 425–434. doi:10.1083/jcb.200412022.

Lee, J.-A. (2012). Neuronal Autophagy: A Housekeeper or a Fighter in Neuronal Cell Survival? *Exp. Neurobiol.* 21, 1. doi:10.5607/en.2012.21.1.1.

Liu, G.-S., Zhu, H., Cai, W.-F., Wang, X., Jiang, M., Essandoh, K., et al. (2017). Regulation of BECN1-Mediated Autophagy by HSPB6: Insights from A Human HSPB6 ^{S10F} Mutant. *Autophagy* 8627, 01–56. doi:10.1080/15548627.2017.1392420.

Martinez-Vicente, M., and Cuervo, A. M. (2007). Autophagy and neurodegeneration: when the cleaning crew goes on strike. *Lancet Neurol.* 6, 352–361. doi:10.1016/S1474-4422(07)70076-5.

Matsumoto, T., Urushido, M., Ide, H., Ishihara, M., and Hamada-, K. (2015). Small Heat Shock Protein Beta-1 (HSPB1) Is Upregulated and Regulates Autophagy and Apoptosis of Renal Tubular Cells in Acute Kidney Injury. 1, 1–22. doi:10.1371/journal.pone.0126229.

Menzies, F. M., Fleming, A., Caricasole, A., Bento, C. F., Andrews, S. P., Ashkenazi, A., et al. (2017). Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. 2. doi:10.1016/j.neuron.2017.01.022.

Mizushima, N., and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. *Autophagy* 3, 542–545. doi:10.4161/auto.4600.

Mymrikov, E. V., Seit-Nebi, a. S., and Gusev, N. B. (2011). Large Potentials of Small Heat Shock Proteins. *Physiol. Rev.* 91, 1123–1159. doi:10.1152/physrev.00023.2010.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., et al. (2003). HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol. Cell. Biol.* 23, 5790–5802. doi:10.1128/MCB.23.16.5790.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi:10.1038/nprot.2013.143.

Salmon, P., and Trono, D. (2006). Production and Titration of Lentivial Vectors. *Curr. Protoc. Neurosci.* 4, 1–24. doi:10.1002/0471142301.ns0421s37.

Sarkar, S., Davies, J. E., Huang, Z., Tunnacliffe, A., and Rubinsztein, D. C. (2007). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and ??-synuclein. *J. Biol. Chem.* 282, 5641–5652. doi:10.1074/jbc.M609532200.

Sun, P., Zhang, S., Qin, X., Chang, X., Cui, X., Li, H., et al. (2017). Foot-and-mouth disease virus capsid protein VP2 activates the cellular EIF2S1-ATF4 pathway and induces autophagy via HSPB1. *Autophagy* 8627, 00–00. doi:10.1080/15548627.2017.1405187.

Wong, P.-M., Feng, Y., Wang, J., Shi, R., and Jiang, X. (2015). Regulation of autophagy by coordinated action of mTORC1 and protein phosphatase 2A. *Nat. Commun.* 6, 8048. doi:10.1038/ncomms9048.

Yang, Z., and Klionsky, D. J. (2010). Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* 12, 814–822. doi:10.1038/ncb0910-814.

Zhang, H., Rajasekaran, N. S., Orosz, A., Xiao, X., Rechsteiner, M., and Benjamin, I. J. (2010). Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin-proteasome pathways. *J. Mol. Cell. Cardiol.* 49, 918–930. doi:10.1016/j.yjmcc.2010.09.004.

Zhang, X., Chen, S., Huang, K., and Le, W. (2013). Why should autophagic flux be assessed? *Acta Pharmacol. Sin.* 34, 595–9. doi:10.1038/aps.2012.184.

CHAPTER 4

An interactomics study of HSPB1 wild-type and mutant links it to the autophagy receptor P62/SQSTM1

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Abstract

HSPB1 has the ability of influencing various cellular processes and signaling pathways by interacting with a wide range of binding partners. Disruption of such interactions is at the basis of many pathomechanisms caused by mutations in *HSPB1*. After revealing that HSPB1 is necessary for the autophagy process and that mutations disrupt this function we decided to explore the interactome of HSPB1 in relation to autophagy. We performed a label-free LC-MS/MS analysis on the various HSPB1 variants (wild type and mutants) and we identified interactors associated with the autophagy process. Furthermore, we reveal the autophagy receptor P62/SQSTM1 as an interactor of HSPB1. We show that mutant HSPB1 might cause abnormal interaction with P62/SQSTM1 affecting its autophagy function. Our study shows that cells expressing mutant HSPB1 have a decreased capacity to form P62-bodies which are crucial for the P62/SQSTM1 function in autophagy, and lead to decrease in phagophore formation. These findings propose HSPB1 as a regulator of autophagy through interaction with P62/SQSTM1.

Introduction:

Macroautophagy, hereafter referred to as autophagy, is a homeostatic cellular process which targets and degrades protein aggregates and cellular organelles in vesicles called autophagosomes. Autophagy consists of multiple steps: induction, nucleation of the isolation membrane (or phagophore), elongation of the phagophore to form fully closed, double-membraned autophagosomes, and the fusion of autophagosomes with lysosomes for cargo degradation (Parzych and Klionsky, 2014). The various autophagy steps are commanded by a wide array of proteins and protein complexes (Behrends et al., 2010). Disruption of one or more of these regulatory complexes and proteins can lead to defects in autophagy (Komatsu et al., 2005). Such disruption can result from disease-causing mutations, especially in neuropathic and neurodegenerative diseases where a healthy autophagy machinery is crucial for the health and maintenance of neurons (Frake et al., 2015). We have shown before that mutations in *HSPB1* impair autophagy (see Chapter 3). Similarly knock-out of *HSPB1* disrupts autophagy suggesting that it is necessary for the autophagy process. While our data pointed out to the involvement of HSPB1 in the formation of autophagosomes, the exact role of HSPB1 in autophagy and the way mutations impair this role remained unclear.

HSPB1 has been shown to interact with a wide variety of other proteins, mediating different cellular processes including cytoskeletal dynamics and apoptosis through such interactions (Arrigo and Gibert, 2014; Katsogiannou et al., 2014b). In addition, mutations in *HSPB1* have been shown to alter its interactions with other proteins and hinder its relative function such as 1) the R127W and S135F mutations which lead to increased binding to actin and overstabilisation of microtubules and 2) the P182L mutant which disturbs the translational function of PCBP1 by increased binding (Almeida-Souza et al., 2011; Geuens et al., 2017). Interestingly, other small heat shock proteins, HSPB6 and HSPB8 have been shown to play a pivotal role in autophagy by binding to other autophagy-related proteins. HSPB6 can bind to the autophagy regulator BECN1 to stimulate autophagy (Liu et al., 2017). HSPB8 forms a complex with the co-chaperone BAG3, Hsc70 (Heat shock cognate 70) and CHIP (C-terminus of the Hsc70-interacting protein) to activate the autophagic degradation of protein aggregates (Carra et al., 2008b). Mutations in *HSPB6* and in *HSPB8*, which lead to disease conditions, have been shown to disrupt autophagy by disturbing the interactions of these HSPBs with their autophagy-related partners (Carra et al., 2010; Liu et al., 2017). Therefore, it is not far-fetched to assume that HSPB1 might play a role in autophagy by interacting with autophagy-associated proteins, and that mutations in *HSPB1* affect such interaction.

To further elucidate the involvement of HSPB1 in autophagy, we performed a protein interaction study using label-free LC-MS/MS analysis (liquid chromatography followed by mass spectrometry) and we looked for different autophagy-related interactors of HSPB1. We identified the autophagy receptor, sequestosome-1 (SQSTM1/P62) as an interactor of HSPB1. We show that HSPB1 binds to the Phox and Bem-1 (PB1) domain of P62, necessary for its oligomerization. The oligomerization of P62 is necessary for forming P62-bodies and scaffolds that allows the formation of isolation membranes destined to become autophagosomes. We show that mutations in *HSPB1* impair the ability of P62 to form P62-bodies and lead to a decreased formation of isolation membranes upon starvation.

Materials and Methods:

Creation of constructs and stables cell lines:

Constructs used to generate stable HeLa and SH-SY5Y cell lines were designed using the Gateway recombination system (Life Technologies). The open reading frames of HSPB1 and EGFP were amplified by PCR using specific primers flanked by attB recombination sites to allow the insertion of the PCR product in a pDONR221 vector. The HSPB1 mutations used in this study (HSPB1-R127W, HSPB1-S135F, and HSPB1-P182L) were generated by site-directed mutagenesis. Sequence validated pDONRs were transferred by recombination to a pLenti6/V5 destination vector (Life Technologies) to generate constructs where the ORF is fused to a V5-tag. All the finally obtained plasmids were validated by Sanger sequencing. Stable cell lines were generated by lentiviral transduction of HeLa cell lines or the neuroblastoma cell line SH-SY5Y according to the method described previously (Salmon and Trono, 2006). The expression levels and growth rates of the generated cell lines were monitored and shown to be similar (data not shown).

Cell culture material and conditions:

Unless otherwise mentioned, all the cell culture media and supplements were purchased from Life Technologies. The HeLa cell line was purchased from ATCC and grown at 37ºC and 5% CO2 in MEM supplemented with 10% fetal calf serum (FCS), and 1% Glutamine and Penicillin-Streptomycin. The human neuroblastoma cell line SH-SY5Y was purchased from ATCC and cultured at 37ºC and 5% CO_2 in DMEM supplemented with 10% FCS, non-essential amino acids, glutamine and Penicillin-Streptomycin. From the patients we obtained lymphocytes through venepuncture and lymphoblastoid cell lines were EBV-transformed and cultivated at 37°C and 6% CO_2 in Gibco® RPMI 1640 supplemented with 15% FCS, Sodium Pyruvate and Penicillin-Streptomycin. Lymphocytes were obtained from an HSPB1-P182L (cmt391.21), an HSPB1-R127W patient (cmt751.01) and a healthy control individual (ceph1454.14). Medical ethical approval was obtained from our local medical ethical committees to perform our studies using patient derived material.

Co-immunoprecipitation:

Cells were lysed in a buffer containing 20 mM Tris-HCL; pH 7.4, 2.5 mM $MgCl_2$, 100 mM KCL, 0.5% Nonidet P-40, together with complete protease inhibitor (Roche Applied Science)

and left on ice for 20 mins. Sepharose 6B (Sigma Aldrich) and V5-coupled protein G beads (Sigma Aldrich) were used in 3:1 ratio and incubated overnight at 4ºC together with the protein lysate. Afterwards beads were pulled down by centrifugation and were repeatedly washed with lysis buffer. Co-immunoprecipitated proteins were sent for LC-MS/MS analysis or together with total lysates were resolved by SDS-PAGE.

LC-MS/MS analysis:

After HSPB1 pull-down, washed beads were re-suspended in 150 µl trypsin digestion buffer and incubated for 4 hours with 1 µg trypsin (Promega) at 37 ˚C. Beads were removed by centrifugation and another 1 µg of trypsin was added to the supernatants to complete digestion overnight at 37 ˚C. Peptides were purified on Omix C18 tips (Agilent), dried and re-dissolved in 20 µl loading buffer (0.1% trifluoroacetic acid in water/acetonitrile (98:2, v/v)) of which 2 µl was injected for LC-MS/MS analysis on an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific, Bremen, Germany) in-line connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). The peptides were first loaded on a trapping column (made in-house, 100 µm internal diameter (I.D.) × 20 mm, 5 µm beads C18 Reprosil-HD, Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing the trapping column, peptides were loaded in solvent A (0.1% formic acid in water) on a reverse-phase column (made in-house, 75 µm I.D. x 250 mm , 3 µm beads C18 Reprosil-Pur, Dr. Maisch, packed in the needle and eluted by an increase in solvent B (0.1% formic acid in acetonitrile) in a linear gradient from 2% solvent B to 55% solvent B in 120 minutes, followed by a washing step with 99% solvent B, all at a constant flow rate of 300 nl/min. The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant peaks in a given MS spectrum. The source voltage was set at 3.4 kV, and the capillary temperature at 275°C. One MS1 scan (m/z 400−2,000, AGC target 3×10^6 ions, maximum ion injection time 80 ms), acquired at a resolution of 70,000 (at 200 m/z), was followed by up to 10 tandem MS scans (resolution 17,500 at 200 m/z) of the most intense ions fulfilling predefined selection criteria (AGC target 5×10^4 ions, maximum ion injection time 60 ms, isolation window 2 Da, fixed first mass 140 m/z, spectrum data type: centroid, underfill ratio 2%, intensity threshold $1.7xE^4$, exclusion of unassigned, 1, 5-8, >8 positively charged precursors, peptide match preferred, exclude isotopes on, dynamic exclusion time 20 s). The HCD collision energy was set to 25% Normalized Collision Energy and the polydimethylcyclosiloxane background ion at 445.120025 Da was used for internal calibration (lock mass).

Protein Identification and statistical analysis:

Data analysis was performed with MaxQuant (version 1.5.3.30) (Cox and Mann, 2008) using the Andromeda search engine with default search settings including a false discovery rate set at 1% on both the peptide and protein level. Spectra were searched against the human proteins in the Uniprot/Swiss-Prot database (database release version of April 2015 containing 20,193 human protein sequences (www.uniprot.org) expanded with the eGFP sequence. The mass tolerance for precursor and fragment ions were set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to arginine and lysine, also allowing cleavage at proline bonds with a maximum of two missed cleavages. Variable modifications were set to oxidation of methionine residues and acetylation of protein N-termini, phosphorylation of serine, threonine and tyrosine residues and biotinylation of lysine residues.

Proteins were quantified by the MaxLFQ algorithm integrated in the MaxQuant software (Cox et al., 2014). A minimum ratio count of two unique or razor peptides was required for quantification. Further data analysis was performed with the Perseus software (version 1.5.2.6) after loading the proteingroups file from MaxQuant. Proteins only identified by site, contaminants and reverse database hits were removed and replicate samples were grouped. Proteins with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution around the detection limit.

For each identified protein, a two-way ANOVA test calculated a -log p-value. Proteins with a -log p-values > 2 were then considered significantly up- or downregulated. The intensities of these significantly regulated proteins were then visualised on a heatmap after non-supervised hierarchical clustering.

Transient transfection

HeLa cell lines were transfected using polyethylenimine (PEI) according to an in-house optimized protocol. Briefly, cells were seeded out in a 24-well plate at $7x10^4$ cells per well 24 hrs before transfection. On the day of transfection, 500 ng plasmid DNA was diluted in 36 µl Opti-MEM (Life Technologies) and in parallel, 2.5 µl PEI (1 ug/ul) was diluted in 36 µl Opti-MEM. The diluted PEI was then added to the DNA and mixed by vortexing for 10 seconds. Afterwards, the PEI-DNA mix was incubated for 10 mins at room temperature, and then added drop-wise onto the cells. Cells were used 24 hours post-transfection for plasmid DNA and 48 hours post-transfection for shRNA treatment.

Generation of HSPB1 CRISPR knock-out cell lines

Knock-out cell lines for HSPB1 were generated according to the protocol of Ran, et al, 2013 (Ran et al., 2013). In brief, two guide RNAs (gRNAs) targeting the first nucleotides after the start codon, each selected based on its specificity score and number of off-targets (online tool MIT, http://crispr.mit.edu). The gRNAs were ordered as phosphorylated primers from IDT (www.idtdna.com) and cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene). gRNA efficiency was verified by quantifying the reduction of HSPB1 protein expression in HEK293T cells after transfecting the gRNAs with polyethylenimine and performing a western blot after 72 h. Next, HeLa cells were transfected in 6-well dishes with the PX459 plasmids using Lipofectamine LTX Plus (Life Technologies) and puromycin was added (1µg/ml) for 72 h. Surviving cells were then serially diluted in a 96-well plate in order to isolate single colonies. After expansion of the single colonies, complete knock-out of HSPB1 was assessed by western blotting and the presence of premature stop codons was verified by Sanger sequencing.

Autophagy induction and treatment

Autophagy was induced by serum starvation by culturing cells in culture medium deprived of FCS for the indicated amount of time. Where necessary, the lysosomal inhibitor Bafilomycin A1 (Enzo Life Sciences) was used at 10nM concentration to block the lysosomal degradation.

Western blotting

Cells were lysed in lysis buffer (0.5% Nonidet P-40, 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 4mM Sodium orthovanadate, 20mM Glycerol-2-Phosphate, 10 mM Sodium Fluoride, 1 mM Sodium Pyrophosphate, together with complete protease and Phospho-STOP inhibitor mixtures - Roche Applied Science) for 30 min on ice and cleared by centrifugation for 10 min at 14.000RPM. After protein concentration was determined, by using the Pierce BCA protein assay kit (ThermoFisher Scientific), cell lysates were boiled for 5 min at 95°C in reducing Laemmli buffer (Life Technologies) supplemented with 100mM 1,4-Dithiothreitol (DTT). Proteins were separated on NuPAGE gels (Life Technologies) and transferred to a nitro-cellulose-membrane (Hybond^{TM}-P, GE Healthcare). Blocking of the membrane was performed using 5% milk powder diluted in PBS, supplemented with 0.1% Tween 20. Afterwards, membranes were incubated with a primary antibody over night at 4°C and one hour with a secondary horseradish peroxidase conjugated antibody. Blots were developed by using the Enhanced Chemiluminescence ECL Plus^{TM} detection system (Thermoscientific) and imaged with an ImageQuant imager (GE Healthcare).

Band intensities were determined by quantifying the mean pixel grey values using the ImageJ software (Eliceiri et al., 2012). Mean pixel grey values were measured in a rectangular region of interest.

Immunocytochemistry and immunofluorescence microscopy

Immunostainings were performed according to a standardized protocol. Briefly, cells were seeded out on glass coverslips the day before fixation. Fixation was performed by incubating the cells with 4% paraformaldehyde or Ice Cold Methanol for 20 min. Afterwards cells were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS). Blocking was performed with 5% BSA diluted in PBS for 1 hour, primary and secondary antibodies were incubated for 1 hour diluted in 1% BSA in PBS supplemented with 0.1% Tween 20. Nuclear staining was done by using Hoechst33342 (Life Technologies), afterwards cells were mounted with fluorescent mounting medium (Dako). Images were taken on a LSM700 confocal fluorescence microscope using a 40x/1.40 plan-apochromatic objective. For assays using automated image acquisition, cells were seeded (15.000 cells per well) in Ibidi µ-Plate IbiTreat 96-well plates and fixed 24h later in 4% paraformaldehyde. Three replicate wells were used per genotype and immunocytochemical staining was performed as described above. Images were acquired with an automated Nikon Eclipse TiE microscope, equipped with DS-Qi2 camera, PFS3, 40x Plan Fluor 0.75 NA objective (DIC M N2), Spectra X epifluorescence excitation source, motorized stage (Prior) and using the JOBS module of Nikon Elements software (Nikon Instruments Europe B.V.). Per well, 30 randomly positioned multichannel image sets (1608 x 1608 pixels, 4 channels, 0.183 µm pixels) were acquired using the PS3 hardware autofocus.

Image analysis and quantification was done automatically using CellProfiler software (Kamentsky et al., 2011). An image analysis pipeline was designed in CellProfiler to allow for the segmentation and quantification of individual cells, punctate structures inside the cells, and their relative intensities. For the colocalization experiment, regions of interest (ROIs) containing P62 puncta were selected from each image and the intensisty-based colocalisation was measure in ImageJ using the Coloc-2 plugins. The Pearson's colocalisation ratio R, was calculated and compared between different cell lines. Statistical analysis was then performed in R and GraphPad Prism.

