Identification and functional characterization of BICD2 mutations causing SMALED2, a congenital dominant form of spinal muscular atrophy

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for Jared and Markus

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1. Aim, thesis structure and major findings

Homozygous alterations in *SMN1* are the most common genetic cause of spinal muscular atrophy (SMA). However, the causing gene of many other cases with SMA remain unknown.

My PhD project aimed to identify and analyze functionally novel genes causing motor neuropathies. To this end, the current work led to the discovery of *BICD2* as the causing gene of spinal muscular atrophy, lower extremity-predominant, autosomal dominant, type 2 (SMALED2 MIM 615290).

This PhD thesis starts with an introduction (**Section 2**) about the different SMA forms, with emphasis on autosomal dominant SMA and focus on the lower extremity-predominant form (SMALED). In the last part of the introduction, the cellular functions of BICD2 are described including the role of this protein in neuronal tissue.

The major findings of my work are presented in **Section 3**, in conjunction with my individual contributions for each scientific publication.

I summarized in **Section 3.1** the discovery of *BICD2* variants in affected individuals with SMALED2 (Neveling; Martinez-Carrera et al. 2013, Synofzik et al. 2014). In addition, a review is included where I elaborated on the clinical features of the affected individuals, the locations of the mutations within the protein domains, and possible consequences at the molecular level (Martinez-Carrera and Wirth 2015). Reading this review is highly recommendable for a better overview of this part of the work.

Section 3.2 describes the pathological consequences that the different mutations exert at cellular level: These were Golgi fragmentation in some cases (Neveling; Martinez-Carrera et al. 2013) and alterations in the interaction with the dynein-dynactin complex in others (Martinez-Carrera et al. 2018) . However, the striking finding was that regardless of where the mutation is located in the protein, all of them altered the microtubule array (arrangement), which led to aberrations in axon development in motor neurons, the disease relevant cell type in SMALED2 (Martinez-Carrera et al. 2018). Furthermore, I also include in this section, the findings from the characterization of the first *in vivo Drosophila* model for SMALED2. I generated transgenic *Drosophila* lines that carry the *BICD2* variants found in individuals with SMALED2. By using the UAS-GAL4, I was able to allow the expression of the transgenic construct (either wild type or mutant) in a tissue-restricted manner. When expressed in neuronal tissue, but not in muscle, the

BICD2 mutations caused impaired locomotion with reduced neuromuscular junction size, a hallmark of developmental impairment (Martinez-Carrera et al. 2018).

In **Section 3.3**, I expand the knowledge about the spectrum of disorders that are associated with variants in *BICD2*. The clinical presentation in such disorders differed from the typical SMALED2. Two of the *BICD2* variants that cause SMALED2, were also identified in affected individuals with chronic myopathy (Unger et al. 2016), and novel variants were associated with congenital arthrogryposis multiplex with respiratory failure and early lethality, the most severe clinical presentation observed in association with *BICD2* (Storbeck et al. 2017).

In the next part of my thesis (**Section 4**), I include unpublished findings obtained during functional investigation of endocytosis, centrosomes, and aggregate formation.

At last, I discuss the compilation of findings of my work (**Section 5**), correlate with the findings of others, underline the novel insights into the disease and suggest possible molecular mechanisms. **Sections 6 and 7** include the summary and highlights of this work. **Section 8** includes an additional contribution (Komlosi et al. 2014)

2. Introduction

2.1. Spinal muscular atrophies

Spinal muscular atrophies (SMAs) comprise a group of genetic disorders characterized by aberrant development and/or loss of spinal motor neurons. The clinical features of spinal muscular atrophy are wasting and weakness of muscles supplied by the affected motor neurons (Emery 1971).

SMAs present a broad clinical spectrum, and differential diagnosis from other disorders can be challenging due to the overlapping of symptoms. However, SMAs are often classified based on the mode of inheritance (autosomal recessive, autosomal dominant, X-linked), age of onset, and pattern of muscle weakness (i.e. proximal or distal).