Antibodies and plasmids used

The following antibodies were used in this study: anti-HSPB1 (Enzo Life Sciences), anti-V5 (Life Technologies), anti-LC3 (Sigma-Aldrich), anti-GFP (Abcam), anti-P62 (Cell Signaling), anti-FLAG (Sigma-Aldrich), Anti-beta-Actin (Sigma-Aldrich), anti-ATG9A (Bio-Techne), anti-TRAPPC8 (Sigma-Aldrich), anti-GM130 (BD Bioscience). The pEGFP-ATG14L and the pMXs-IP GFP-WIPI-1 plasmids were purchased from Addgene Inc. The pCS2-FLAG-WT-p62, pCS2-FLAG-deltaUBA-p62, and pCS2-FLAG-deltaPB1-p62 constructs were a kind gift from the group of Prof. Aaron Ciechanover, Technion-Israel Institute of Technology, Haifa, Israel. The mCherry-p62 WT, mCherry-p62 LIR mutant (335 DDDW 338 -> AAAA), and mCherry-p62 ΔBP1 (Δ2-102) plasmids were a kind gift from the lab of Prof. Sascha Martens, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Vienna, Austria. shRNAs against SQSTM1 were purchased from Sigma Aldrich and contained the following TRC

numbers and sequences:
(TRCN0000007234,CCGGCGAGGAATTGACAATGGCCATCTCGAGATGGCCATTGT
CAATTCCTCGTTTTT);(TRCN0000007236,CCGGCCGAATCTACATTAAAGAGAACT
CGAGTTCTCTTTAATGTAGATTCGGTTTTT);(TRCN0000007237,CCGGCCTCTGGGC
ATTGAAGTTGATCTCGAGATCAACTTCAATGCCCAGAGGTTTTT)

Results

Identification of HSPB1 interactors

To identify protein interaction partners of wild type (WT) HSPB1 and 3 different HSPB1 mutants: P182L, R127W and S135F, HeLa cells stably expressing V5-tagged WT or mutant proteins were used. Cells were grown with or without serum and V5 pull-down was performed, followed by LC-MS/MS analysis. Label-free quantitation was used to compare the different variants and conditions. As negative control, pull down of V5-tagged eGFP was performed in parallel (Fig 1, A). Afterwards, data analysis with MaxQuant (version 1.5.3.30) (Cox and Mann, 2008) followed by statistical analysis was then performed and proteins significantly upregulated in the HSPB1 expressing samples were identified as interaction partners. For visualization, the intensities of these significantly regulated proteins were then shown on a heatmap after non-supervised hierarchical clustering (Fig 1, B and C; Fig S1) and associated

proteins are listed in Table S1 and Table S3. When grouped according to treatment condition, 133 proteins were upregulated with starvation and 107 were downregulated (Fig S1, Table S2). When grouped according to the different HSPB1 variant, 3 different clusters were identified. Cluster 1 represented proteins that interact with the R127W and S135F mutant forms and contained 67 proteins. Cluster 2 contained 28 proteins that interact with all the different HSPB1 variants (WT and mutants), and Cluster 3 contained 26 proteins that showed interaction with the P182L mutant form of HSPB1 (Fig1 B and C). Afterwards, the interactors from each of the 3 clusters were filtered using a literature study according to their role in autophagy. The interactors with known roles in autophagy are listed in table 1 (15 from cluster 1, 2 from cluster 2, and 7 from cluster 3). Selected autophagy-associated interactors were then subjected to validation by immunoprecipitation.

HSPB1 binds to P62 and the mutant HSPB1 forms show increased binding to P62:

Sequestosome 1 (SQSTM1) also known as P62 is a well-established autophagy receptor (Bjørkøy et al., 2005; Katsuragi et al., 2015). We performed immunoprecipitation of V5-tagged HSPB1 in HeLa cells expressing WT HSPB1 or the mutant forms (R127W, S135F, and P182L). Afterwards, we performed immunoblotting to detect if P62 was co-immunoprecipitated with HSPB1. We saw that P62 was indeed immunoprecipitated with all the different forms of HSPB1 (WT and mutant) (Fig 2 A and B). This observation was also confirmed in SH-SY5Y cell lines stably expressing the WT or mutant forms of HSPB1 (Fig S2). To make sure that the interaction of HSPB1 with P62 is not an artefact of V5-tagging, we performed immunoprecipitation of endogenous HSPB1 in lymphoblasts from patients carrying the R127W mutation and patients carrying the P182L mutation, and from a healthy control. All the different lymphoblast lines showed co-immunoprecipitation of P62 with the pull down of endogenous HSPB1 confirming that the interaction between these two proteins is not due to an artefact (Fig 2 C). Interestingly, the mutant forms of HSPB1 showed tendency for increased binding to P62 compared to the WT in HeLa cells (Fig 2 A and B). The increased binding feature of HSPB1 mutants to interacting proteins has been reported to be at the centre of their functional pathogenicity before (Almeida-Souza et al., 2011; Geuens et al., 2017).

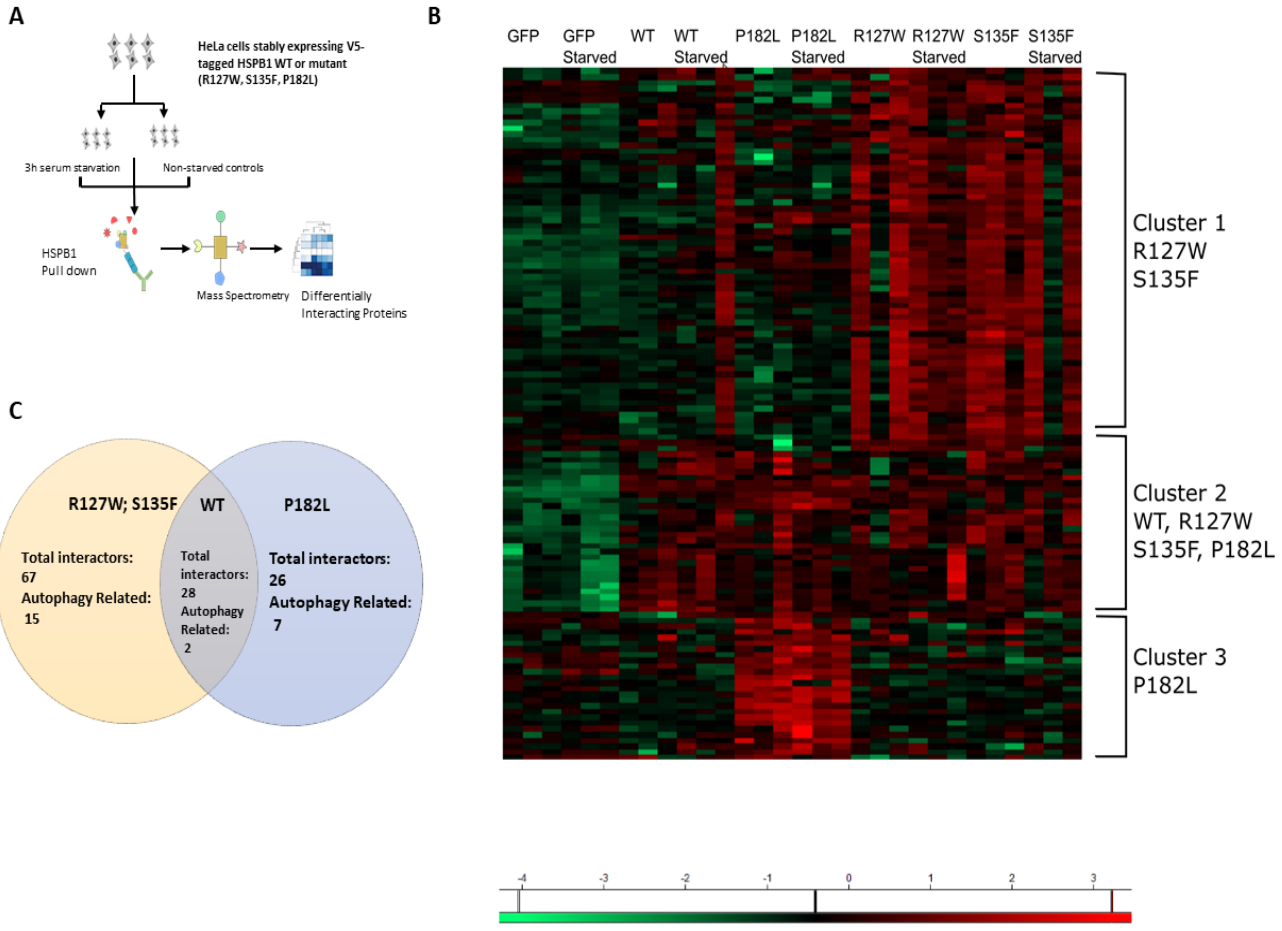

Fig 1. Identification of Interaction partners of WT and mutant HSPB1

(A) Design of the experiment. (**B)** Cluster analysis of the different HSPB1 variants revealing 3 clusters corresponding to the different HSPB1 variants. Upregulated proteins are shown in Red, down regulated proteins are shown in Green. (**C)** A venn diagram summarizing the number of interactors per cluster and the number of interactors linked to autophagy.

Cluster	-Log Two-way ANOVA p value	Protein name	Gene name	Link to Autophagy
1	5.4241	Clusterin	CLU	facilitates LC3-Atg3 stability and LC3 lipidation
1	3.8526	Emerin	EMD	involved in ceramide-induced autophagy (binds to LC3 after phosphorylation)
1	3.6812	Glutamate dehydrogenase 1	GLUD1;GLUD2	Knock-down leads to Autophagy increase
1	2.8573	Protein transport protein Sec16A	SEC16A	interacts with Ulk1
1	2.7696	Translational activator GCN1	GCN1L1	involved in cell response to amino acid starvation
1	2.5735	LETM1 and EF-hand domain-containing protein 1	LETM1	Knock-down leads to LC3II increase
1	2.5332	Microtubule-associated protein 1B	MAP1B	links microtubules to autophagy receptor nbr1
1	2.1026	Transferrin receptor protein 1	TFRC	colocalizes with phagophores
1	2.0613	Sequestosome-1	SQSTM1	Selective autophagy marker
1	1.7504	Acyl-CoA desaturase	SCD	Is required for autophagosome formation
1	1.6138	Protein-S-isoprenylcysteine O-methyltransferase	ICMT	Inhibition induces autophagy
1	1.3832	Ancient ubiquitous protein 1	AUP1	Required for mitophagy in yeast
2	2.5583	Proteasome subunit beta type-5	PSMB5	decreased expression leads to decreased proteasome activation and increased autophagy
2	2.4775	Prohibitin	PHB	increased expression leads to decreased autophagy
3	7.6804	AP-1 complex subunit gamma-1	AP1G1	essential for transport from PM or TG
3	5.9430	Trafficking protein particle complex subunit 8	TRAPPC8	Necessary of ATG9 recruitment to phagophores
3	2.7361	TGF-beta-activated kinase 1 and MAP3K7-binding protein 2	TAB2	negative regulator of autophagy
3	2.2412	Collagen alpha-1(I) chain	COL1A1	increased after starvation in fibroblasts
3	1.6145	Ras-related protein Rab-32	RAB32	is required for the formation of autophagic vacuoles under basal conditions
3	1.5771	Kinesin-like protein KIF2A	KIF2A	is required for the formation of autophagic vacuoles under basal conditions
3	1.3689	Exocyst complex component 2	EXOC2	Binds RALB, BECN1, MTORC1, ULK1 and PIK3C3 under nutrient-rich conditions, inhibiting macroautophagy

Table 1. List of autophagy-associated interactors of HSPB1 variants and their link to autophagy.

Table showing the significantly upregulated proteins per cluster, their p-value, gene and protein name, and their link to autophagy. The autophagy-related function is based on genecards information followed by literature search.

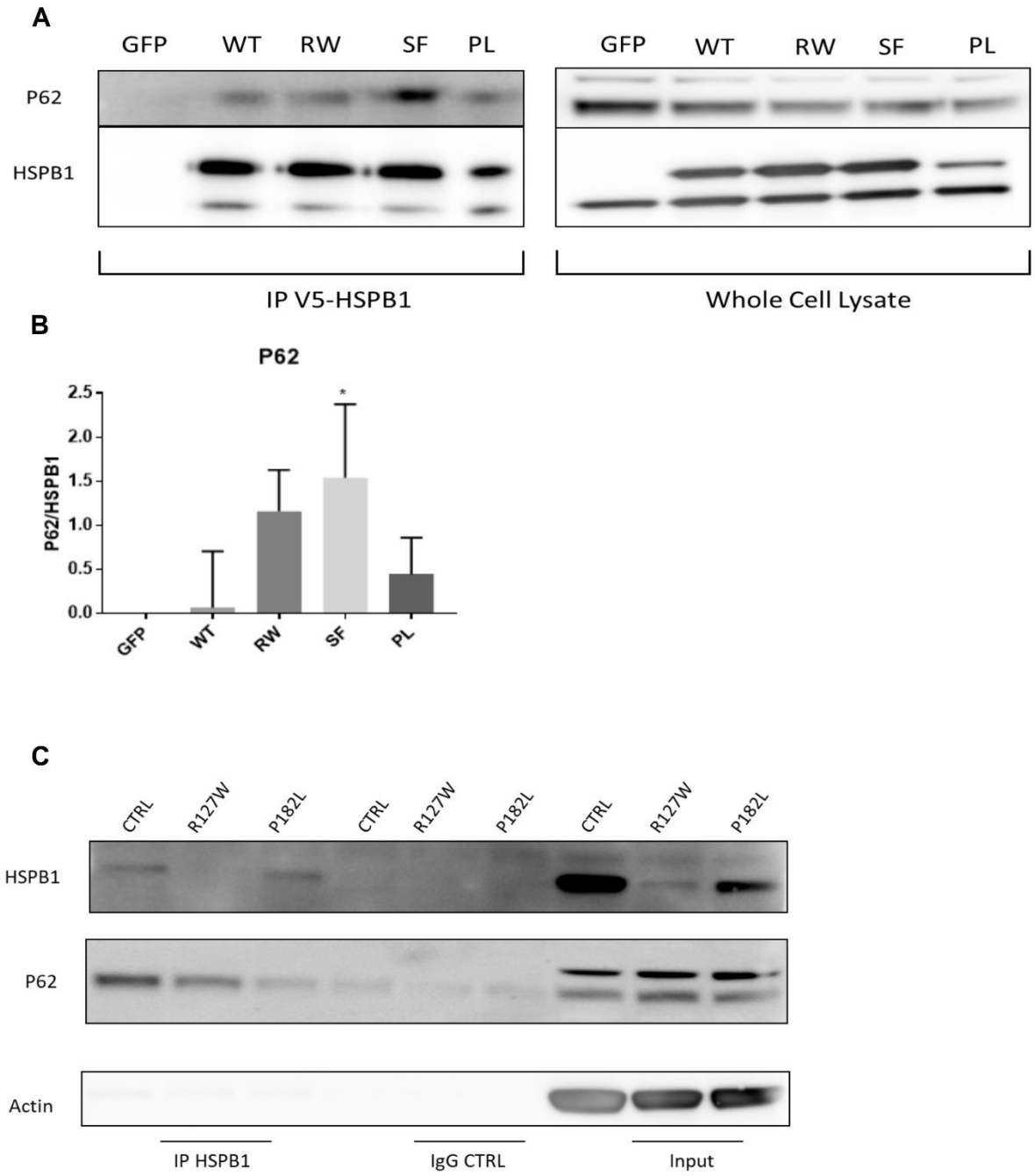

Fig 2. HSPB1 interacts with P62.

(**A**) Co-Immunoprecipitation analysis of HSPB1 in HeLa cells expressing GFP or V5-Tagged HSPB1 WT or mutant (R127W, S135F, and P182L). (**B**) Quantification of band intensities of P62 from 3 different co-immunoprecipitation experiments show tendency for mutant HSPB1 to show increased binding to P62. N=3, One-way ANOVA with Tukey's correction test. *=P<0.05. (**C**) Co-immunoprecipitation analysis of HSPB1 in lymphoblasts derived from patients carrying the HSPB1 mutations R127W or P182L and from a healthy control.

HSPB1-P182L mutant interacts with TRAPPC8 but does not disrupt its function in autophagy

The trafficking protein particle complex subunit 8 (TRAPPC8) belongs to the TRAPP complex and can modulate autophagy and secretory trafficking (Imai et al., 2016; Lamb et al., 2016). Recent studies have shown that TRAPPC8 is necessary for the trafficking of the autophagy related protein 9 (ATG9) between the Golgi apparatus and the recycling endosomes and to the forming autophagosomes (Imai et al., 2016; Lamb et al., 2016). ATG9 is the only transmembrane protein in autophagy and is required for the formation of phagophores (Reggiori and Tooze, 2012). Since our data hinted to a deficit in the formation of autophagosomes, we also performed immunoprecipitation analysis to validate TRAPPC8 as an interactor of HSPB1. TRAPPC8 was only co-immunoprecipitated with the P182L mutant (Fig 3 A). The P182L mutant has been shown to form large assemblies inside the cells that might trap other interacting proteins and prevent their function (Ackerley et al., 2006). We checked whether the P182L mutant, by binding to TRAPPC8, binds to ATG9. However we were not able to pull down ATG9 together with P182L in HeLa cells (Fig 3 A). Knock-down of TRAPPC8 was shown to disrupt the translocation of ATG9 from the Golgi apparatus upon starvation and to disrupt Golgi structure and function (Lamb et al., 2016). We used immunofluorescence to detect the cellular localisation of ATG9 in HeLa cells expressing WT HSPB1 and the P182L mutant. We could not detect a difference in the cellular dispersion of ATG9 in fed and starved conditions between the WT and the mutant cell lines (Fig 3B). In addition, we did not detect any difference in the Golgi structure among these cell lines (Fig 3B). These results indicate that the interaction between the P182L mutant and TRAPPC8 does not have an effect on the autophagy process.

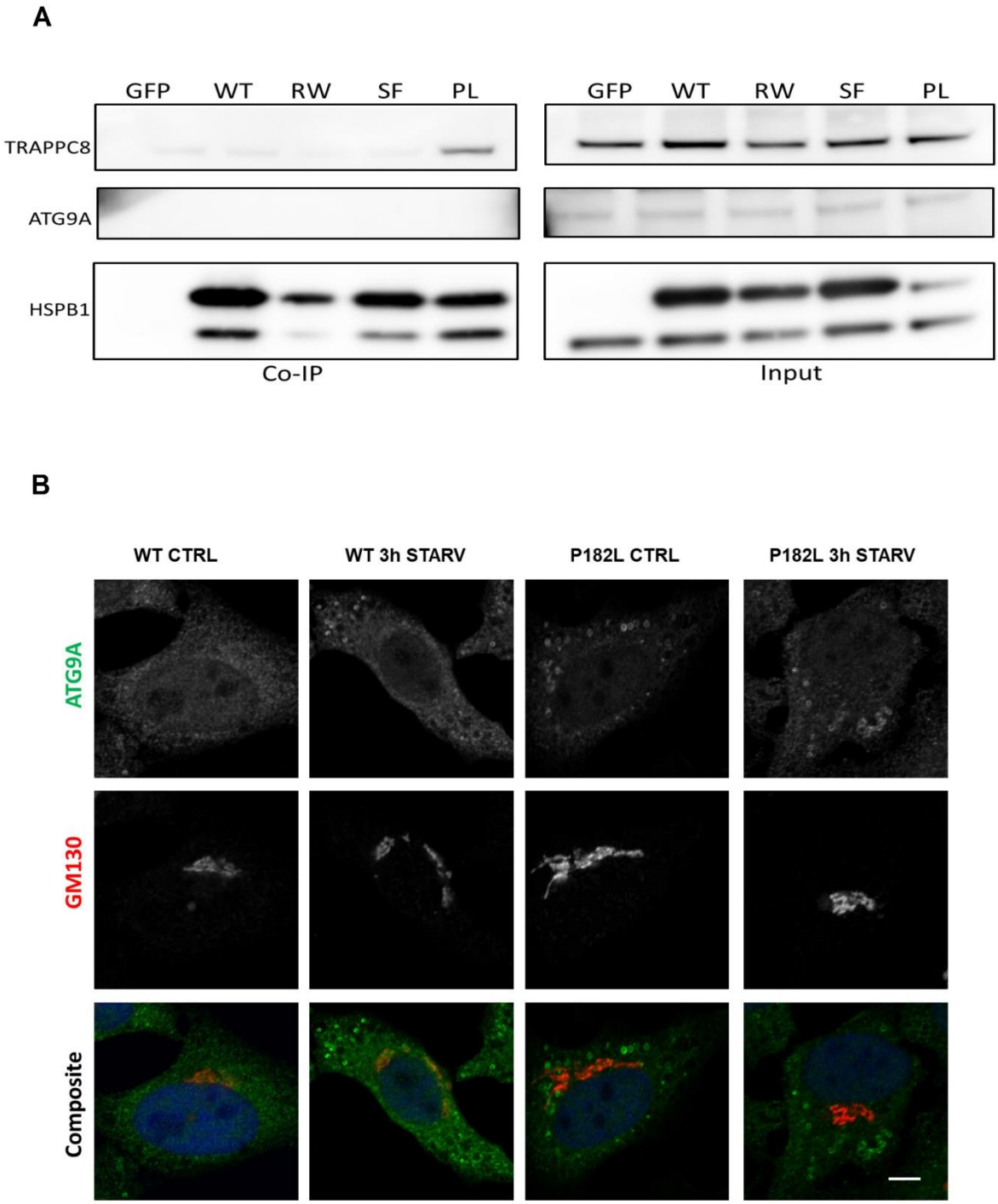

Fig 3. HSPB1-P182L binds to TRAPPC8 but does not affect its autophagy-linked function

(**A**) Co-immunoprecipitation analysis of HSPB1 in HeLa cells showing that TRAPPC8 is detected with the pull-down of HSPB1-P182L mutant only, but ATG9A is not pulled down. (**B**) Immunofluorescence analysis of the cellular distribution of ATG9A with and without starvation treatment and Golgi structure in HeLa cells expressing HSPB1-WT or HSPB1-P182L. The staining shows ATG9A (Green), Golgi (Red), and DAPI (blue). Scale bar = 20 μm.