2.1.1. Autosomal recessive and X-linked forms of SMA

The majority of the SMA cases are autosomal recessive linked to chromosome 5 (5q-SMA), and caused by homozygous deletion/mutation of the *survival motor neuron 1* (*SMN1*) gene, localized on chromosome 5q12-q13 (Lefebvre et al. 1995, Wirth et al. 1999, Wirth 2000). The muscle weakness in 5q-SMA is usually symmetrical, more proximal than distal, the lower limbs are more affected than upper. The 5q-SMA is classified into four subtypes based on age of onset and severity of symptoms (MIM 253300, 253550, 253400, 271150).

X-linked forms of SMA (SMAX) affect mainly men and are considered of rare incidence. The X-linked type I (SMAX1, MIM 313200) is characterized by adult onset of weakness and atrophy of the limb and bulbar muscles, with fasciculations, and an increase in the size of breast tissue (Harding et al. 1982). SMAX1 is associated with trinucleotide repeat expansion CAG(n) in the *androgen receptor* gene (La Spada et al. 1991, Lund et al. 2001). In contrast to SMAX1, cases with X-linked SMA type II (SMAX2, MIM 301830) show a neonatal onset of severe hypotonia, areflexia, contractures (muscle shortening) and/or fractures. Death occurs in infancy due to respiratory failure (Ramser et al. 2008). Variants in the gene encoding ubiquitin-activating enzyme-1 (UBA1) have been described as causative of SMAX2 (Ramser et al. 2008). A less frequent X-linked SMA type III (SMAX3, MIM 300489) affects distal muscles of lower limbs and later of upper limbs. The symptoms appear in the first decade of life and are slowly progressive. Variants in the *ATP7A* gene, which encodes a transmembrane copper-transporting ATPase, have been identified in individuals with SMAX3 (Kennerson et al. 2010).

2.1.2. Autosomal dominant SMAs

Autosomal dominant forms of SMA are considered highly heterogeneous and show high variability of clinical features. It is estimated that less than 2% of the total cases of SMAs are dominantly inherited (Farrar and Kiernan 2015).

In contrast to 5q-SMA, autosomal dominant SMAs are considered milder and without or slow progression. Most of the dominant SMAs affect lower and upper limbs, with proximal and/or distal pattern of muscle weakness and the age of onset ranges from congenital to late adulthood (Table 1).

In the great majority of individuals with autosomal dominant SMA, the genetic causes are unknown. However, the rapid advance in genetic screening due to massive parallel sequencing and improvement in phenotypical classification have contributed remarkably to the discovery and characterization of novel causative genes (Table 1).

2.1.2.1. Autosomal dominant SMAs that affect lower and upper extremities

This group of autosomal dominant SMAs that affects lower and upper limbs includes:

The scapuloperoneal SMA (SPSMA MIM 181405) is characterized by weakness of the scapular (shoulder blade) and peroneal (lower leg) muscles. The onset of the symptoms is congenital or early childhood. Variants in *TRPV4*, have been described as the genetic cause of SPSMA (Auer-Grumbach et al. 2010). TRPV4 is a transient receptor potential cation channel that plays a role in neuronal signalling (Liedtke 2008). Variants in *TRPV4* have been also associated with Charcot-Marie-Tooth type 2C (CMT2C MIM 606071) (Landoure et al. 2010).

The Finkel SMA with late adult onset (SMAFK MIM 182980), presents proximal muscle weakness and fasciculations. Variants in *VAPB* are associated to SMAFK (Nishimura et al. 2004). *VAPB* encodes a vesicle-associated membrane protein that plays a role in the unfolded protein response (UPR) and has been associated with amyotrophic lateral sclerosis type 8 (ALS8 MIM 608627) (Kanekura et al. 2006).

The distal SMAs, also known as distal hereditary motor neuropathies (dHMN), cause weakness and atrophy of lower and upper limbs with distal predominance. dHMN are clinically and genetically diverse. The phenotypes of various dHMN overlap with other disorders such as Charcot-Marie-Tooth and amyotrophic lateral sclerosis. Several types and subtypes of dHMN have been associated with variants in genes that encode for heat-shock proteins like HSPB8 (Irobi et al. 2004), HSPB1 (Houlden et al. 2008) and

HSPB3 (Kolb et al. 2010), also the choline transporter SLC6A7 (Barwick et al. 2012) and dynactin-1 (Puls et al. 2003), among others (Table 1).

Other two dominant SMAs with distal predominance are the hereditary neuropathy with or without age-related macular degeneration (HNARMD MIM 608895) and the peripheral neuropathy, myopathy, hoarseness, and hearing loss (PNMHH MIM 614369). HNARMD has been associated with variants in the *FBLN5* gene, which encodes the fibulin-5 that might promote the deposit formation in macular degeneration (Mullins et al. 2007). Regarding PNMHH, the identified causing-gene *MYH14* encodes a member of the myosin II family, which interact with cytoskeletal actin (Choi et al. 2011).

Table 1. Current known disease-causing genes for autosomal dominant spinal muscular atrophies.
Limbs affected LL: lower limbs; UL: upper limbs; +++: majority of the cases; ++: some of the cases; +: few cases; -: no cases; CMT: Charcot-Marie-Tooth; HSP: Hereditary spastic paraplegia; ALS: Amyotrophic lateral sclerosis; ADCL2: autosomal dominant cutis laxa-2

Type (MIM#)	Gene	Function	Age of onset	LL	UL	Proximal	Distal	Progressive	Other features	Allelic disorders
SPSMA (181405) Scapuloperoneal	*TRPV4*	Cation channel receptor	congenital, early childhood	+++	++	-	+++	Non or slowly	Scapuloperoneal muscle weakness, laryngeal palsy, arthrogryposis	CMT(2C)
SMALED1 (158600) Lower-extremity predominant, 1	*DYNC1H1*	Subunit of the cytoplasmic dynein complex	congenital to adult	+++	-	+++	+	Non or very slowly	One case with cognitive delay	CMT (20)
SMALED2 (615290) Lower-extremity predominant, 2	*BICD2*	Dynein-dynactin adaptor	congenital, early childhood	+++	+	+++	++	Non or slowly	± Arthrogryposis	Late onset HSP
SMAFK (182980) Finkel late-adult	*VAPB*	Vesicle-associated membrane protein	median age at 37 years	+++	+++	+++	-	Progressive	Muscle cramps and fasciculations	ALS
HMN (Hereditary motor neuropathies)										
HMN2A (158590)	*HSPB8*	Heat-shock proteins	childhood to	+++	++	-	+++	Rapid	Muscle paresis of the big toe	Overlapping
HMN2B (608634)	*HSPB1*		early	+++	+	-	+++	Slowly		phenotype with
HMN2C (613376)	*HSPB3*		adulthood	+++	+++	-	+++	Slowly	Calf weakness	CMT
HMN2D (615575)	*FBXO38*	F-box family		+++	+	+++	+++	Slowly		
HMN5A (600794)	*GARS*	Glycyl-tRNA synthetase	~18 years	-	+++	-	+++	Slowly		
	BSCL2	lipid droplet formation								
HMN5B (614751)	*REEP1*	G-protein-couple receptor	childhood	+++	+++	-	+++		Vocal cord paralysis	
HMN7A (158580)	*SLC6A7*	Choline transporter	>20 years	++	++	-	+++	Slowly	Vocal cord paralysis	
HMN7B (607641)	*DCTN1*	Dynactin-1 or p150 (Glued)	adulthood	+++	+++	+++	+++	Slowly		ALS
HNARMD (608895) Hereditary neuropathy with or without age-related macular degeneration	*FBLN5*	Adhesion of endothelial cells	childhood to adulthood	+++	+	-	+++	Slowly	Macular degeneration in some cases. Demyelinating neuropathy.	ADCL2
PNMHH (614369) Peripheral neuropathy, myopathy, hoarseness, and hearing loss	*MYH14*	Myosin II, ATP-dependent molecular motor	childhood	+++	+	-	+++	Progressive	Hoarseness and hearing loss	Deafness

2.1.2.2. Autosomal dominant SMA that affects lower extremities predominantly, type 1 (SMALED1)

The term SMALED was first used by Harms and colleagues, to distinguish a dominant SMA with clear lower limb predominance from other dominant SMA forms with upper extremity involvement (Harms et al. 2010). The first gene to be identified as causative of SMALED was *DYNC1H1* (SMALED1 MIM 158600).