HSPB1 binds P62 at the oligomerization domain (PB1)

The autophagy receptor P62 is a multi-domain protein containing, among other domains, three domains crucial for its function in autophagy. An N-terminal PB1 domain that mediates its homo- and hetero-oligomerization, an LIR (LC3 Interacting Region) motif that mediates its interaction with the autophagy protein LC3, and a C-terminal UBA domain that binds ubiquitin (Johansen and Lamark, 2011; Pankiv et al., 2007). Knock-down of P62 has been shown to disrupt starvation-induced autophagy in HeLa cells (Bjørkøy et al., 2005). Furthermore, deletions or mutations in one of these domains can affect the formation of P62-bodies (Fig S3) (Lin et al., 2013; Wurzer et al., 2015), the recruitment of P62 to autophagy membranes (Itakura and Mizushima, 2011) or the recognition of ubiquitinated protein aggregates by P62 (Cohen-Kaplan et al., 2016). To test whether HSPB1 binds to one of these main domains of P62, HeLa cells stably-expressing V5-tagged HSPB1-WT or the mutants (R127W, S135F, P182L) were subjected to P62 knock-down using shRNA followed by transient transfection of shRNA resistant, FLAG-tagged wild type P62 (P62-WT) or one of deletion constructs of the PB1 or UBA domains (delPB1, delUBA). Afterwards, HSPB1 was pulled-down by immunoprecipitation and P62 was detected by immunoblotting. While P62-WT and P62-delUBA co-immunoprecipitated with HSPB1, we were not able to detect P62-delPB1 (Fig 4). This shows that the deletion of the PB1 domain prevented the binding of HSPB1 to P62 and that HSPB1 binds to P62 at the PB1 domain. We also used a similar approach to check for the binding of HSPB1 to P62 with a point mutation in its LIR domain, in this case we used mCherry-tagged P62 constructs (P62-WT, P62-LIR-MUT). In both cases P62 co-immunoprecipitated with HSPB1, ruling out the LIR domain as an interaction domain between P62 and HSPB1 (Fig S4).

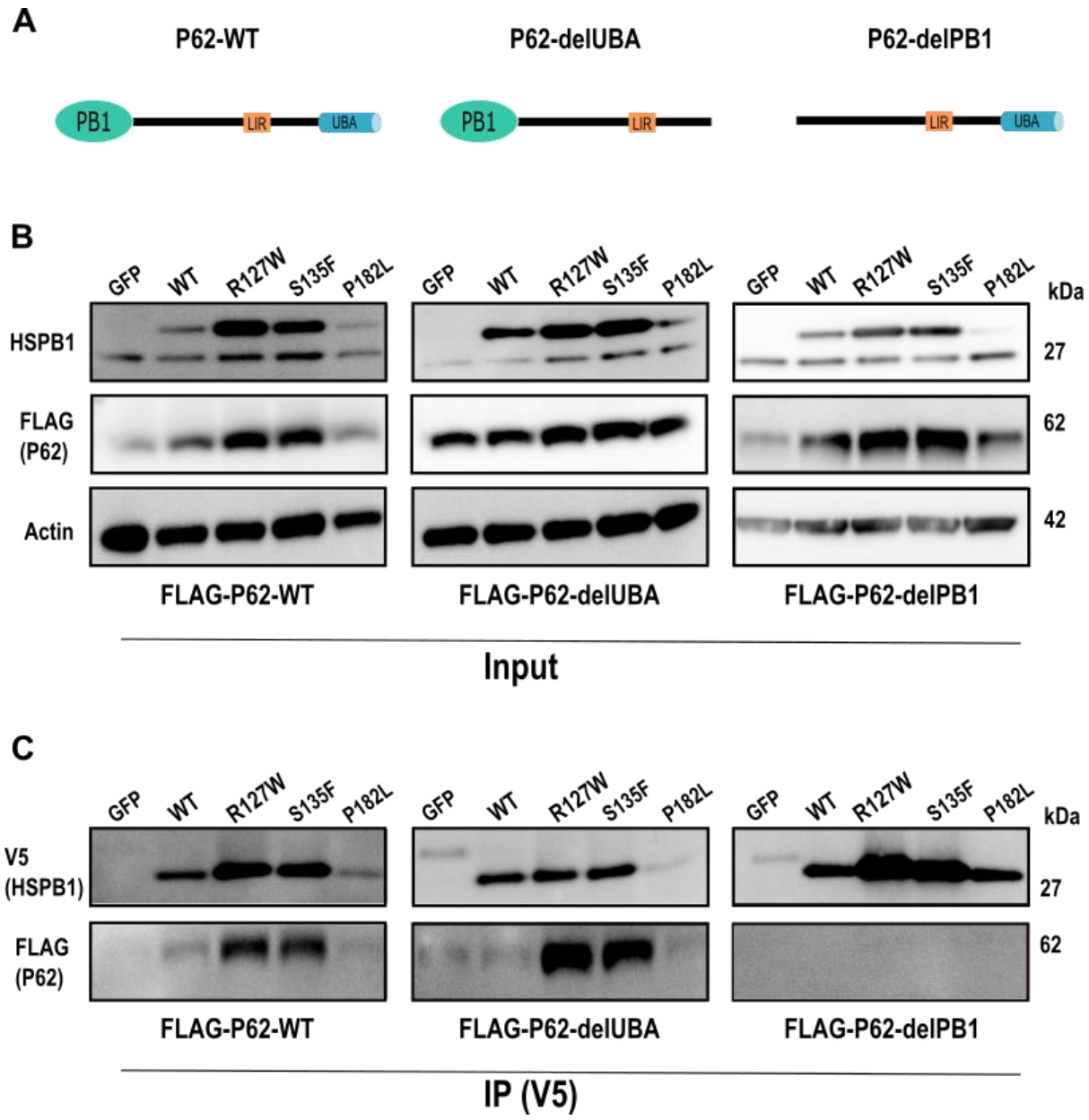

Fig 4. HSPB1 binds to P62 at the oligomerization domain PB1

(**A**) An overview of P62 deletion constructs used. (**B**) and (**C**) co-immunoprecipitation analysis of HeLa cells expressing V5-tagged HSPB1 WT or mutant (R127W, S135F, P182L) and FLAG-tagged P62 WT, delPB1 or delUBA constructs.

The formation of P62 bodies but not their recruitment to autophagosomes is disrupted by HSPB1 mutants

P62 forms punctate structures in the cells referred to as P62 bodies which are increased upon the induction of autophagy (Bjørkøy et al., 2005). The deletion of the PB1 domain was shown to abolish the formation of these punctate structures and disrupt the function of P62 in autophagy (Fig S3) (Pankiv et al., 2007; Wurzer et al., 2015). We performed automated immunofluorescence analysis of P62 puncta on HeLa cells expressing WT or mutant (S135F, P182L) HSPB1. The P62-bodies were quantified by automated counting using the CellProfiler software and compared between the WT and the mutants. Cells expressing the mutant forms of HSPB1 showed a significantly lower number of P62-bodies compared to the WT (Fig 5 A and B). This trend was also seen after serum starvation treatment. Similarly, HeLa cells with CRISPR knock-out of HSPB1 showed lower number of P62 puncta compared to WT expressing cells (Fig 5). These results show that the interaction of HSPB1 with P62 can have an effect on the function of P62 and that mutations in *HSPB1* can disrupt this function.

While the LIR domain of P62 is crucial for its recruitment to the autophagosomes (Pankiv et al., 2007), some studies suggested that the PB1 domain is required for the translocation of P62 to some of the autophagic structures (Itakura and Mizushima, 2011). To test whether mutations in *HSPB1* affect the recruitment of P62 to the autophagosomes, we performed an immunofluorescence analysis of P62 and LC3 in HeLa cells expressing WT and mutant HSPB1. Fluorescence intensities from LC3 positive and P62 positive puncta were measured for colocalization and the colocalization ratios were compared between the different cell lines. We did not observe any significant difference in the colocalization ratios between the mutant and the WT expressing cells (Fig 6 A and B). Our results show that while mutations in HSPB1 decrease ability of P62 to form P62-bodies they do not prevent them from localizing to the autophagosomes.

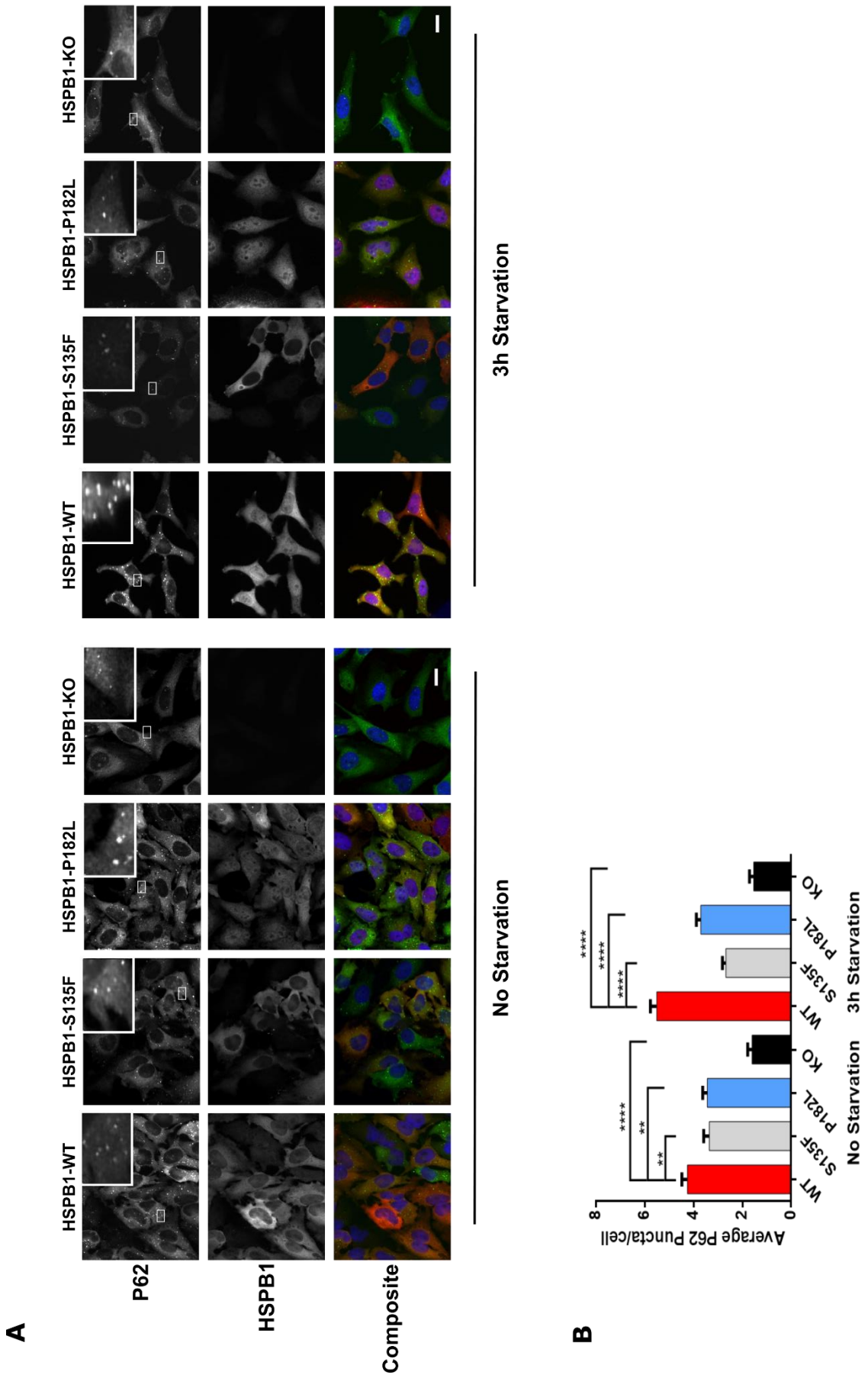
A
HSPB1-WT
HSPB1-S135F
HSPB1-P182L
HSPB1-KO
P62
HSPB1
Composite
No Starvation
3h Starvation
B
Average P62 Puncta/cell
0
2
4
6
8
WT
S135F
P182L
KO
No Starvation
3h Starvation
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Figure 5. HSPB1 mutations lead to decreased formation of P62 bodies

Immunofluorescence microscopy analysis of HeLa cells expressing HSPB1-WT or the mutants (S135F and P182L). **A-** Cells were stained for HSPB1 (Red), P62 (Green) and DAPI (Blue), Scale bar = 20 µm. **B-** Quantification of P62 puncta per cell. N>2000 cells from 2 independent experiments. One-way ANOVA analysis with Tukey's correction test. **=P<0.01, ****=p<0.0001 Error bars are Mean with 95% C.I.

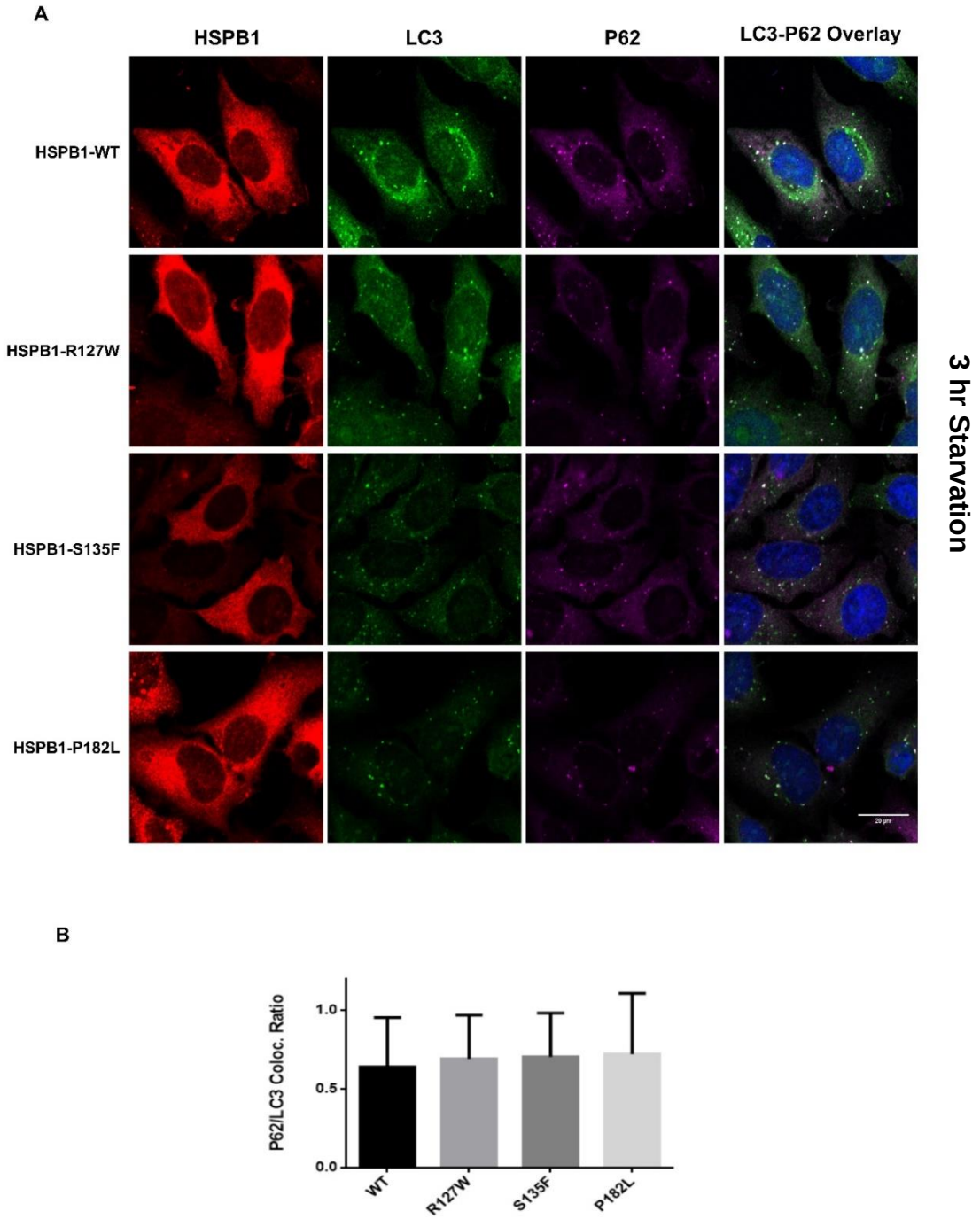

Fig 6. HSPB1 mutations do not affect the recruitment of P62-bodies to autophagosomes

A- Immunofluorescence microscopy analysis of HeLa cells expressing HSPB1 WT or mutant (R127W, S135F, P182L). Cells were treated with serum starvation for 3 hrs and then stained for HSPB1 (Red), LC3 (Green), P62 (Magenta) and DAPI (Blue). Scale Bar = 20 µm. **B-** The Ratio of colocalisation between LC3 and P62 based on signal intensities in the Green and Magenta channels compared between the different cell lines. N>400 ROIs from >100 cells from 3 independent experiments. One-way ANOVA with Tukey's correction test.

HSPB1 mutants display a lower number of phagophores

Phagophores, or isolation membranes, are membrane structures that are nucleated after the induction of autophagy and are then elongated to become mature autophagosomes. The source that provides the building material for the forming phagophores is a matter of debate, but it seems that different compartments of the cell including the ER, the plasma membrane, and the endosomes might contribute to the formation of phagophores (Hamasaki et al., 2013; Hayashi-Nishino et al., 2009; Ravikumar et al., 2010). P62 has been shown to form scaffolds of oligomers using its PB1 domain (Ciuffa et al., 2015). These scaffolds have been suggested to be important hubs for the nucleation of phagophores. To test whether HSPB1 mutants disrupt the formation of phagophores, we transfected the stable HeLa cell lines with ATG14 (a known phagophore marker) (Klionsky et al., 2016) tagged to GFP. Then using fluorescence microscopy we quantified the number of GFP-ATG14 puncta formed after 1.5 hrs of starvation as recommended by the study of Itakura and Mizushima (Itakura and Mizushima, 2010) (Fig. 7). Interestingly, mutant cell lines displayed a significantly lower number of phagophores compared to the WT. This indicated that HSPB1 mutations disrupt the formation of phagophores leading to a lower autophagy levels. These results were confirmed using another phagophore marker: WD repeat domain phosphoinositide interacting protein 1 (WIPI-1) tagged to GFP (Fig S5) (Proikas-Cezanne et al., 2007). This suggests that the interaction between HSPB1 and P62 might be important for the function of P62 in nucleation of phagophores destined to become autophagic vesicles. By disturbing this function, mutations in HSPB1 can lead to the reduced autophagy levels seen in our cell models.

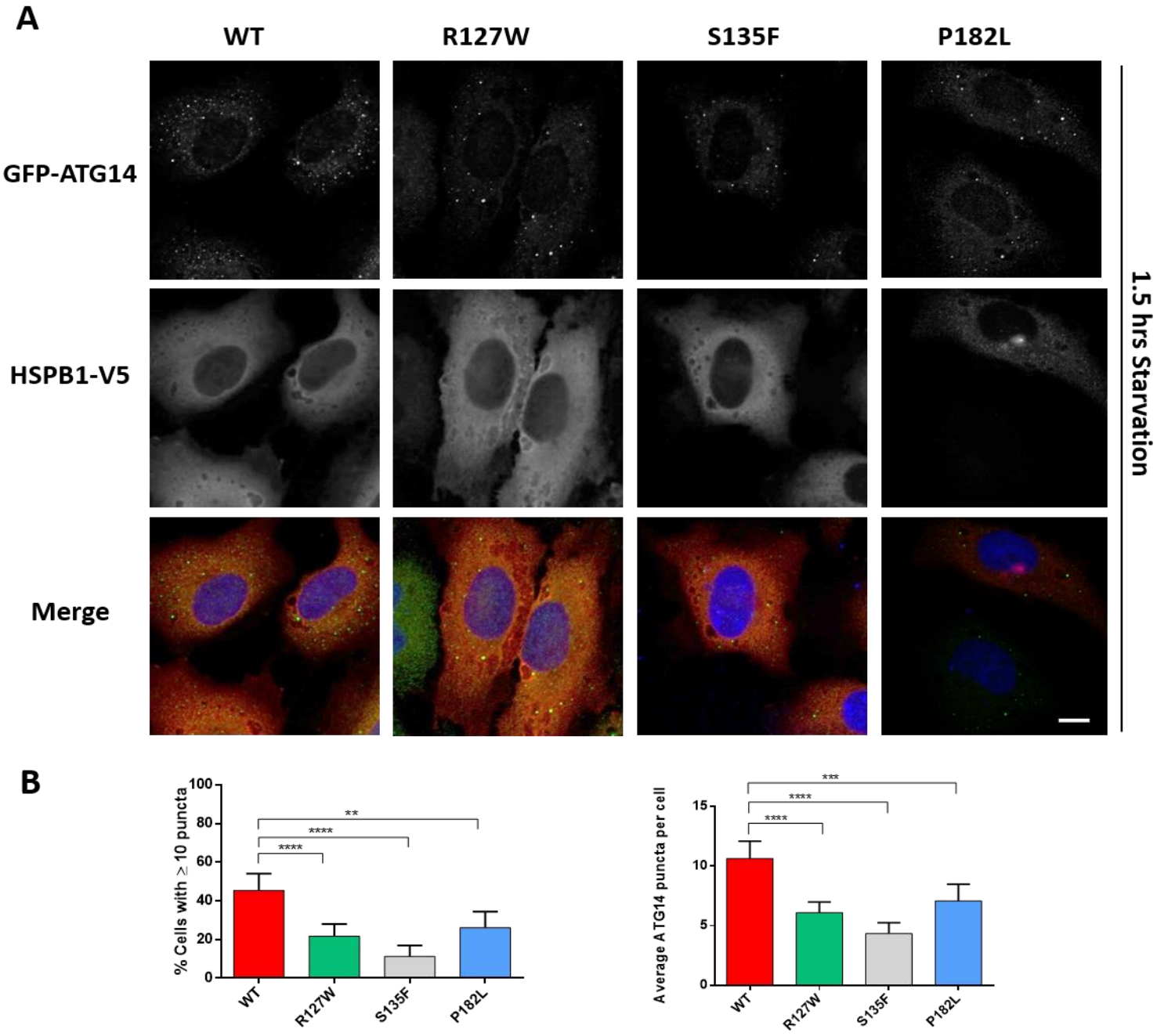

Fig 7. HSPB1 mutants display a lower number of phagophores.

HeLa cells expressing or not HSPB1 WT or the mutants were transfected with GFP-ATG14 and then subjected to immunofluorescence microscopy. (**A**) The staining shows ATG14 (Green), HSPB1 (Red), and the DAPI nucleus stain (Blue) (Scale bar = 10 µm). (**B**) Comparison of the percentage of cells with 10 or more ATG14-positive puncta (Left graph) and the average number of puncta per cell (Right graph). One-way ANOVA, with Tukey's correction range test. N>200, Error bars= Mean with 95% CI, **=p<0.01, *** = p<0.001, **** = p<0.0001

Discussion:

In this study, we have shown that HSPB1 interacts with proteins with known functions in autophagy. HSPB1 has been reported to modulate many vital cellular functions through interaction with different proteins and protein complexes (Arrigo and Gibert, 2014; Katsogiannou et al., 2014b). Studies of the interactome of HSPB1 have aided in identifying more of its potential functions and presented it as a regulatory protein with actions beyond its molecular chaperoning role (Doshi et al., 2009; Kennedy et al., 2017; Mymrikov et al., 2011; Sun et al., 2013). Our results show that the autophagy-regulating protein P62 is an interactor of HSPB1 and that mutations in *HSPB1* can lead to increased binding to P62. In fact, as the nature of interactions of HSPB1 with other proteins can sometimes be transient and go undetected in proteomic analysis, mutations that increase the binding affinity of HSPB1 might increase the detectability of binding partners of HSPB1. Our findings are in line with previous studies showing that mutations in HSPB1 can affect their binding to other interactors and lead to detrimental effects on the functions of these proteins (Almeida-Souza et al., 2011; Geuens et al., 2017). We have previously shown that mutations in HSPB1 produce defects in autophagy in different cellular models (Chapter 3). Here we have shown that the binding of HSPB1 to P62 is relevant to the function of the latter in autophagy and that disruption of this interaction by mutations or by knocking-out *HSPB1* can lead to detrimental effects on autophagy. The PB1 domain of P62 has been reported to play a crucial role in P62 performing its pro-autophagy functions (Ciuffa et al., 2015; Itakura and Mizushima, 2011; Wurzer et al., 2015). The main function of this domain is to allow P62 to form homo- and hetero-oligomers (Lamark et al., 2003), which are needed for the involvement of P62 in autophagy including the selection of cargo and isolation membrane and the formation of scaffolds for nucleation of autophagosomes (Ciuffa et al., 2015; Wurzer et al., 2015). Our results suggest that HSPB1 might be needed for the formation of isolation membranes (phagophore) that later develop to form the autophagosomes. The phagophore formation step in autophagy is still not fully understood (Lamb et al., 2013).Whether HSPB1 plays a role in stabilizing the P62 oligomers or the scaffolds they form, remains to be investigated. Interestingly, P62 and HSPB1 share similar characteristics in being able to bind to several other proteins in the cell and to modulate different pathways. Whether the association between HSPB1 and P62 is limited to autophagy also remains obscure, but maintaining proteostasis seems to be a common function of both proteins. One compelling fact is that mutations in both HSPB1 and P62 have been linked to

neurodegenerative diseases such as ALS and ataxia (Capponi et al., 2016; Haack et al., 2016; Teyssou et al., 2013). This study comes in line with recent evidence of the involvement of HSPBs in autophagy and their interaction with other autophagy regulating proteins. Other studies have established that HSPB8 acts in a complex with BAG3 and Hsc70 to modulate autophagy (Carra et al., 2008b), and HSPB6 has been shown to be an essential binding partner of BECN1, aiding in the stimulation of autophagy (Liu et al., 2017). This study puts us a step closer to establishing HSPB1 as another player in the autophagy pathway through its interaction with P62.

Acknowledgements:

The proteomics analysis and the LC-MS/MS runs was outsourced to the VIB Proteomics Core, Ghent, Belgium. We thank Prof. Dr. W. De Vos and Prof. Dr. J.P. Timmermans from the Antwerp Centre for Advanced Microscopy (ACAM) of the University of Antwerp for access to the Nikon High throughput microscope used in this study and supported by a HERCULES grant for microscopy infrastructure. This research was supported in part by the University of Antwerp, the Fund for Scientific Research (FWO-Flanders), the Medical Foundation Queen Elisabeth (GSKE), the "Association Belge contre les Maladies Neuromusculaires" (ABMM), the EC 7th Framework Programme under grant agreement number 2012-305121, 'Integrated European–omics research project for diagnosis and therapy in rare neuromuscular and neurodegenerative diseases (NEUROMICS)' and the H2020 grant Solve-RD, 'Solving the unsolved rare diseases' under grant agreement 2017-779257.

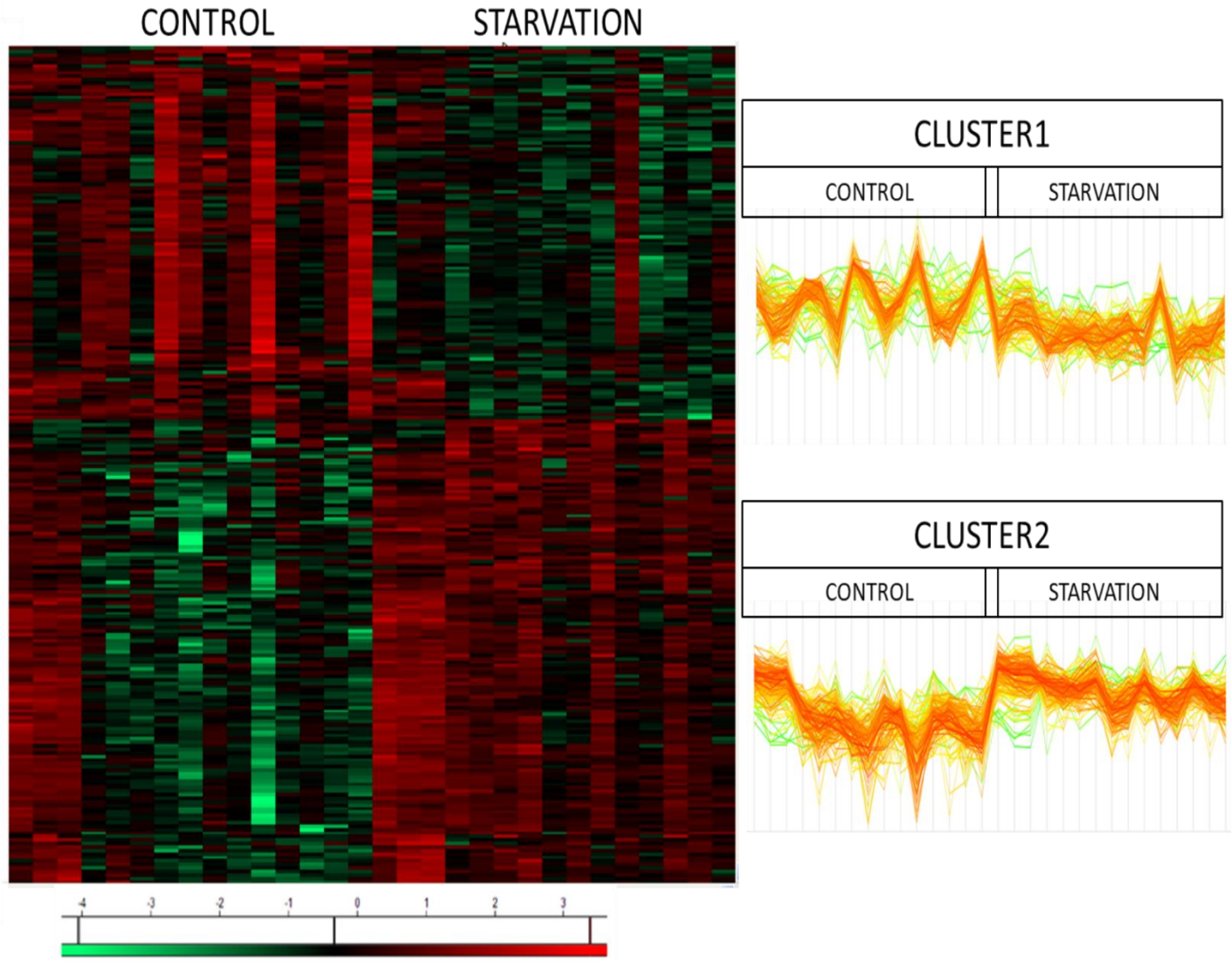

Fig S1. Cluster analysis protein expression in HeLa cells under starvation

Unsupervised hierarchical cluster analysis of upregulated proteins under control and starvation conditions reveals 2 clusters of upregulated proteins for each treatment (Red).

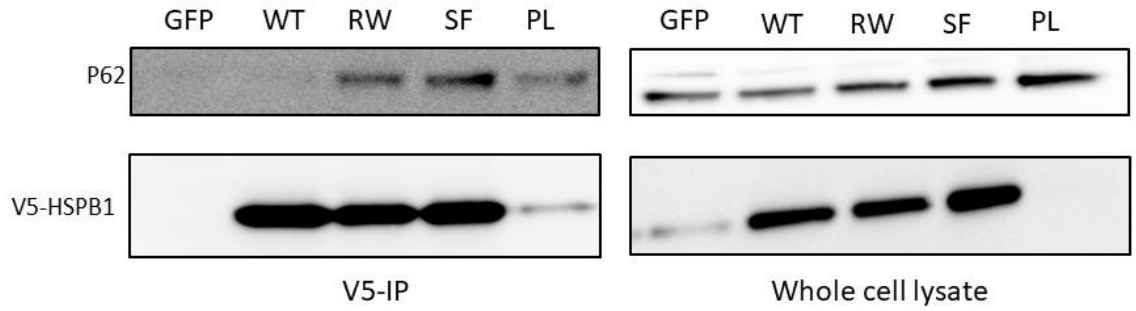

Fig S2. Validation of HSPB1-P62 interaction in neuroblastoma SH-SY5Y cell lines expressing HSPB1-WT, Mutant (R127W, S135F, P182L) or GFP.

Fig S3 Disruption of different domains of P62 affects the pattern of P62 distribution in cells

HeLa cells were transiently transfected with mCherry-tagged P62 constructs expressing Wild type P62 (mCherry-P62-WT), P62 with the (335 DDDW 338 -> AAAA) mutation in the LIR region (mCherry-P62-LIR Mut.), and P62 where the PB1 domain was deleted (mCherry-P62-del. PB1). Note that deletion of the PB1 domain completely abolishes the ability of P62 to form punctate P62 bodies.

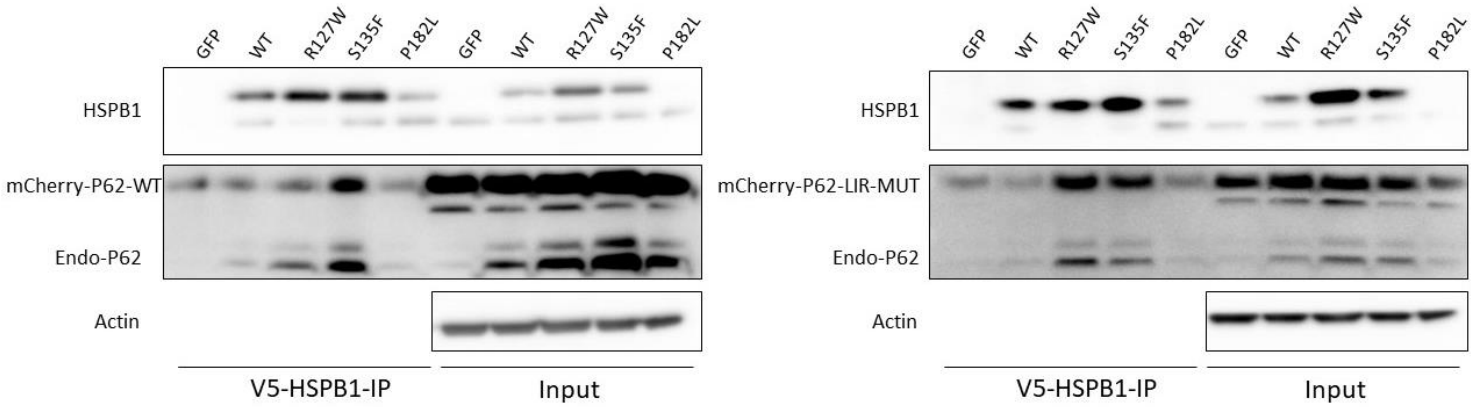

Fig S4. Disruption of the LC3 Interacting Region (LIR) motif of P62 does not hinder the binding of HSPB1.

V5-tagged HSPB1 pull-down of HeLa cells expressing HSPB1 WT, mutant (R127W, S135F, P182L) or GFP expressing mCherry-tagged P62-Wild type or P62 with mutation in the LIR motif (P62-LIR-MUT: 335 DDDW 338 -> AAAA).

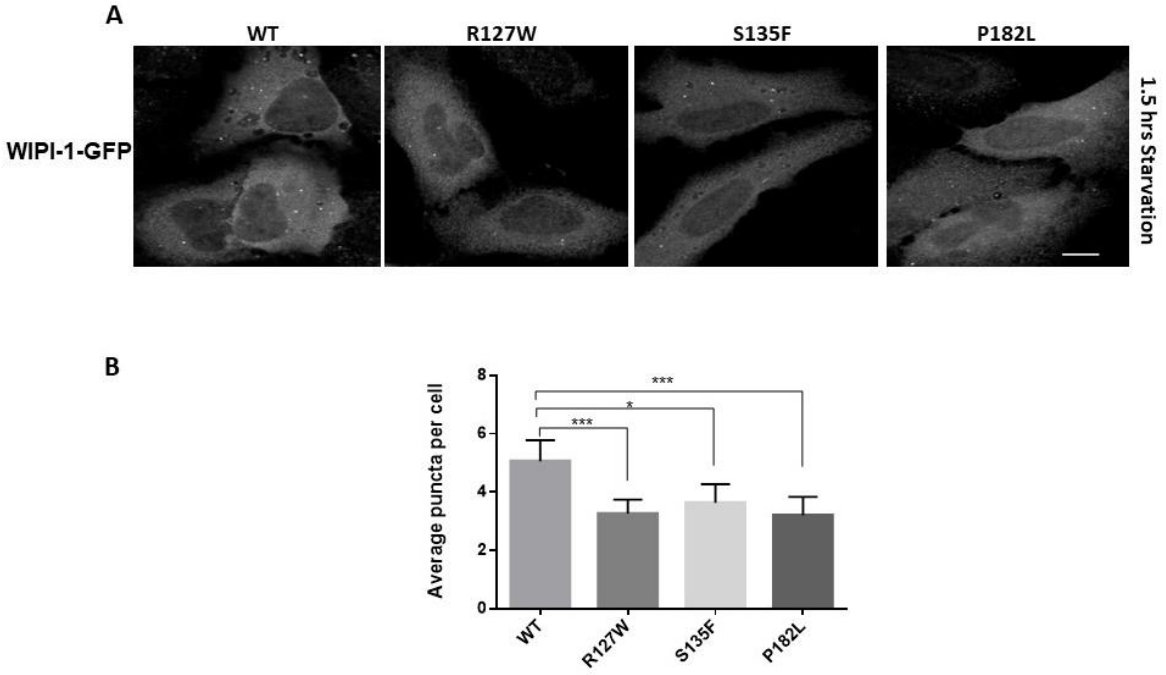

Fig S5. HSPB1 mutants display a lower number of WIPI-1 puncta

(**A**) Immunofluorescence microscopy of GFP-WIPI-1 in HeLa cells expressing HSPB1 WT or the mutants (Scale bar = 10 µm). (**B**) Quantification of GFP-WIPI-1 puncta in cells from two independent experiments (N>100). One-way ANOVA with Tukey's correction tests. Error bars = Mean with 95% CI, *P<0.05, ***P<0.001

Table S1: Full list of HSPB1 WT and/or mutant interactomes which are significantly upregulated in the different clusters of figure 1.