Individuals with SMALED1 present difficulties to walk and show proximal leg weakness with muscular atrophy without sensory involvement. The weakness and atrophy are prominent in quadriceps muscles, and mild atrophy of distal leg muscles has been also reported. In very few cases, the upper limbs are mildly affected. Reduced or absent reflexes in the lower limbs are also reported. The symptoms appear in early childhood and the course of the disease is non-progressive or very slowly progressive (Harms et al. 2010, Harms et al. 2012, Tsurusaki et al. 2012). In many cases of SMALED1, electromyography (EMG) and skeletal muscle biopsy show signs of chronic denervation (Harms et al. 2010, Harms et al. 2012). Very few individuals with SMALED1 present contractures.

Regarding the molecular basis of SMALED1, several heterozygous mutations in the tail domain of the heavy chain of cytoplasmic dynein (DYNC1H1) have been described, and experimental evidence suggested that these mutations disrupt dynein complex stability, and/or affect its function (Harms et al. 2012, Hoang et al. 2017). Interestingly, it was recently reported that the majority of the mutations do not affect binding of dynein to dynactin and to its cargo adaptor BICD2 (Hoang et al. 2017). However, those mutations decrease the travel distances of moving dynein-dynactin complex, presumably by changes in the microtubule-binding domain of dynein (Hoang et al. 2017). Mutations in the motor domain of DYNC1H1 are described as to possess the strongest effects on dynein motility *in vitro*, and have been associated with malformations of cortical development (MCD, MIM 614563) (Schiavo et al. 2013, Hoang et al. 2017).

2.1.2.3. Autosomal dominant SMA that affects lower limbs predominantly, type 2 (SMALED2)

In 1994, Frijns et al. described a family of Dutch origin, in which the affected individuals exhibited congenital nonprogressive atrophy and weakness predominantly of lower limb muscles, in association with contractures in ankles and feet (Frijns et al. 1994). Tendon reflexes were reduced or absent. The affected individuals presented difficulties when walking, such as the ability to walk only on toes and waddling gait.

Histological studies of the affected muscles revealed evidence of a neurogenic disorder, and signs of denervation and reinnervation (Frijns et al. 1994).

The pattern of inheritance was dominant and linkage to the 5q12-q13 region was excluded. The phenotype of the disease observed in family 1 was described as dominant non-5q SMA with lower limb predominance. However, the genetic cause was unknown.

This family described by (Frijns et al. 1994), constituted the starting point of my project. We identified variants in the *BICD2* gene as disease-causing (Section 3.1). The next section includes an overview of the cellular functions of BICD2, which are widely investigated.

2.2. BICD2: structure and function

Bicaudal-D (*BicD*) was initially identified in *Drosophila melanogaster* through a characterization of two dominant lethal maternal mutations, which disrupt the establishment of anterior and posterior polarity giving rise to bicaudal (two tails) embryos (Mohler and Wieschaus 1986, Steward 1987, Schupbach and Wieschaus 1991).

Only one *BicD* gene is present in invertebrates, while mammals have two homologs *BICD1* and *BICD2*. In humans, *BICD1* is localized in the chromosomal region 12p11.2-p11.1 and is mainly expressed in brain, skeletal muscle and heart (Baens and Marynen 1997), while *BICD2* localizes in the chromosomal region 9q22.3 and is ubiquitously expressed.

Structural analysis in *Drosophila* revealed that more than half of the BicD protein consists of heptad repeats. A heptad repeat is defined as a repeating pattern of seven amino acids of which hydrophobic residues are preferentially located at positions 1 and 4 (McLachlan and Karn 1983). These heptad repeats are responsible of mediating the packaging of one helix against another, resulting in the formation of coiled-coil (CC) structures (Bruccoleri et al. 1986). The N-terminal domain contains the coiled-coil segment 1 (CC1) and the coiled-coil segment 2 (CC2), and the C-terminal domain contains the coiled-coil segment 3 (CC3).