C: Cluster	T: Gene names	N: -Log Two-way ANOVA p value mutant
cluster 1	GNB2;GNB4;GNB1	4.12857
cluster 1	SERPINB12	2.41056
cluster 1	JUP	2.12393
cluster 1	DSP	2.22819
cluster 1	POF1B	2.03988
cluster 1	CAT	2.12859
cluster 1	PKP1	2.41603
cluster 1	DSC1	3.1919
cluster 1	ARG1	2.30912
cluster 1	DSG1	3.2271
cluster 1	KRT2	4.33576
cluster 1	ATP5O	6.08905
cluster 1	CRYAB	5.33274
cluster 1	HSPB1	12.1951
cluster 1	UBA52;RPS27A;UBB;UBC	5.61418
cluster 1	PEG10	6.36761
cluster 1	DNAJA3	5.61775
cluster 1	CPS1	4.53355
cluster 1	BZW1	3.01671
cluster 1	LRPPRC	2.03182
cluster 1	SRPRB	2.51809
cluster 1	PSMA5	2.44501
cluster 1	PSMB5	2.55839
cluster 1	SLC25A24	2.2801
cluster 1	DNAJC19	2.28183
cluster 1	ATP5C1	4.03706
cluster 1	SUB1	3.04433
cluster 1	SUPT16H	2.26442
cluster 1	PAM16	2.67311
cluster 1	ZRANB2	2.90439
cluster 2	ABCD3	2.02007
cluster 2	HAX1	2.22456
cluster 2	CLU	5.42413
cluster 2	TFRC	2.10264
cluster 2	NNT	2.24034
cluster 2	LETM1	2.57356
cluster 2	PDE3A	3.3354
cluster 2	CYC1	2.06481

cluster 2	NUP93	2.52342
cluster 2	BAG2	3.11737
cluster 2	RUVBL2	2.47166
cluster 2	RUVBL1	2.41526
cluster 2	MCM7	2.58849
cluster 2	HADHA	3.0322
cluster 2	GARS	2.20143
cluster 2	GCN1L1	2.76967
cluster 2	AKAP8	4.92739
cluster 2	SQSTM1	2.06131
cluster 2	PANK4	2.02983
cluster 2	RCN2	2.29619
cluster 2	TUBA1B	2.44656
cluster 2	SLC25A13	3.02537
cluster 2	PRKDC	3.83714
cluster 2	XPOT	2.84331
cluster 2	MSH6	2.96024
cluster 2	MCCC1	2.38818
cluster 2	ATP2A2	2.709
cluster 2	SEC16A	2.85734
cluster 2	AIFM1	2.05902
cluster 2	TUBA4A	3.28534
cluster 2	MAP1B	2.5332
cluster 2	AKAP8L	3.56335
cluster 2	MCCC2	2.92618
cluster 2	TOMM40	2.03661
cluster 2	STT3A	2.66541
cluster 2	TIMM21	2.14389
cluster 2	ERLIN2	2.73373
cluster 2	PHB	2.47754
cluster 2	PHB2	2.27762
cluster 2	MTCH2	3.50929
cluster 2	SLC25A6	2.95273
cluster 2	GLUD1;GLUD2	3.68125
cluster 2	SLC25A5	3.65268
cluster 2	CDK1	2.3717
cluster 2	ATP5A1	2.10344
cluster 2	CHCHD3	3.98698
cluster 2	DNAJC11	3.2603
cluster 2	ERLIN1	3.79579
cluster 2	EMD	3.85269
cluster 2	LBR	3.59828
cluster 2	IMMT	3.92826
cluster 2	ACADM	3.34962
cluster 2	GEMIN4	3.77032
cluster 2	SFXN3	2.30381
cluster 2	KIAA1217	3.94194
cluster 2	SLC25A12	2.74379

cluster 2	MLF2	3.08942
cluster 2	SLC25A3	2.8631
cluster 2	UQCRC2	2.01487
cluster 2	SLC25A1	3.29748
cluster 2	HSPB8	2.21373
cluster 2	PC	2.72208
cluster 2	SUCLG2	2.74104
cluster 2	YME1L1	2.5001
cluster 2	PTPN1	2.53873
cluster 2	GFPT1	2.51622
cluster 3	PPIL1	2.07226
cluster 3	CEP170	2.01457
cluster 3	ANLN	2.23488
cluster 3	CAPZA1	3.58968
cluster 3	SEP7	2.01051
cluster 3	ARHGDIA	2.24234
cluster 3	VASP	3.06528
cluster 3	TSEN34	3.57697
cluster 3	TRAPPC8	5.94308
cluster 3	ARPC4	2.07413
cluster 3	USP32	6.47589
cluster 3	AURKB	4.19346
cluster 3	FAM83D	5.93469
cluster 3	AP1G1	7.6804
cluster 3	HCFC1	2.82172
cluster 3	FAM96B	2.63321
cluster 3	ARPC5	9.83386
cluster 3	UBXN1	12.1777
cluster 3	CHORDC1	5.77389
cluster 3	TAB2	2.7361
cluster 3	PCBP2	2.13332
cluster 3	RPA1	7.71601
cluster 3	COL1A1	2.24127
cluster 3	MAP3K7	3.1413
cluster 3	EIF5B	2.42151
cluster 3	TAB1	6.16965
cluster 3	PCBP1	2.30968
cluster 4	IGHM	3.80379
cluster 4	EIF3K	2.44471
cluster 4	MRPL23	2.88236
cluster 4	ASCC3	3.47617
cluster 4	IKBKAP	2.63258
cluster 4	DST	2.54012
cluster 4	EGFR	5.05998
cluster 4	VAMP3;VAMP2	2.61679
cluster 4	HDAC2	3.80539
cluster 4	EPS15L1	2.18002

cluster 4	RAB11FIP1;RAB11FIP2	2.02731
cluster 4	YARS2	3.67178
cluster 4	RPL22	2.47342
cluster 4	SPCS2	2.80281
cluster 4	SMARCD2	2.12305
cluster 4	UACA	2.89277
cluster 4	MRPL17	2.15403
cluster 4	ZNF281	2.05492
cluster 4	CUL3	2.68701
cluster 4	SUN2	2.04624
cluster 4	RPUSD4	5.52302
cluster 4	PDLIM7	5.12376
cluster 4	NMD3	2.84742
cluster 4	TBL1XR1	2.03894
cluster 4	FNDC3A	2.98099
cluster 4	FAM98A	2.5983
cluster 4	KPNA6;KPNA5	2.84179
cluster 4	MRGBP	3.4727
cluster 4	NUP214	2.35525
cluster 4	CCDC137	2.69233
cluster 4	HNRNPLL	2.51361
cluster 4	MED12	2.21149
cluster 4	RANBP9	2.1631
cluster 4	RPN2	2.02229
cluster 4	MAP4K4	2.91813
cluster 4	TPM1	2.10443
cluster 4	IKBIP	3.41662
cluster 4	TGFBI	3.71513
cluster 4	CYR61	3.35395
cluster 4	SGPL1	2.08239
cluster 4	TK1	2.19042
cluster 4	SMNDC1	3.28562
cluster 4	P4HA1	2.3407
cluster 4	DNM1L	2.05976
cluster 4	FNDC3B	3.48828
cluster 4	ZCCHC8	2.69896
cluster 4	TRIM38	2.82019
cluster 4	ACTR2	2.28193
cluster 4	UFD1L	2.81738
cluster 4	USP15	2.84022
cluster 4	ITGB1	2.98807
cluster 4	EIF2D	2.89318
cluster 4	GPX8	2.73843
cluster 4	MED10	2.14676
cluster 4	TRAM1	2.57238
cluster 4	SFPQ	2.45755
cluster 4	NONO	3.3332
cluster 4	C14orf166	2.43463

cluster 4	DDX1	2.46176
cluster 4	EIF3C;EIF3CL	2.72405
cluster 4	TRIP4	2.04322
cluster 4	MRPL45	2.10995
cluster 4	PPP1R12C	2.09691
cluster 4	MTERF3	2.22676
cluster 4	SRP54	2.18217
cluster 4	TPM4	2.06585
cluster 4	MRPS6	2.80741
cluster 4	RTN4	2.36977
cluster 4	FAM103A1	2.00872
cluster 4	CAP1	2.00268
cluster 4	LASP1	3.07186
cluster 4	PDLIM5	3.73019
cluster 4	SMAP1	3.91592
cluster 4	PHLDB1	3.94299
cluster 4	MORF4L2	2.99924
cluster 4	PAWR	6.3182
cluster 4	FLNB	4.76675
cluster 4	FLNA	4.10151
cluster 4	SND1	8.03329
cluster 4	RBM4;RBM4B	3.63595
cluster 4	ZNF622	3.61336
cluster 4	PPP4R2	3.92435
cluster 4	GBE1	2.73189
cluster 4	CARM1	3.20697
cluster 4	LOX	2.74511
cluster 4	ERP44	2.03151
cluster 4	WDR48	2.40372
cluster 4	PDE12	2.01126
cluster 4	NOTCH1	2.22873
cluster 4	MRPL9	4.949
cluster 4	BOLA2	3.61242
cluster 4	PFN2	2.6008
cluster 4	PCBD1	3.76676
cluster 4	ITCH	2.0583
cluster 4	PHLPP1	3.76644
cluster 4	DNTTIP1	3.44133
cluster 4	LMO4	3.47864
cluster 4	CTNNB1	3.00729
cluster 4	HSPG2	4.11737
cluster 4	CSTF1	2.52593
cluster 4	SS18	3.35834
cluster 4	ELMSAN1	3.89224
cluster 4	LAMB3	2.02802
cluster 4	MRPL24	2.30365
cluster 4	GOLGA2	4.69839

cluster 4	APC	3.13163
cluster 4	SNRNP70	3.01078
cluster 4	EIF3A	2.22267
cluster 4	HNRNPH3	4.3084
cluster 4	TRIOBP	3.22959
cluster 4	DDX42	3.29766
cluster 4	RBM17	2.61823
cluster 4	SF3A2	2.64795
cluster 4	CHERP	2.83493
cluster 4	SF3A1	2.24393
cluster 4	SF3B3	2.22546
cluster 4	RPS15A	2.11585
cluster 4	U2SURP	3.18394
cluster 4	SF3B1	2.89401
cluster 4	NOA1	2.14279
cluster 4	CCDC97	2.44827
cluster 4	TTC33	2.72044
cluster 4	KARS	2.23257
cluster 4	PDCD6	3.13167
cluster 4	HDAC1	3.6735
cluster 4	KTN1	3.18564
cluster 4	CTTN	2.56352
cluster 4	PPP1R12A	2.12224
cluster 4	MPDZ	2.90826
cluster 4	SSBP2	2.1913
cluster 4	RFX5	2.54876
cluster 4	PPP1CB	2.90998
cluster 4	TAF15	2.24108
cluster 4	LARP1	3.45269
cluster 4	GIGYF2	3.1738
cluster 4	MAP7D1	3.66291
cluster 4	MTDH	3.45392
cluster 4	PHAX	2.38449
cluster 4	MYO9A	3.60964
cluster 4	CTNNA1	2.76169
cluster 4	SPECC1	7.26222
cluster 4	SEC24D	6.14521
cluster 4	FKBP10	3.86535
cluster 4	MN1	5.69569
cluster 4	PARD3B	3.28878
cluster 4	ATP6V1D	4.13576
cluster 4	PRPS1;PRPS1L1	9.21723
cluster 4	LDB1	5.46456
cluster 4	DCTPP1	9.19996
cluster 4	NUP155	5.95381
cluster 4	BRD8	6.77643
cluster 4	UGGT1	3.415
cluster 4	PRPF39	4.44222

cluster 4	AGO3	3.13396
cluster 4	MYO5A	5.56656
cluster 4	USP9X	4.52687
cluster 4	PTCD1	2.84549
cluster 4	CLTC	6.07564
cluster 4	MPP7	5.07804
cluster 4	MAST4	4.05156
cluster 4	BAG4	2.74325
cluster 4	BCL9L	2.18359
cluster 4	HTATSF1	2.10199
cluster 4	TNKS1BP1	5.44013
cluster 4	UBL7	3.8036
cluster 4	HNRNPF	2.7288
cluster 4	ZNF609	2.99759
cluster 4	HELZ	3.16347
cluster 4	RNMT	2.74425
cluster 4	SEC24A	2.82978
cluster 4	SEC13	3.22778
cluster 4	SEC23B	2.354
cluster 4	QSER1	2.23748
cluster 4	SETD2	2.58577
cluster 4	SLC4A1AP	2.93467
cluster 4	PRCC	4.76938
cluster 4	PALLD	2.65962
cluster 4	UBE4B	4.64669
cluster 4	TIAL1	4.36055
cluster 4	AMOTL2	6.19713
cluster 4	HDAC4	3.08728
cluster 4	SPAG5	5.53422
cluster 4	MYOF	3.12706
cluster 4	MED25	5.34982
cluster 4	SF3B2	4.33121
cluster 4	CASK	4.1733
cluster 4	DLG1	4.12218
cluster 4	SEC24B	4.6361
cluster 4	HNRNPUL1	3.2878
cluster 4	TNRC6A	3.2231
cluster 4	VCP	3.2262
cluster 4	SART3	4.21118
cluster 4	MRPS22	2.99563
cluster 4	MOV10	5.70589
cluster 4	AGO2	2.5071
cluster 4	SNX9	3.18297
cluster 4	AGL	3.24242
cluster 4	SEC24C	3.4259
cluster 4	SEC31A	3.61766
cluster 4	SEC23A	3.76387

cluster 4	MRPL41	2.55383
cluster 4	SEP15	2.91102
cluster 4	YTHDF1	3.66832
cluster 4	AHCYL1	2.11116
cluster 4	RPL21	2.49844
cluster 4	LNX2	3.71012
cluster 4	STAU2	2.01534
cluster 4	DNMT1	2.50188
cluster 4	RPS12	2.54429
cluster 4	PTRF	3.30189
cluster 4	RBM3	4.47666
cluster 4	MYL12A;MYL12B	2.01885
cluster 4	MYL6	2.47896
cluster 4	MORF4L1	3.89697
cluster 4	KPNA2	2.02746
cluster 4	ACTN4	2.19278
cluster 4	ACTN1	4.36901
cluster 4	BANF1	4.67673
cluster 4	DAZAP1	3.06068
cluster 4	PDXK	3.71574
cluster 4	RPL10;RPL10L	2.23349
cluster 4	HNRNPD	2.40499
cluster 4	HNRNPAB	2.79932
cluster 4	RPS24	2.45312
cluster 4	CDC42BPB	2.32491
cluster 4	GRSF1	2.37862
cluster 4	RPS15	2.23104
cluster 4	HBS1L	2.30546
cluster 4	DNAJC13	2.77041
cluster 4	XRN2	3.8182
cluster 4	UPF1	2.95712
cluster 4	NCBP2	2.18814
cluster 4	DDX55	2.04689
cluster 4	FNBP4	3.1201
cluster 4	NCKAP1	3.17063
cluster 4	PCNP	3.26875
cluster 4	ETF1	2.55344
cluster 4	NARS	4.91409
cluster 4	ACLY	2.82948
cluster 4	CRTAP	4.26134
cluster 4	MED23	2.1196
cluster 4	PSPC1	3.64007
cluster 4	IQGAP3	5.2876
cluster 4	SEMA3C	3.29655
cluster 4	SDC2	4.91543
cluster 4	DROSHA	5.13434
cluster 4	EP400	4.95476
cluster 4	TMPO	4.74008

cluster 4	ARID1A	2.65484
cluster 4	TMPO	3.81926
cluster 4	TXNDC5	2.94636
cluster 4	YTHDF3	5.02369
cluster 4	CSDE1	2.88788
cluster 4	MRPL3	2.15001
cluster 4	CCDC80	4.93793
cluster 4	YAP1	7.53323
cluster 4	XIAP	3.15004
cluster 4	RBM5	2.06584
cluster 4	RPLP1	8.80856
cluster 4	TRIO	8.77388
cluster 4	FKBP9	4.44107
cluster 4	GPATCH11	7.44012
cluster 4	CNN3	9.06668
cluster 4	SMEK1	7.10306
cluster 4	HDAC7	8.51357
cluster 4	COL12A1	8.42239
cluster 4	IVNS1ABP	9.79024
cluster 4	NUDT1	4.27906
cluster 4	ZYX	7.57391
cluster 4	PXDN	3.34689
cluster 4	TIA1	3.40316
cluster 4	MRPL21	2.89758
cluster 4	WIBG	3.54507
cluster 4	RPL32	2.72546
cluster 4	PRMT9	7.58296
cluster 4	CTNNA2	2.65482
cluster 4	GTPBP1	3.77383
cluster 4	AP2A1	4.99605
cluster 4	CNN2	3.85518
cluster 4	OASL	3.83922
cluster 4	CSRP2	4.43618
cluster 4	LOXL2	2.48167
cluster 4	SFN	2.32554
cluster 4	SRP68	5.77123
cluster 4	SF3B5	3.90325
cluster 4	FAU	5.48115
cluster 4	TRIP6	3.3068
cluster 4	AGO1	3.12485
cluster 4	FAM120A	6.86321
cluster 4	MRPS30	2.97877
cluster 4	PWP1	3.08515
cluster 4	DDX6	4.01438
cluster 4	RECQL	2.73115
cluster 4	TRIM25	2.05195
cluster 4	C7orf50	4.32294

cluster 4	CKAP5	4.19653
cluster 4	TRIM56	3.34469
cluster 4	PLEKHA2	4.14447
cluster 4	AP2M1	4.2357
cluster 4	FN3KRP	3.08244
cluster 4	MED14	2.98314
cluster 4	RPS21	3.53343
cluster 4	COL5A1	3.06308
cluster 4	MSI2	2.6208
cluster 4	HNRNPH1	2.04949
cluster 4	PPP4C	2.54441
cluster 4	SEH1L	2.36558
cluster 4	YTHDC2	4.34275
cluster 4	DHX36	3.34799
cluster 4	NOB1	2.39042
cluster 4	RPS8	2.48726
cluster 4	RPS13	3.20387
cluster 4	RPS7	2.26742
cluster 4	RPLP0;RPLP0P6	2.55706
cluster 4	RPLP2	2.86057
cluster 4	SRP14	3.28436
cluster 4	IGF2BP1	2.76426
cluster 4	SNRPA	2.44369
cluster 4	IGF2BP3	3.28453
cluster 4	RPL13A	2.88375
cluster 4	RRBP1	2.20899
cluster 4	RPS14	3.15333
cluster 4	GNB2L1	2.94455
cluster 4	DHX57	2.86024
cluster 4	CREBBP	2.35596
cluster 4	GTPBP10	3.34533
cluster 4	FUS	4.83368
cluster 4	EWSR1	3.2938
cluster 4	MAP4	3.68729
cluster 4	CKAP4	2.65602
cluster 4	IFI16	2.64539
cluster 4	DDX17	3.69117
cluster 4	CDKN2AIP	2.05204
cluster 4	DDX3X;DDX3Y	3.29569
cluster 4	DHX30	2.40374
cluster 4	YBX1	3.34209
cluster 4	RPL31	2.39777
cluster 4	YBX3	3.28704
cluster 4	RPS20	2.56115
cluster 4	RPL26	2.11627
cluster 4	RPL15	2.39238
cluster 4	RPL3	2.33753
cluster 4	RPL28	4.08557

cluster 4	RPL18	2.08359
cluster 4	RPL6	2.59433
cluster 4	RPL24	2.70119
cluster 4	RPL8	2.15905
cluster 4	RPL34	2.02103
cluster 4	RPL7	2.56966
cluster 4	RPL13	2.55168
cluster 4	RPL4	2.66106
cluster 4	RPL7A	2.22629
cluster 4	DDX5	2.98076
cluster 4	HDLBP	3.68452
cluster 4	RPS25	3.25146
cluster 4	RPS18	2.94589
cluster 4	RPS3A	2.81284
cluster 4	RPS5	2.40653
cluster 4	PABPC1	5.34667
cluster 4	RPS3	2.597
cluster 4	RPS19	3.48938
cluster 4	RPS16	2.79371
cluster 4	EIF2S1	2.33294
cluster 4	RPS2	2.19972
cluster 4	RPSA	3.21978
cluster 4	WDR82	2.39747
cluster 4	NCBP1	2.09506
cluster 4	PPP2CA;PPP2CB	2.6458
cluster 4	TRIM28	3.83687
cluster 4	LRRC47	5.03938
cluster 4	PURB	2.8878
cluster 4	MRPL11	2.05055
cluster 4	EXOSC9	2.08349
cluster 4	MRPS27	2.22927
cluster 4	EXOSC7	2.8916
cluster 4	NEMF	2.38356
cluster 4	CTNND1	4.3985
cluster 4	MRPS16	2.32774
cluster 4	MAPK1	2.47574
cluster 4	PRMT1	6.80219
cluster 4	MRPS7	2.09192
cluster 4	CLTA	2.45161
cluster 4	MRPL44	2.37073
cluster 4	TXLNA	2.34859
cluster 4	IGHG1;IGHG3	2.20453
cluster 4	TF	6.86277
cluster 4	HPX	3.63295
cluster 4	SRP72	2.03372
cluster 4	THUMPD1	2.18029

Table S2. Full list of proteins significantly upregulated under starvation (Cluster 1) and under non-treated conditions (Cluster 2).

C: Cluster	T: Gene names	N: -Log Two-way ANOVA p value Starvation
cluster 1	FAM76B	2.39
cluster 1	AURKB	2.27
cluster 1	SARS	2.76
cluster 1	PRPF38A	2.15
cluster 1	PYCRL	4.38
cluster 1	ATIC	2.02
cluster 1	PPIB	3.79
cluster 1	RPL22	4.13
cluster 1	TMOD3	2.90
cluster 1	KPNA2	2.83
cluster 1	CDC73	2.02
cluster 1	TPR	2.06
cluster 1	HIST2H2BE;HIST1H2BB;HIST1H2BO;HIST1H2BJ	2.38
cluster 1	FAM207A	2.34
cluster 1	PRPF4B	2.57
cluster 1	SNW1	3.08
cluster 1	NOC4L	2.59
cluster 1	RRP12	2.05
cluster 1	BYSL	2.12
cluster 1	DDX47	2.02
cluster 1	CDC5L	2.05
cluster 1	UTP14A	2.11
cluster 1	TP53BP1	2.77
cluster 1	PABPN1	2.89
cluster 1	MPRIP	3.00
cluster 1	SPTAN1	3.09
cluster 1	SPTBN1	2.36
cluster 1	RBM34	2.28
cluster 1	MTA2	2.15
cluster 1	HMGA1	2.61
cluster 1	NOLC1	2.17
cluster 1	ACIN1	2.34
cluster 1	DNTTIP2	2.21
cluster 1	GTPBP4	2.22
cluster 1	SRSF6	2.65
cluster 1	EFTUD2	3.53
cluster 1	PRPF8	

cluster 1	SNRNP200	3.10
cluster 1	SART1	2.90
cluster 1	NUP98	2.26
cluster 1	DDX18	2.43
cluster 1	NOP14	3.03
cluster 1	MISP	2.25
cluster 1	HEATR1	3.35
cluster 1	H1F0	2.65
cluster 1	RRP15	2.66
cluster 1	DBN1	2.51
cluster 1	WDR3	3.16
cluster 1	WDR43	2.85
cluster 1	LIMA1	2.61
cluster 1	CORO1C	3.19
cluster 1	EIF4A3	2.92
cluster 1	SRSF10	3.83
cluster 1	NOL7	2.37
cluster 1	ZNF326	3.07
cluster 1	HIST1H1E	2.04
cluster 1	AHCTF1	2.12
cluster 1	TBL3	2.12
cluster 1	BAZ1B	3.02
cluster 1	LMO7	2.50
cluster 1	CBX3	2.33
cluster 1	NUMA1	2.37
cluster 1	MYH10	2.17
cluster 1	TOP2A	2.14
cluster 1	HMGN1	2.61
cluster 1	KIF23	2.04
cluster 1	H2AFY	2.40
cluster 1	HIST1H1C	2.34
cluster 1	HIST1H1B	2.25
cluster 1	HIST2H2AC;HIST2H2AA3;HIST1H2AJ;HIST1H2AH;H2AFJ;HIST1H2AG;HIST1H2AD;HIST1H2AA;H2AFX	2.26
cluster 1	HIST1H4A	2.10
cluster 1	HIST1H2BL;HIST1H2BM;HIST1H2BN;HIST1H2BH;HIST2H2BF;HIST1H2BC;HIST1H2BD;H2BFS;HIST1H2BK;HIST1H2BA	2.61
cluster 1	NPM1	2.45
cluster 1	NAT10	2.42
cluster 1	KIAA0020	2.72
cluster 1	NOP58	2.11
cluster 1	PLEC	2.46
cluster 1	VIM	3.46
cluster 1	DDX21	3.26
cluster 1	NOP2	2.24
cluster 1	MKI67	2.14
cluster 1	SMARCA5	3.01
cluster 1	NOC3L	2.15
		2.95

cluster 1	THOC2	2.58
cluster 1	PWP2	2.46
cluster 1	DDX27	2.16
cluster 1	PPAN	2.32
cluster 1	FN1	2.52
cluster 1	LUZP1	2.19
cluster 1	MYO1B	2.17
cluster 1	NUP35	2.03
cluster 1	DDX39B	3.49
cluster 1	NUP205	3.18
cluster 1	PDS5A	2.10
cluster 1	DMAP1	2.61
cluster 1	RRP1B	2.16
cluster 1	RBM3	2.80
cluster 1	PTRF	2.04
cluster 1	MYL6	3.30
cluster 1	MYH9	2.90
cluster 1	NUP50	2.16
cluster 1	HSPA9	2.51
cluster 1	CELF1	2.13
cluster 1	RBM8A	3.23
cluster 1	ACTG1	4.93
cluster 1	CHMP4B	2.07
cluster 2	MYL12A;MYL12B	2.48
cluster 2	IMPDH2	2.71
cluster 2	TECR	2.54
cluster 2	GPD2	2.15
cluster 2	TFRC	2.40
cluster 2	NDUFS7	2.29
cluster 2	PARP4	2.39
cluster 2	MVP	3.23
cluster 2	MMTAG2	2.73
cluster 2	PCBP1	2.74
cluster 2	IQGAP1	2.22
cluster 2	MRPL47	2.62
cluster 2	MRPL50	3.63
cluster 2	PDCD4	2.14
cluster 2	MRPL38	2.88
cluster 2	FASN	2.64
cluster 2	MRPS17	2.28
cluster 2	MRPL22	2.76
cluster 2	PSME1	3.49
cluster 2	TXLNA	4.51
cluster 2	TMED10	2.13
cluster 2	DNAJB11	4.21
cluster 2	CFI	4.19
cluster 2	HPX	3.95
cluster 2	MRPL49	4.59

cluster 2	MRPS15	3.85
cluster 2	TF	8.57
cluster 2	IGHG2	3.28
cluster 2	IGHG1;IGHG3	2.03
cluster 2	MRPL15	3.94
cluster 2	MRPL44	4.59
cluster 2	MICU2	2.79
cluster 2	GIGYF2	2.30
cluster 2	PDCD6IP	2.04
cluster 2	ACACA	2.87
cluster 2	MRPS34	2.03
cluster 2	DAP3	2.70
cluster 2	LARP1	3.01
cluster 2	MRPS9	2.08
cluster 2	UBR5	2.18
cluster 2	LANCL1	6.46
cluster 2	QARS	2.65
cluster 2	EPRS	2.03
cluster 2	MAP7	4.39
cluster 2	PRDX1	2.70
cluster 2	CD83	4.73
cluster 2	PPP2R2A	2.53
cluster 2	CLINT1	2.35
cluster 2	SDHA	2.38
cluster 2	DNAJC10	3.12
cluster 2	PHAX	2.49
cluster 2	DNTTIP1	3.44
cluster 2	PRMT1	2.03
cluster 2	LRRC47	2.80
cluster 2	ITCH	2.10
cluster 2	MYO5A	3.80
cluster 2	PARD3B	2.69
cluster 2	SEC24D	2.70
cluster 2	SPECC1	2.73
cluster 2	TTC33	2.01
cluster 2	PTCD1	2.70
cluster 2	MRPS7	3.45
cluster 2	RFX5	3.08
cluster 2	TRUB2	3.06
cluster 2	PICALM	2.04
cluster 2	PTCD3	2.99
cluster 2	AGO3	4.09
cluster 2	UBXN7	2.88
cluster 2	KPNA1	2.44
cluster 2	MYOF	2.26
cluster 2	PPP1CB	2.04
cluster 2	PUM1	2.22

cluster 2	SEC23B	3.13
cluster 2	SEC13	3.12
cluster 2	SEC24A	2.84
cluster 2	LDB1	4.17
cluster 2	PCBD1	4.22
cluster 2	TNKS1BP1	2.63
cluster 2	PRPF39	3.39
cluster 2	CLTC	6.16
cluster 2	PRPS1;PRPS1L1	7.83
cluster 2	DCTPP1	3.66
cluster 2	NUP155	3.09
cluster 2	PDCD6	2.60
cluster 2	PRCC	2.01
cluster 2	SEC24B	4.28
cluster 2	CHERP	2.73
cluster 2	RNMT	2.61
cluster 2	SPAG5	2.14
cluster 2	SF3B2	2.64
cluster 2	DLG1	2.78
cluster 2	TIAL1	2.32
cluster 2	BCL9L	2.20
cluster 2	MAST4	2.63
cluster 2	MRPS2	3.80
cluster 2	MRPS22	5.23
cluster 2	MRPS18B	5.48
cluster 2	TNRC6A	3.25
cluster 2	HNRNPUL1	4.23
cluster 2	SART3	3.66
cluster 2	AGL	3.75
cluster 2	MOV10	6.72
cluster 2	AGO2	4.47
cluster 2	SNX9	4.39
cluster 2	SEC23A	4.81
cluster 2	SEC24C	3.75
cluster 2	SEC31A	3.88
cluster 2	CPSF6	2.97
cluster 2	GRB2	2.27
cluster 2	CTTN	2.02
cluster 2	TFG	2.62
cluster 2	HDAC5	2.95
cluster 2	MTMR4	2.74
cluster 2	TNRC6B	3.72
cluster 2	RERE	2.36
cluster 2	SEC23IP	3.81
cluster 2	PPP1R12A	3.51
cluster 2	CLTA	4.22
cluster 2	MRPS26	2.84
cluster 2	MRPL4	3.03

cluster 2	ADRM1	2.20
cluster 2	SRP68	2.29
cluster 2	MRPL23	2.22
cluster 2	THUMPD1	2.35
cluster 2	SRP72	3.44
cluster 2	YTHDC2	2.76
cluster 2	DHX36	2.17
cluster 2	C8orf33	2.74
cluster 2	DHX30	3.79
cluster 2	CDKN2AIP	2.43
cluster 2	EWSR1	2.59
cluster 2	FUS	2.34
cluster 2	RPL28	2.10
	DDX3X;DDX3Y	2.25

REFERENCES

Ackerley, S., James, P. A., Kalli, A., French, S., Davies, K. E., and Talbot, K. (2006). A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. *Hum. Mol. Genet.* 15, 347–354. doi:10.1093/hmg/ddi452.

Almeida-Souza, L., Asselbergh, B., d'Ydewalle, C., Moonens, K., Goethals, S., de Winter, V., et al. (2011). Small Heat-Shock Protein HSPB1 Mutants Stabilize Microtubules in Charcot-Marie-Tooth Neuropathy. *J. Neurosci.* 31, 15320–15328. doi:10.1523/JNEUROSCI.3266-11.2011.

Arrigo, A. P., and Gibert, B. (2014). *HspB1, HspB5 and HspB4 in human cancers: Potent oncogenic role of some of their client proteins*. doi:10.3390/cancers6010333.

Behrends, C., Sowa, M. E., Gygi, S. P., and Harper, J. W. (2010). Network organization of the human autophagy system. *Nature* 466, 68–76. doi:10.1038/nature09204.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., et al. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* 171, 603–614. doi:10.1083/jcb.200507002.

Capponi, S., Geuens, T., Geroldi, A., Origone, P., Verdiani, S., Cichero, E., et al. (2016). Molecular Chaperones in the Pathogenesis of Amyotrophic Lateral Sclerosis: The Role of HSPB1. *Hum. Mutat.* 37, 1202–1208. doi:10.1002/humu.23062.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., et al. (2010). Identification of the Drosophila ortholog of HSPB8: Implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* 285, 37811–37822. doi:10.1074/jbc.M110.127498.

Carra, S., Seguin, S. J., and Landry, J. (2008). HspB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 4, 237–239. doi:5407 [pii].

Ciuffa, R., Lamark, T., Tarafder, A. K., Guesdon, A., Rybina, S., Hagen, W. J. H., et al. (2015). The Selective Autophagy Receptor p62 Forms a Flexible Filamentous Helical Scaffold. *Cell Rep.* 11, 748–758. doi:10.1016/j.celrep.2015.03.062.

Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., et al. (2016). p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci.* 113, E7490–E7499. doi:10.1073/pnas.1615455113.

Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., and Mann, M. (2014). Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol. Cell. Proteomics* 13, 2513–2526. doi:10.1074/mcp.M113.031591.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi:10.1038/nbt.1511.

Doshi, B. M., Hightower, L. E., and Lee, J. (2009). The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock. *Cell Stress Chaperones* 14, 445–457. doi:10.1007/s12192-008-0098-1.

Eliceiri, K., Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ : 25 years of image analysis HISTORICAL commentary NIH Image to ImageJ : 25 years of image analysis. *Nat. Methods* 9, 671–675. doi:10.1038/nmeth.2089.

Frake, R. A., Ricketts, T., Menzies, F. M., and Rubinsztein, D. C. (2015). Autophagy and neurodegeneration. *J. Clin. Invest.* 125, 65–74. doi:10.1172/JCI73944.

Geuens, T., De Winter, V., Rajan, N., Achsel, T., Mateiu, L., Almeida-Souza, L., et al. (2017). Mutant HSPB1 causes loss of translational repression by binding to PCBP1, an RNA binding protein with a possible role in neurodegenerative disease. *Acta Neuropathol. Commun.* 5, 5. doi:10.1186/s40478-016-0407-3.

Haack, T. B. B., Iuso, A., Kremer, L. S. S., Hartig, M., Strom, T. M. M., Meitinger, T., et al. (2016). Absence of the Autophagy Adaptor SQSTM1/p62 Causes Childhood-Onset Neurodegeneration with Ataxia, Dystonia, and Gaze Palsy. *Am. J. Hum. Genet.* 99, 735–743. doi:10.1016/j.ajhg.2016.06.026.

Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., et al. (2013). Autophagosomes form at ER-mitochondria contact sites. *Nature* 495, 389–93. doi:10.1038/nature11910.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. (2009). A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nat. Cell Biol.* 11, 1433–1437. doi:10.1038/ncb1991.

Imai, A. K., Hao, F., Fujita, N., Tsuji, Y., and Oe, Y. (2016). Atg9A Trafficking Through the Recycling Endosomes is Required for Autophagosome Formation.

Itakura, E., and Mizushima, N. (2010). Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* 6, 764–776. doi:10.4161/auto.6.6.12709.

Itakura, E., and Mizushima, N. (2011). p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. *J. Cell Biol.* 192, 17–27. doi:10.1083/jcb.201009067.

Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 7, 279–296. doi:10.4161/auto.7.3.14487.

Kamentsky, L., Jones, T. R., Fraser, A., Bray, M. A., Logan, D. J., Madden, K. L., et al. (2011). Improved structure, function and compatibility for cellprofiler: Modular high-throughput image analysis software. *Bioinformatics* 27, 1179–1180. doi:10.1093/bioinformatics/btr095.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al. (2014). The functional landscape of Hsp27 reveals new cellular processes such as DNA repair and alternative splicing and proposes novel anticancer targets. *Mol. Cell. Proteomics*, 3585–3601. doi:10.1074/mcp.M114.041228.

Katsuragi, Y., Ichimura, Y., and Komatsu, M. (2015). P62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J.* 282, 4672–4678. doi:10.1111/febs.13540.

Kennedy, D., Mnich, K., Oommen, D., Chakravarthy, R., Almeida-Souza, L., Krols, M., et al. (2017). HSPB1 facilitates ERK-mediated phosphorylation and degradation of BIM to attenuate endoplasmic reticulum stress-induced apoptosis. *Cell Death Dis.* 8, e3026.

doi:10.1038/cddis.2017.408.

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi:10.1080/15548627.2015.1100356.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., et al. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* 169, 425–434. doi:10.1083/jcb.200412022.

Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., et al. (2003). Interaction Codes within the Family of Mammalian Phox and Bem1p Domain-containing Proteins. *J. Biol. Chem.* 278, 34568–34581. doi:10.1074/jbc.M303221200.

Lamb, C. a, Nühlen, S., Judith, D., Frith, D., Snijders, A. P., Behrends, C., et al. (2016). TBC1D14 regulates autophagy via the TRAPP complex and ATG9 traffic. *EMBO J.* 35, 281–301. doi:10.15252/embj.201592695.

Lamb, C. a, Yoshimori, T., and Tooze, S. a (2013). The autophagosome: origins unknown, biogenesis complex. *Nat. Rev. Mol. Cell Biol.* 14, 759–74. doi:10.1038/nrm3696.

Lin, X., Li, S., Zhao, Y., Ma, X., Zhang, K., He, X., et al. (2013). Interaction domains of p62: a bridge between p62 and selective autophagy. *DNA Cell Biol.* 32, 220–7. doi:10.1089/dna.2012.1915.

Liu, G.-S., Zhu, H., Cai, W.-F., Wang, X., Jiang, M., Essandoh, K., et al. (2017). Regulation of BECN1-Mediated Autophagy by HSPB6: Insights from A Human HSPB6 ^{S10F} Mutant. *Autophagy* 8627, 01–56. doi:10.1080/15548627.2017.1392420.

Mymrikov, E. V., Seit-Nebi, a. S., and Gusev, N. B. (2011). Large Potentials of Small Heat Shock Proteins. *Physiol. Rev.* 91, 1123–1159. doi:10.1152/physrev.00023.2010.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., et al. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. *J. Biol. Chem.* 282, 24131–24145. doi:10.1074/jbc.M702824200.

Parzych, K. R., and Klionsky, D. J. (2014). An overview of autophagy: morphology, mechanism, and regulation. *Antioxid. Redox Signal.* 20, 460–73. doi:10.1089/ars.2013.5371.

Proikas-Cezanne, T., Ruckerbauer, S., Stierhof, Y. D., Berg, C., and Nordheim, A. (2007). Human WIPI-1 puncta-formation: A novel assay to assess mammalian autophagy. *FEBS Lett.* 581, 3396–3404. doi:10.1016/j.febslet.2007.06.040.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi:10.1038/nprot.2013.143.

Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., and Rubinsztein, D. C. (2010). Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nat. Cell Biol.* 12, 747–757. doi:10.1038/ncb2078.

Reggiori, F., and Tooze, S. a. (2012). Autophagy regulation through Atg9 traffic. *J. Cell Biol.* 198, 151–153. doi:10.1083/jcb.201206119.

Salmon, P., and Trono, D. (2006). Production and Titration of Lentivial Vectors. *Curr. Protoc. Neurosci.* 4, 1–24. doi:10.1002/0471142301.ns0421s37.

Sun, X., Zhou, Z., Fink, D. J., and Mata, M. (2013). HspB1 silences translation of PDZ-RhoGEF by enhancing miR-20a and miR-128 expression to promote neurite extension. *Mol. Cell. Neurosci.* 57, 111–119. doi:10.1016/j.mcn.2013.10.006.

Teyssou, E., Takeda, T., Lebon, V., Boillée, S., Doukouré, B., Bataillon, G., et al. (2013). Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: Genetics and neuropathology. *Acta Neuropathol.* 125, 511–522. doi:10.1007/s00401-013-1090-0.

Wurzer, B., Zaffagnini, G., Fracchiolla, D., Turco, E., Abert, C., Romanov, J., et al. (2015). Oligomerization of p62 allows for selection of ubiquitinated cargo and isolation membrane during selective autophagy. *Elife* 4, 1–28. doi:10.7554/eLife.08941.

CHAPTER 5

General Discussion

General Discussion

State of the art

Inherited peripheral neuropathies (IPNs) are marked with length-dependent degeneration affecting the motor and/or sensory nerves (Stojkovic, 2016). One of the major complications of hereditary IPNs is that they comprise a genetically heterogeneous disease population with over 80 affected genes reported so far (Baets et al., 2014; Timmerman et al., 2014). This heterogeneity might explain in a way why a treatment for IPN conditions does not exist yet. Finding the functional link between the disruption at the gene level and the development of pathological symptoms has been the focus of many research groups studying the biology behind IPNs. In 2004, our research group identified mutations in the gene coding for the small heat shock protein HSPB1, causing distal hereditary motor neuropathy (dHMN) and axonal CMT (CMT2F) (Evgrafov et al., 2004). Since then, studying this molecular chaperone and how mutations in it can lead to neurodegeneration has been a major focus of the research group. The main aim of my doctoral thesis was to study the implications of mutations in *HSPB1* in the autophagy process. The idea of studying autophagy in the context of *HSPB1* mutations came mainly from i) the emergence of autophagy deficits as a pathomechanism in different IPN-causing mutations in multiple genes (Haidar and Timmerman, 2017), and ii) the fact that our previous studies showed that mutations within the alpha-crystallin domain (ACD) of HSPB1 lead to overstabilisation of microtubules (Almeida-Souza et al., 2011), an effect which can be detrimental to autophagy (Köchl et al., 2006). In the first part of this thesis we found that autophagy is disrupted by mutations in and outside the of ACD of HSPB1, hinting at a more integral role for HSPB1 in autophagy than the link to microtubules. The importance of HSPB1 for the autophagy mechanism was confirmed by the fact that knocking-out *HSPB1* caused a drastic impairment in autophagy. Interestingly, the effects of mutations in *HSPB1* on autophagy were also seen in patient-derived motor neurons differentiated from iPSC. While the role of HSPB1 in proteostasis extends to its refolding activity and its ability to direct certain protein substrates to the proteasome, this research study suggests that it is mainly the autophagy function that is affected by mutations leading to IPNs. In the second part of this thesis we tried to follow up the function of HSPB1 in autophagy. Knowing that HSPB1 has been shown to interact with various other proteins and play a role in different cellular pathways (Arrigo, 2017; Katsogiannou et al., 2014a), we performed an interactomics study and revealed that HSPB1 interacts with the autophagy regulator P62. We hypothesize that HSPB1, through binding to

P62 at its oligomerization domain, can aid its function in autophagy and that mutations in *HSPB1* disrupt this function and the autophagy process. The main findings of this thesis are listed in the Box below, and are discussed in more detail in the following paragraphs.

Key Findings

- Mutations in HSPB1 disrupt autophagy
- Autophagy impairment is seen in patient derived motor neurons
- HSPB1 interacts with the autophagy receptor P62 and mutant HSPB1 shows increased binding to P62
- HSPB1 binds to the PB1 domain of P62
- Mutations in HSPB1 affect the formation of P62-bodies
- Mutations in HSPB1 impair the formation of early autophagic membranes (phagophores)

Is the disruption of autophagy by HSPB1 mutants responsible for the neuropathy phenotype?