In the case of BICD2, experimental evidence supports that the CC1 binds to CC3 and undergo intramolecular interactions forming homodimers (Hoogenraad et al. 2001). It is further proposed that once the CC3 engages in an interaction with other proteins, the CC1 becomes available for interaction with other proteins. These protein interactions seem to determine the cellular localization and function of BICD2 (Hoogenraad et al. 2001).

2.2.1. BICD2 is an adaptor of dynein and functions in different cellular processes

Motor adaptor proteins that link motors to cargo are commonly implicated in controlling motor coordination and cargo movement (Schlager and Hoogenraad 2009, Akhmanova and Hammer 2010, Jolly and Gelfand 2011, Fu and Holzbaur 2014). BICD2 is a widely studied cargo adaptor of cytoplasmic dynein, the major contributor to minus end-directed microtubule transport (Figure 1) (Hoogenraad et al. 2001, Matanis et al. 2002, Splinter et al. 2010, Splinter et al. 2012).

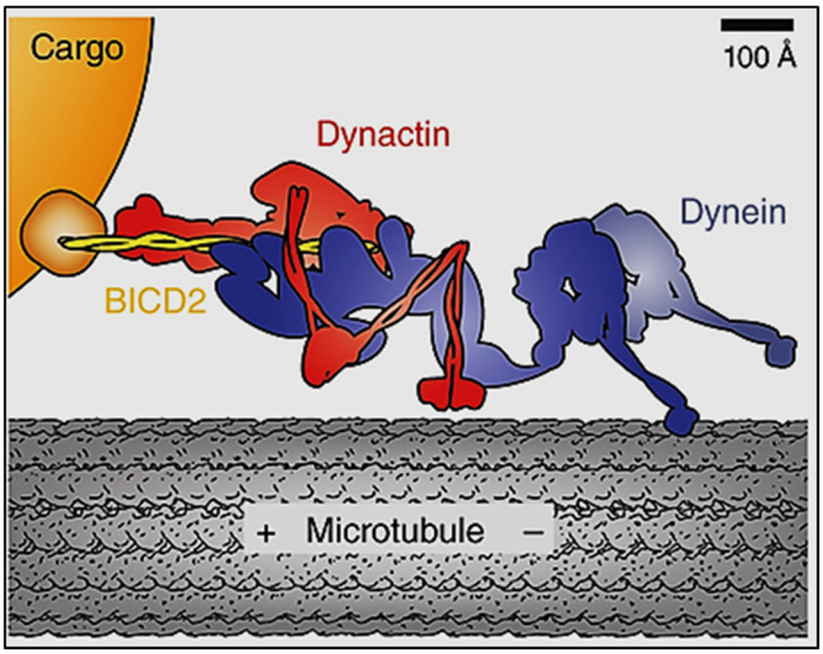

Figure 1. Cartoon of dynein-dynactin-BICD2 complex.
Dynein (blue) moves toward the minus end of the microtubule. Dynein associates with dynactin (red) and with BICD2 (yellow) to link to cargo (orange). Source: (Reck-Peterson 2015).

Dynein requires dynactin (name is derived from dynein activator) for nearly all of its cellular functions (Holleran et al. 1998, Karki and Holzbaur 1999, Schroer 2004). Dynein and dynactin bind to each other via the interaction of dynein intermediate chain and the dynactin subunit p150 (Vaughan et al. 1995, Karki and Holzbaur 1999, King et al. 2003). Several studies *in vitro* described that these two complexes exist as separate pools that bind transiently, suggesting that additional factors must be present in cells to strengthen this association in order to achieve long distance transport (Quintyne et al. 1999, Habermann et al. 2001, Quintyne and Schroer 2002). Studies have suggested that the N-terminal domain of BICD2 binds to the dynein-dynactin complex, not only to function as a linker for cargos, but also to promote a stable interaction between dynein and dynactin (Splinter et al. 2012).

The C-terminal domain of BICD2 shows the highest degree of conservation among metazoans and is the cargo-binding domain (Hoogenraad et al. 2001, Terenzio and Schiavo 2010). BICD2 links the dynein-dynactin complex to cargos that are involved in different dynein-mediated processes such as retrograde COPI-independent Golgi transport, centrosome and nuclear positioning during mitotic entry, mRNA localization, lipid droplet transport, endocytosis, and microtubule organization (Figure 2).