One of the main questions in trying to understand how mutations in certain genes lead to neuropathy, is how mutations affecting ubiquitously expressed proteins such as small heat shock proteins can lead to a neuropathic or neuromuscular-specific disease phenotype. Due to their post-mitotic nature and morphological features, including an extended cytoplasm into axons and dendrites, neurons are more sensitive to autophagic impairment (Ariosa and Klionsky, 2016). Interestingly, autophagy deficient mouse models present with neurodegeneration and phenotypic features of peripheral neuropathy (Komatsu et al., 2006). With studies indicating a direct correlation between autophagy deficits and neurodegeneration, we can suggest that the impairment in autophagy that we saw (caused by *HSPB1* mutations) can contribute to the neuropathic pathology seen in patients. The fact that the consequences of a failing autophagy system worsen with aging, and the neuropathy condition deteriorates with age also backs this theory. The findings of this thesis are also of interest due to the fact that autophagy disruption have been seen in mutantions affecting both the ACD and the C-terminus of HSPB1. Recent work have also suggested that mutations in *HSPB1* can impair axonal

transport and mitochondrial homeostasis which are also vital processes for the health and maintenance of neurons (d'Ydewalle et al., 2011; Kalmar et al., 2017b). While degeneration of neurons is probably a compounding effect of many factors, several previous studies have shown functional features specific to either mutations inside the ACD or outside it (Almeida-Souza et al., 2011; Geuens et al., 2017). On the other hand, autophagy impairment presents itself as a unifying pathomechanism in different *HSPB1* mutations. Interestingly, autophagy impairment has also been seen in patient cells and transgenic animal models of IPN-causing mutations in HSPB8 (Bouhy et al., 2017; Carra et al., 2010; Kwok et al., 2011). Yet, while we observed impairment of autophagy in our motor neuron models, to establish a direct cause-effect relationship between the impairment of autophagy and neurite health, a more elaborate approach is required. Such a direct relationship has been shown in models of spinal cord injury, where induction of autophagy promotes neurite outgrowth, decreases the formation of retraction bulbs, and increases axonal regeneration (He et al., 2016). Interestingly, the study by He et al. on spinal cord injury, also reports that cultured mouse cortical neurons treated with autophagy stimulators show higher neurite outgrowth compared to untreated ones, extending the role of autophagy beyond spinal cord injury.

HSPB1 interactors in the context of autophagy

Previous studies by our team and others have portrayed HSPB1 as an effective interactor in many cellular pathways, and that demonstrated that mutations in *HSPB1* can modify the aspects of such interactions (Almeida-Souza et al., 2010; Geuens et al., 2017; Katsogiannou et al., 2014a). After revealing that not only mutations in *HSPB1* lead to autophagy impairment, but also knocking-out HSPB1, and that expression of HSPB1 in the knock-out cell lines rescues the autophagy defects, we wanted to understand the interaction spectrum of HSPB1 wild-type and mutant lines. Looking at the interactors of each variant of HSPB1 (wild-type, R127W, S135F, P182L) we did not notice a huge difference in interactors between the untreated conditions and the ones treated with autophagy-inducing serum starvation. Another interesting observation was that the cluster analysis revealed 3 different clusters: one for all the variants, one for the ACD mutants (R127W and S135F) and one for the C-terminal mutant (P182L). Looking at the broader spectrum of interactions, the interactors of the ACD mutants showed many mitochondrial proteins while the P182L mutant showed tendency to interact with proteins involved in translation confirming previous work published by our group (Geuens et

al., 2017). Due to the characteristically increased binding to other proteins by HSPB1 mutants, which might stabilize such interactions (Almeida-Souza et al., 2010, 2011; Geuens et al., 2017), the use of HSPB1 mutants for studying the interactome of HSPB1 helped us recognize a larger spectrum of potential HSPB1 interactors that might have been missed using only the wild-type variant. Indeed, some of the interactors ranked in mutant-specific clusters were shown by immunoprecipitation to bind to the wild-type HSPB1 as well.

Zooming in on the autophagy-specific proteins, we found that several protein interactors which are involved in the autophagy pathway appeared in the different clusters. A great deal of time and effort during this study has been spent on trying to validate the different autophagy-associated interactors of HSPB1 and the consequences of such interactions for the autophagy process. Of main interest to us were interactors which do not affect the initiation of autophagy per se but rather its procession, in line with what we saw in our initial experiments. Therefore, proteins necessary for the nucleation and maturation of autophagosomes such as Clusterin (CLU) (Zhang et al., 2014), Emerin (EMD) (Deroyer et al., 2014), sequestosome-1 (SQSTM1) (Pankiv et al., 2007), TRAPPC8 (Imai et al., 2016), and MAP1B (Marchbank et al., 2012) were of major interest to us. Nonetheless, the autophagy regulator sequestosome-1 (SQSTM1) known commonly as P62 (Katsuragi et al., 2015) was the main interactor which we could validate in different cell lines, with different tags and in patient cell lines. More importantly, we could see an effect on P62 caused by mutations in *HSPB1* which gave more meaning to this protein-protein interaction. Another protein that we managed to consistently validate as an interactor is TRAPPC8 (Trafficking Protein Particle Complex 8) which interacted chiefly with the P182L mutant. The interest in TRAPPC8 was driven by i) the knowledge that the P182L mutant can behave differently from the other mutants (Ackerley et al., 2006; Almeida-Souza et al., 2011; Geuens et al., 2017) and ii) the fact that TRAPPC8 is required for the trafficking of the autophagy protein ATG9A and for the autophagy process to proceed but not for its initiation (Imai et al., 2016; Lamb et al., 2016) which is in line with the deficits we saw in our initial experiments. However, we could not link the interaction of the P182L mutant with TRAPPC8 to the role of the latter in autophagy, neither in the maintenance of Golgi structure. More importantly, the interaction of the P182L mutant with P62 was in line with what we saw in the other mutants, hence the HSPB1-P62 interaction became our main focus.

Consequences of the interaction of HSPB1 with P62:

Due to its multi-domain structure, P62 is capable of binding several other proteins and regulating a number of cell signalling functions (Moscat and Diaz-Meco, 2009). Both P62 and HSPB1 have been reported to play roles in signalling pathways involving apoptosis, carcinogenesis and protein aggregation (Katsogiannou et al., 2014a; Katsuragi et al., 2015; Parcellier et al., 2003; Sanz, 2000). In addition to modulating similar cellular pathways, both HSPB1 and P62 are involved in the HSF1 (heat shock factor 1) stress response pathway. While HSPB1 is under the transcriptional control of HSF1 (De Thonel et al., 2012), the proteostatic activity of P62 and its phosphorylation status have been recently shown to be regulated by the HSF1 stress response pathway (Watanabe et al., 2017). Intriguingly, mutations in both HSPB1 and P62 have been associated with ALS (Capponi et al., 2016; Teyssou et al., 2013) pointing to a possible common pathomechanism.

Our results show that the binding of HSPB1 to P62 requires an intact PB1 domain. Through its PB1 domain, P62 has been shown to bind to other kinases containing the PB1 domain and to form oligomers (Saio et al., 2009). The PB1 domain may also mediate signalling that do not necessarily involve PB1-PB1 interactions, such is the case with ERK5 kinase which lacks a PB1 domain yet requires an intact PB1 region of the kinase MEK5 for their interaction (Nakamura et al., 2006). The P62 cytosolic speckles or P62-bodies are formed of PB1-driven P62 oligomers and P62-aPKC complexes (Jin et al., 2009; Pankiv et al., 2007; Sanz, 2000). Deletion of the PB1 domain abolishes the formation of P62 oligomers and the formation of intracellular P62-positive speckles (Bjørkøy et al., 2005; Wurzer et al., 2015). Similarly, depletion of P62 or introduction of mutations affecting the oligomerisation of P62 have been reported to abolish the formation of autophagosomes (Bjørkøy et al., 2005). Along the same line, our results also point out to a decrease in P62-bodies and in LC3 puncta in cells expressing mutant HSPB1, an interactor of P62. Several studies have shown that the disruption of autophagy, independently from P62, can lead to accumulation of P62-puncta (Korolchuk et al., 2009). However, the exact opposite was seen in our mutant cell lines, where a decrease in P62-puncta formation was seen. This also indicates that in our study, the impairment of autophagy is linked to the interaction of HSPB1 with P62.

The exact way of how the oligomerization of P62 contributes to the formation of autophagosomes remains unclear. Recently, it has been reported that through its PB1 domain, P62 forms flexible helical filaments that might be key to the scaffolding of the autophagosomal

membrane and to forming the template for the forming membranes (Ciuffa et al., 2015). A putative model might be that HSPB1 plays a role in the oligomerisation, stability or flexible assembly and disassembly of the P62 scaffolds and that mutations in HSPB1 disrupt this role. However, more experimental evidence is required for such conclusions (Fig1).

Do the different HSPB1 mutants behave similarly in relation to autophagy impairment?

The three HSPB1 mutants which are the focus of this thesis have been studied before in different contexts, and have been chosen based on two factors: i) the fact they have been well characterised in previous research from our group, and ii) that they represent the most common mutation sites in *HSPB1* (ACD and the C-terminus) (Almeida-Souza et al., 2010, 2011; Geuens et al., 2017; Holmgren et al., 2013). In this study we observed a similar impairment of autophagy by all the three different mutations marked by reduced autophagy levels and disruption in the formation of phagophores and P62-bodies. Previous studies have shown that mutations targeting the ACD of HSPB1 lead to increased chaperone activity and hence abnormally increased binding to interacting proteins, but not mutations outside the ACD (Almeida-Souza et al., 2010). In our own study we observed that the HSPB1-S135F mutant located inside the ACD showed significantly higher binding to P62, while the C-terminal P182L mutant, showed a trend of increased binding compared to the wild-type yet less so than the S135F mutant. Indeed, recently the P182L mutant has been revealed to have an increased binding to PCBP1 leading to reduction in translational repression (Geuens et al., 2017). The C-terminal mutations in HSPB1 have been reported to cause a more aggressive phenotype in patients and to possess the tendency to form aggregates and large assemblies which can trap the wild-type protein and hinder its function (Ackerley et al., 2006; Echaniz-Laguna et al., 2017). In either case, both increased binding or trapping of an interactor might cripple its function. What we saw in this study is that all different mutations in HSPB1 resulted in similar impairment levels of autophagy and no difference was observed between the different mutations. More importantly, knock-out of HSPB1 recapitulated the same damage to the autophagy process. We hypothesize that all HSPB1 mutations lead to a similar effect regarding the autophagy process, mainly by disrupting the function of HSPB1 in this pathway.

The role of HSPB1 in autophagy

A few studies have suggested a role for HSPB1 in autophagy. Earlier studies have suggested that HSPB1 is required for the autophagic clearance of dysfunctional mitochondria. It was proposed that HSPB1 mediates the downstream actions of the high mobility group box 1 (HMGB1) in maintaining mitochondrial homeostasis and autophagic surveillance (Tang et al., 2011). More recently, it was shown that overexpression of HSPB1 in renal tubular cells increased the autophagic flux and inhibited apoptosis in case of kidney injury (Matsumoto et al., 2015b). A very recent study has also shown, that the capsid protein VP2 of the foot-and-mouth disease virus, affecting cloven-hoofed animals, interacts with HSPB1 and induces autophagy. This study revealed that this interaction activates the EIF2S1-ATF4 (eukaryotic pathway translation initiation factor 2 subunit 1-activating transcription factor 4) pathway and in turn inhibits the mTORC1 pathway leading to autophagy activation (Sun et al., 2017). Autophagy regulation by other small heat shock proteins have revealed that they can work in a protein-protein binding fashion to regulate autophagy. HSPB8 has been shown to interact with BAG3 and HSC70 to stimulate the autophagic degradation of aggregates (Carra et al., 2008b). Interestingly, mutations in *HSPB8* leading peripheral neuropathies hinder its autophagy-related function (Carra et al., 2010). Similarly, a recent study has shown that HSPB6 also plays a role in autophagy by binding to the autophagy regulating protein BECN1 leading to induction of autophagy and inhibition of apoptosis (Liu et al., 2017). Also in the case of HSPB6, cardiomyopathy-causing mutations disrupt its autophagy function and highlights the disease pathomechanism (Liu et al., 2017). In line with the above mentioned studies, our study shows HSPB1 as a player in the autophagy pathway. In the same way as HSPB6 and HSPB8, we propose that HSPB1 fulfils its function in autophagy through interaction with a binding partner, in this case P62. Similarly, disease-causing mutations in *HSPB1* disrupt this function which might underlie the neuropathic conditions associated with them. It is worth noting that in our study we used starvation-induced autophagy as a template for detecting changes in the autophagy levels. While this is the mostly used approach to study autophagy in cultured cell lines, there are other ways of stimulating autophagy (Klionsky et al., 2016). Interestingly, starvation represents a stressful treatment to cells, and HSPB1 is involved in cellular stress response. This may indicate that the role of HSPB1 in autophagy may be more amplified or mostly needed in cases of stress-induced autophagy. Similarly P62 is involved in autophagy due to stressful conditions (Cohen-Kaplan et al., 2016). Indeed, in preliminary experiments using the stress-independent autophagy activator trehalose we saw much less pronounced

autophagy impairment among HSPB1 mutant cell lines (data not shown). Taking into consideration previous studies on HSPB1 and autophagy, and the multifunctionality of HSPB1, the role played by HSPB1 in autophagy may yet vary according to the physiological and pathological context (Kidney cell injury, viral infection, and neuropathy).

The bigger picture and concluding remarks

More than a decade after the identification of the first hereditary neuropathy causing mutations in HSPB1 (Evgrafov et al., 2004), 32 mutations have been described (Adriaenssens et al., 2017; Echaniz-Laguna et al., 2017). Meanwhile several publications, studying the functions of HSPB1 and the impact of mutations in it have emerged,, providing novel insight to its diverse role in cellular functions and at the same time exposing the great complexity surrounding it. At the same time, the field of inherited peripheral neuropathies has been expanding with new genes discovered adding to the heterogeneous spectrum of the disease (Baets et al., 2014). We, as well as others in the field, have tried to understand the common pathomechanisms that might underlie hereditary neuropathies, in order to provide a better picture of the disease and clearer targets for therapy. Recently, we suggested the impairment of autophagy as an emerging common pathomechanism in hereditary neuropathies (Haidar and Timmerman, 2017). Later, research done by our group on a neuropathic mouse model carrying a mutation in HSPB8, confirmed the presence of autophagy deficits in the muscles and nerves of these mice (Bouhy et al., 2017). This study was set up with the goal of finding out whether impairment of autophagy is also an underlying pathomechanism in mutations in *HSPB1*, which we managed to reveal. Autophagy is already a drug target in several neurodegenerative diseases and cancers (Towers and Thorburn, 2016). Thus, identifying autophagy disruption as a common pathomechanism in hereditary neuropathies, brings us a step closer to finding treatments for these debilitating conditions. In the case of treating mutant HSPB1-induced neuropathy, the most important advancement has been the use of HDAC6 inhibitors in transgenic mouse models (d'Ydewalle et al., 2011). While a visible restoration of axonal loss and axonal transport was observed in these mouse models upon HDAC6 inhibitor treatment, autophagy levels were not tested. Interestingly, HDAC6 inhibitors have been shown to increase secretory autophagy of alpha-synuclein in cellular models of Parkinsons' disease (Ejlerskov et al., 2013). Secretory autophagy plays a crucial role in neurodegenerative diseases (Ponpuak et al., 2016). Therefore, the disruption of autophagy by mutations in *HSPB1*, and the restoration of axonal loss by HDAC6 inhibitors hints at a possible involvement of secretory autophagy in the pathomechanism of mutant HSPB1. A study monitoring degradative and secretory autophagy

levels upon treatment with HDAC6 inhibitors in neuronal models of HSPB1-linked neuropathy is therefore of great interest.

On a final note, though a role for HSPB1 in autophagy was suggested before, it remained largely elusive. Through this work we have a clearer idea about the role played by HSPB1 in autophagy. The model proposed in this thesis by which HSPB1 interacts with the autophagy receptor P62 to aid the formation of autophagosomes, fits with the models of action suggested for other HSPBs in modulating autophagy. In the end, while this research leaves us with many open questions, it provides a step in the right direction towards understanding the pathology of peripheral neuropathies and the role of HSPB1 in autophagy.

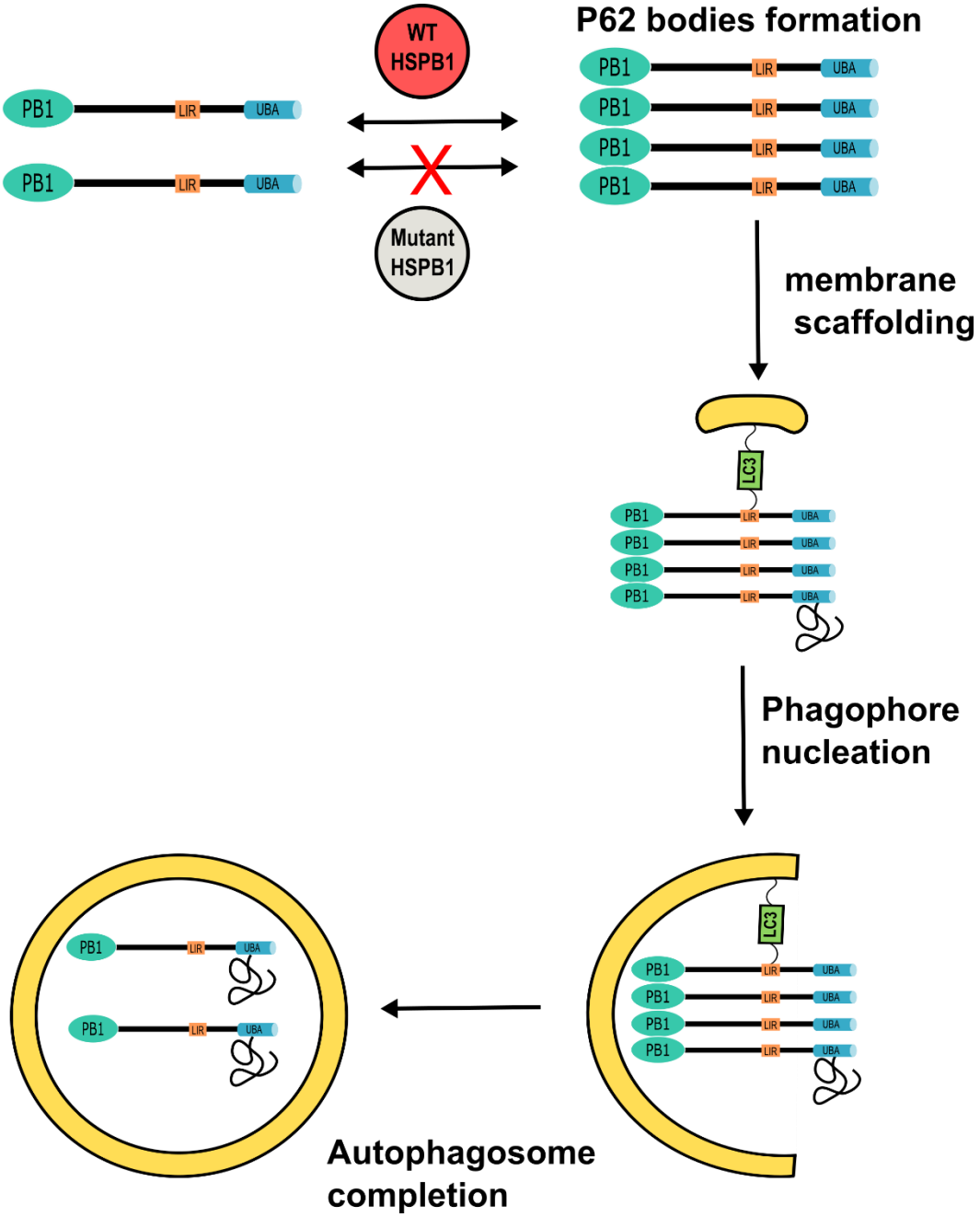

Fig 1. A putative model of HSPB1 involvement in P62-mediated autophagy

Binding of HSPB1 might be required for maintaining the dynamic oligomerization of P62. Once P62 forms large oligomers and P62-bodies it can provide a scaffold for forming membranes destined to become phagophores and eventually autophagosomes. Mutations in *HSPB1* disturb the formation of P62-bodies leading to a decrease in phagophore formation and ultimately in autophagy levels.

REFERENCES

Ackerley, S., James, P. A., Kalli, A., French, S., Davies, K. E., and Talbot, K. (2006). A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. *Hum. Mol. Genet.* 15, 347–354. doi:10.1093/hmg/ddi452.

Adriaenssens, E., Geuens, T., Baets, J., Echaniz-Laguna, A., and Timmerman, V. (2017). Novel insights in the disease biology of mutant small heat shock proteins in neuromuscular diseases. *Brain* 140, 2541–2549. doi:10.1093/brain/awx187.

Almeida-Souza, L., Asselbergh, B., d'Ydewalle, C., Moonens, K., Goethals, S., de Winter, V., et al. (2011). Small Heat-Shock Protein HSPB1 Mutants Stabilize Microtubules in Charcot-Marie-Tooth Neuropathy. *J. Neurosci.* 31, 15320–15328. doi:10.1523/JNEUROSCI.3266-11.2011.

Almeida-Souza, L., Goethals, S., De Winter, V., Dierick, I., Gallardo, R., Van Durme, J., et al. (2010). Increased monomerization of mutant HSPB1 leads to protein hyperactivity in Charcot-Marie-Tooth neuropathy. *J. Biol. Chem.* 285, 12778–12786. doi:10.1074/jbc.M109.082644.

Ariosa, A. R., and Klionsky, D. J. (2016). Autophagy core machinery: overcoming spatial barriers in neurons. *J. Mol. Med.*, 1–11. doi:10.1007/s00109-016-1461-9.

Arrigo, A. P. (2017). Mammalian HspB1 (Hsp27) is a molecular sensor linked to the physiology and environment of the cell. *Cell Stress Chaperones* 22, 517–529. doi:10.1007/s12192-017-0765-1.

Baets, J., Jonghe, P. De, and Timmerman, V. (2014). Recent advances in Charcot – Marie – Tooth disease. doi:10.1097/WCO.0000000000000131.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., et al. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* 171, 603–614. doi:10.1083/jcb.200507002.

Bouhy, D., Juneja, M., Katona, I., Holmgren, A., Asselbergh, B., de Winter, V., et al. (2017). A knock-in/knock-out mouse model of HSPB8-associated distal hereditary motor neuropathy and myopathy reveals toxic gain-of-function of mutant Hspb8. *Acta Neuropathol.*, 1–18. doi:10.1007/s00401-017-1756-0.

Capponi, S., Geuens, T., Geroldi, A., Origone, P., Verdiani, S., Cichero, E., et al. (2016). Molecular Chaperones in the Pathogenesis of Amyotrophic Lateral Sclerosis: The Role of HSPB1. *Hum. Mutat.* 37, 1202–1208. doi:10.1002/humu.23062.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., et al. (2010). Identification of the Drosophila ortholog of HSPB8: Implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* 285, 37811–37822. doi:10.1074/jbc.M110.127498.

Carra, S., Seguin, S. J., and Landry, J. (2008). HspB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 4, 237–239. doi:5407 [pii].

Ciuffa, R., Lamark, T., Tarafder, A. K., Guesdon, A., Rybina, S., Hagen, W. J. H., et al. (2015).

The Selective Autophagy Receptor p62 Forms a Flexible Filamentous Helical Scaffold. *Cell Rep.* 11, 748–758. doi:10.1016/j.celrep.2015.03.062.

Cohen-Kaplan, V., Livneh, I., Avni, N., Fabre, B., Ziv, T., Kwon, Y. T., et al. (2016). p62- and ubiquitin-dependent stress-induced autophagy of the mammalian 26S proteasome. *Proc. Natl. Acad. Sci.* 113, E7490–E7499. doi:10.1073/pnas.1615455113.

d'Ydewalle, C., Krishnan, J., Chiheb, D. M., Van Damme, P., Irobi, J., Kozikowski, A. P., et al. (2011). HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. *Nat. Med.* 17, 968–974. doi:10.1038/nm.2396.

De Thonel, A., Le Mouël, A., and Mezger, V. (2012). Transcriptional regulation of small HSP - HSF1 and beyond. *Int. J. Biochem. Cell Biol.* 44, 1593–1612. doi:10.1016/j.biocel.2012.06.012.

Deroyer, C., Renert, A. F., Merville, M. P., and Fillet, M. (2014). New role for EMD (emerin), a key inner nuclear membrane protein, as an enhancer of autophagosome formation in the C16-ceramide autophagy pathway. *Autophagy* 10, 1229–1240. doi:10.4161/auto.28777.

Echaniz-Laguna, A., Geuens, T., Petiot, P., Péréon, Y., Adriaenssens, E., Haidar, M., et al. (2017). Axonal Neuropathies due to Mutations in Small Heat Shock Proteins: Clinical, Genetic, and Functional Insights into Novel Mutations. *Hum. Mutat.* 38, 556–568. doi:10.1002/humu.23189.

Ejlerskov, P., Hultberg, J. G., Wang, J., Carlsson, R., Ambjørn, M., Kuss, M., et al. (2015). Lack of Neuronal IFN-β-IFNAR Causes Lewy Body- and Parkinson's Disease-like Dementia. Cell 163, 324–339. doi:10.1016/j.cell.2015.08.069.

Evgrafov, O. V, Mersiyanova, I., Irobi, J., Van Den Bosch, L., Dierick, I., Leung, C. L., et al. (2004). Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat. Genet.* 36, 602–606. doi:10.1038/ng1354.

Geuens, T., De Winter, V., Rajan, N., Achsel, T., Mateiu, L., Almeida-Souza, L., et al. (2017). Mutant HSPB1 causes loss of translational repression by binding to PCBP1, an RNA binding protein with a possible role in neurodegenerative disease. *Acta Neuropathol. Commun.* 5, 5. doi:10.1186/s40478-016-0407-3.

Haidar, M., and Timmerman, V. (2017). Autophagy as an Emerging Common Pathomechanism in Inherited Peripheral Neuropathies. *Front. Mol. Neurosci.* 10, 1–17. doi:10.3389/fnmol.2017.00143.

He, M., Ding, Y., Chu, C., Tang, J., Xiao, Q., and Luo, Z.-G. (2016). Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury. *Proc. Natl. Acad. Sci.* 113, 11324–11329. doi:10.1073/pnas.1611282113.

Holmgren, A., Bouhy, D., De Winter, V., Asselbergh, B., Timmermans, J. P., Irobi, J., et al. (2013). Charcot-Marie-Tooth causing HSPB1 mutations increase Cdk5-mediated phosphorylation of neurofilaments. *Acta Neuropathol.* 126, 93–108. doi:10.1007/s00401-013-1133-6.

Imai, A. K., Hao, F., Fujita, N., Tsuji, Y., and Oe, Y. (2016). Atg9A Trafficking Through the Recycling Endosomes is Required for Autophagosome Formation.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R., et al. (2009). Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling. *Cell* 137, 721–735. doi:10.1016/j.cell.2009.03.015.

Kalmar, B., Innes, A., Wanisch, K., Kolaszynska, A. K., Pandraud, A., Kelly, G., et al. (2017). Mitochondrial deficits and abnormal mitochondrial retrograde axonal transport play a role in the pathogenesis of mutant Hsp27-induced Charcot Marie Tooth Disease. *Hum. Mol. Genet.* 0, 1–14. doi:10.1093/hmg/ddx216.

Katsogiannou, M., Andrieu, C., Baylot, V., Baudot, A., Dusetti, N. J., Gayet, O., et al. (2014). The Functional Landscape of Hsp27 Reveals New Cellular Processes such as DNA Repair and Alternative Splicing and Proposes Novel Anticancer Targets. *Mol. Cell. Proteomics* 13, 3585–3601. doi:10.1074/mcp.M114.041228.

Katsuragi, Y., Ichimura, Y., and Komatsu, M. (2015). P62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J.* 282, 4672–4678. doi:10.1111/febs.13540.

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi:10.1080/15548627.2015.1100356.

Köchl, R., Hu, X. W., Chan, E. Y. W., and Tooze, S. a. (2006). Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. *Traffic* 7, 129–145. doi:10.1111/j.1600-0854.2005.00368.x.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., et al. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441, 880–884. doi:10.1038/nature04723.

Korolchuk, V. I., Mansilla, A., Menzies, F. M., and Rubinsztein, D. C. (2009). Autophagy Inhibition Compromises Degradation of Ubiquitin-Proteasome Pathway Substrates. *Mol. Cell* 33, 517–527. doi:10.1016/j.molcel.2009.01.021.

Kwok, A. S., Phadwal, K., Turner, B. J., Oliver, P. L., Raw, A., Simon, A. K., et al. (2011). HspB8 mutation causing hereditary distal motor neuropathy impairs lysosomal delivery of autophagosomes. *J. Neurochem.* 119, 1155–1161. doi:10.1111/j.1471-4159.2011.07521.x.

Lamb, C. a, Nühlen, S., Judith, D., Frith, D., Snijders, A. P., Behrends, C., et al. (2016). TBC1D14 regulates autophagy via the TRAPP complex and ATG9 traffic. *EMBO J.* 35, 281–301. doi:10.15252/embj.201592695.

Liu, G.-S., Zhu, H., Cai, W.-F., Wang, X., Jiang, M., Essandoh, K., et al. (2017). Regulation of BECN1-Mediated Autophagy by HSPB6: Insights from A Human HSPB6 ^{S10F} Mutant. *Autophagy* 8627, 01–56. doi:10.1080/15548627.2017.1392420.

Marchbank, K., Waters, S., Roberts, R. G., Solomon, E., and Whitehouse, C. A. (2012). MAP1B interaction with the FW domain of the autophagic receptor Nbr1 facilitates its association to the microtubule network. *Int. J. Cell Biol.* 2012. doi:10.1155/2012/208014.

Matsumoto, T., Urushido, M., Ide, H., Ishihara, M., Hamada-Ode, K., Shimamura, Y., et al. (2015). Small Heat Shock Protein Beta-1 (HSPB1) Is Upregulated and Regulates Autophagy and Apoptosis of Renal Tubular Cells in Acute Kidney Injury. *PLoS One* 10, e0126229. doi:10.1371/journal.pone.0126229.

Moscat, J., and Diaz-Meco, M. T. (2009). p62 at the Crossroads of Autophagy, Apoptosis, and Cancer. *Cell* 137, 1001–1004. doi:10.1016/j.cell.2009.05.023.

Nakamura, K., Uhlik, M., and Johnson, N. (2006). PB1 domain-dependent signaling complex

is required for extracellular signal-regulated kinase 5 activation. *Mol. Cell. Biol.* 26, 2065–2079. doi:10.1128/MCB.26.6.2065.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., et al. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. *J. Biol. Chem.* 282, 24131–24145. doi:10.1074/jbc.M702824200.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., et al. (2003). HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol. Cell. Biol.* 23, 5790–5802. doi:10.1128/MCB.23.16.5790.

Ponpuak, M., Mandell, M. a, Kimura, T., Chauhan, S., Cleyrat, C., and Deretic, V. (2015). Secretory autophagy. Curr. Opin. Cell Biol. 35, 106–116. doi:10.1016/j.ceb.2015.04.016.

Saio, T., Yokochi, M., and Inagaki, F. (2009). The NMR structure of the p62 PB1 domain, a key protein in autophagy and NF-κB signaling pathway. *J. Biomol. NMR* 45, 335–341. doi:10.1007/s10858-009-9370-7.

Sanz, L. (2000). The atypical PKC-interacting protein p62 channels NF-kappa B activation by the IL-1-TRAF6 pathway. *EMBO J.* 19, 1576–1586. doi:10.1093/emboj/19.7.1576.

Stojkovic, T. (2016). Hereditary neuropathies: An update. *Rev. Neurol. (Paris).* 172, 775–778. doi:10.1016/j.neurol.2016.06.007.

Sun, P., Zhang, S., Qin, X., Chang, X., Cui, X., Li, H., et al. (2017). Foot-and-mouth disease virus capsid protein VP2 activates the cellular EIF2S1-ATF4 pathway and induces autophagy via HSPB1. *Autophagy* 8627, 00–00. doi:10.1080/15548627.2017.1405187.

Tang, D., Kang, R., Livesey, K. M., Kroemer, G., Billiar, T. R., Van Houten, B., et al. (2011). High-mobility group box 1 is essential for mitochondrial quality control. *Cell Metab.* 13, 701–711. doi:10.1016/j.cmet.2011.04.008.

Teyssou, E., Takeda, T., Lebon, V., Boillée, S., Doukouré, B., Bataillon, G., et al. (2013). Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: Genetics and neuropathology. *Acta Neuropathol.* 125, 511–522. doi:10.1007/s00401-013-1090-0.

Timmerman, V., Strickland, A. V., and Züchner, S. (2014). Genetics of Charcot-Marie-Tooth (CMT) disease within the frame of the human genome project success. *Genes (Basel).* 5, 13–32. doi:10.3390/genes5010013.

Towers, C. G., and Thorburn, A. (2016). Therapeutic Targeting of Autophagy. *EBioMedicine* 14, 15–23. doi:10.1016/j.ebiom.2016.10.034.

Watanabe, Y., Tsujimura, A., Taguchi, K., and Tanaka, M. (2017). HSF1 stress response pathway regulates autophagy receptor SQSTM1/p62-associated proteostasis. *Autophagy* 13, 133–148. doi:10.1080/15548627.2016.1248018.

Wurzer, B., Zaffagnini, G., Fracchiolla, D., Turco, E., Abert, C., Romanov, J., et al. (2015). Oligomerization of p62 allows for selection of ubiquitinated cargo and isolation membrane during selective autophagy. *Elife* 4, 1–28. doi:10.7554/eLife.08941.

Zhang, F., Kumano, M., Beraldi, E., Fazli, L., Du, C., Moore, S., et al. (2014). Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. *Nat. Commun.* 5, 1–13. doi:10.1038/ncomms6775.

LIST OF ABBREVIATIONS

ALS Amyotrophic lateral sclerosis

ATG Autophagy-related gene

BCL2 B-cell lymphoma2 (BCL2) apoptosis regulator

BECN1 Beclin 1

BNIP3 BCL2 interacting protein 3

BSCL2 seipin lipid droplet biogenesis associated

CASA chaperone-assisted selective autophagy

CCT5 chaperonin containing TCP1 subunit 5

CMA chaperone mediated autophagy

CMAP compound muscle action potential

CMT Charcot-Marie-Tooth

DCTN1 dynactin subunit 1

dHMN distal hereditary motor neuropathy

DNAJB2 DnaJ heat shock protein family (Hsp40) member B2

DNM2 dynamin 2

DST dystonin

eIF2α eukaryotic initiation factor 2-alpha

ER endoplasmic reticulum

ERES ER exit sites

FAM134B family with sequence similarity 134 member B

FIG4 FIG4 phosphoinositide 5-phosphatase

GABARAP GABA type A receptor-associated protein

GDAP1 ganglioside induced differentiation associated protein 1

GEF Guanine nucleotide exchange factor

HOPS Homotypic fusion and protein sorting

HSAN Hereditary sensory and autonomic neuropathy

HSP Hereditary spastic paraplegia

HSPB1/2/3/5/6/7/8/9 Heat shock protein B1/2/3/5/6/7/8/9

IPN inherited peripheral neuropathies

iPSCs induced pluripotential stem cells

KIF1A kinesin family member 1A

KO knock-out

LC3 microtubule associated protein 1 light chain 3 (MAP1LC3)

LITAF lipopolysaccharide induced TNF factor

LRSAM1 leucine rich repeat and sterile alpha motif containing 1

MFN2 mitofusin 2

MTMR2/13 myotubularin-related protein 2/13

MTORC1 mechanistic target of rapamycin 1

NCV nerve conduction velocities

NDRG1 N-myc downstream regulated 1

NEFL neurofilament light

P62/SQSTM1 sequestosome 1

PARK2 parkin 2

PERK protein kinase R (PKR)-like endoplasmic reticulum kinase

PHF23 PHD finger protein 23

PIK3C3/VPS34 phosphatidylinositol 3-kinase catalytic subunit type 3

PIK3R4/VPS15 phosphoinositide-3-kinase regulatory subunit 4

PINK1 PTEN induced putative kinase 1

PKA Protein kinase A

PMP22 peripheral myelin protein 22

PtdIns phosphatidyl inositol

PTPIP51 protein tyrosine phosphatase interacting protein 51

Rab11 Ras-related protein Rab-11A

RAB21 Ras-related protein Rab-7

RAB7 Ras-related protein Rab-21

RB1CC1 RB1 inducible coiled-coil 1

SEC24D SEC24 homolog D, COPII coat complex component

SH3TC2 SH3 domain and tetratricopeptide repeats 2

SNARE SNAP (Soluble NSF Attachment Protein) Receptor

SOD1 superoxide dismutase 1

TECPR2 tectonin beta-propeller repeat containing 2

TrkA Tyrosine kinase A

TRPV4 transient receptor potential cation channel subfamily V member 4

ULK1/2 unc-51 like autophagy activating kinase 1/2

UVRAG UV radiation resistance associated

VAPB VAMP associated protein B

VCP Valosin containing protein

WNK1 WNK lysine deficient protein kinase 1

WT Wildtype

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  Authors: Mansour Haidar, Bob Asselbergh, Elias Adriaenssens, Vicky De Winter, Manisha Juneja, Vincent Timmerman. Submitted to *Autophagy*
- Autophagy as an emerging common pathomechanism in inherited peripheral neuropathies. Authors: Mansour Haidar, Vincent Timmerman.
  Published: 11 May 2017. Journal: Frontiers in Molecular Neuroscience
- Axonal Neuropathies due to Mutations in Small Heat Shock Proteins: Clinical, Genetic and Functional Insights into Novel Mutations.
  Authors: Andoni Echaniz-Laguna, Thomas Geuens, Philippe Petiot, Yann Péréon, Elias Adriaenssens, Mansour Haidar, et al., Human mutation. Published on 01 Feb 2017

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- **01/03/2018:** VIB Training course in drug discovery. Leuven, Belgium.
- **22 and 29/01/17:** VIB Introduction to Python, Ghent, Belgium.
- **3-5/12/17:** VIB course on image processing analysis. Location: Leuven, Belgium.
- **17/11/2017:** VIB course on protein structure analysis. Ghent, Belgium.
- **22/09/16:** Flanders Bio Worskshop: From PhD to Job Market. Location: Gent, Belgium.
- **14/12/2015:** VIB course: Initiation GIMP and Inkscape. Leuven, Belgium.
- **10/11/2015:** VIB Training Course BITS 'Basic statistics in GraphPad Prism', Leuven Belgium
- **15-19/06/2015:** VIB 'Summer School on Advanced Light Microscopy', Gent, Belgium.
- **19/12/2014:** VIB Ingenuity Pathway Analysis (IPA) Course. Leuven, Belgium
- **01-04/12/2014:** StatUA workshop: Methods in Data collection, Universiteit Antwerpen
- **06/10/ 2014:** Training Module "Basic Biosafety", Universiteit Antwerpen,
- **10/02-07/07/2014:** Laboratory Animal Science course, Universiteit Antwerpen
- **05/02/2014:** StatUA Day 2014, Universiteit Antwerpen

Meetings and Conferences:

- **07/09/2017**: From Pictures to Numbers: RBSM symposium on high-dimensional fluorescence-based microscopy. University of Antwerp.
  Poster: *Impairment of Autophagy as a Possible Pathomechanism for CMT Causing Mutations in HSPB1*
- **8-12/07/2017**: 2017 Peripheral Nervous System Society (PNS) Meeting Sitges-Barcelona, Spain.

Oral presentation: *Impairment of Autophagy as a Possible Pathomechanism for CMT Causing Mutations in HSPB1*

- **07/02/2017**: 2nd FWO-WOG meeting on the small heat shock proteins. University of Antwerp.
  Talk: *Impairment of Autophagy as a Possible Pathomechanism for CMT Causing Mutations in HSPB1*
- **26-27/01/2017**: VIB Conference; ER Stress, Autophagy & Immune System. Bruges, Belgium.
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  Poster: *Role of HSPB1 in macro-autophagy and its implication in peripheral neuropathy*
- **8-13/05/2015:** EMBO Conference "Molecular chaperones: From molecules to cells and misfolding diseases", Heraklion, Greece.
  Poster: *Role of HSPB1 in macro-autophagy and its implication in peripheral neuropathy*.
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- **18-19/09/2014:** VIBes PhD students symposium, Antwerpen, Belgium.
  Poster: *Role of HSPB1 in macro-autophagy and its implication in peripheral neuropathy*
- **01-04/07/2014:** Training Course on PNS development, Lecce, Italy.
  Poster: *Autophagy deficits as common pathomechanisms leading to CMT caused by HSPB1 and HSPB8 mutations.*
- **28-30/03/2014:** VIB Seminar 2014, Blankenberge, Belgium.
- **25/10/2012:** Neuroscience Network (NEUREX) annual meeting: Strasbourg, France.
- **20/01/2012:** "Neuropathic Pain: A Challenge For The Patient And The Physician" Conference by the Lebanese Society for the Study of Pain (LSSP)

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