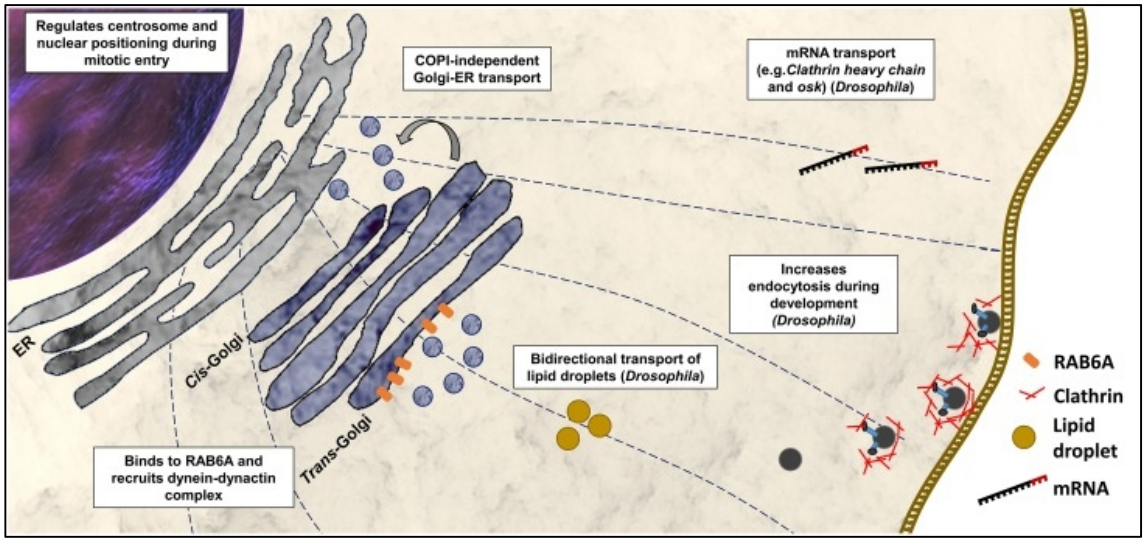

Figure 2. Functions of BICD2 in the cell.
Studies in mammals and/or *Drosophila melanogaster* have shown that BICD2 is involved in several cellular processes. Source: (Martinez-Carrera and Wirth 2015).

2.2.2. COPI/independent Golgi transport

The C-terminal domain of BICD2 binds to the small GTPase RAB6A, which coordinates the retrograde COPI-independent Golgi-ER pathway, a recycling route of Golgi-resident membrane proteins that allows the assembly of functional Golgi stacks (Hoogenraad et al. 2001, Young et al. 2005). The active form of RAB6A (GTP-bound) is associated with the *trans*-Golgi membrane and recruits BICD2 via interaction with the C-terminal domain. The N-terminal domain of BICD2 becomes available and recruits the dynein-dynactin complex to transport RAB6A vesicles from the *trans*-Golgi to the ER (Matanis et al. 2002).

BICD2 is considered a golgin due to its abundant coiled coil structure, localization at the Golgi, and interaction with a member of the RAB family of GTPases (Barr and Short 2003, Short et al. 2005, Goud and Gleeson 2010). Golgins are proteins associated with the Golgi apparatus and contribute to maintain its organization.

2.2.3. Centrosome and nuclear positioning during mitotic entry

In early G2 phase, the C-terminal domain of BICD2 switches from interacting with RAB6A to interact with RANBP2, a component of the nuclear pore (Splinter et al. 2010). BICD2 in turn, recruits the dynein-dynactin complex to the nuclear envelope to facilitate a proper positioning of the nucleus relative to centrosomes prior mitosis. However, the mechanism that controls the shift of interacting partners of BICD2 during the cell cycle is unknown.

2.2.4. mRNA localization

Studies in *Drosophil*a have shown that the C-terminal domain of BicD interacts with Egalitarian (Egl), a RNA-binding protein, whose human ortholog is EXD1 (exonuclease 3´-5´Domain Containing 1) (Bullock and Ish-Horowicz 2001, Delanoue and Davis 2005). BicD recruits the dynein-dynactin complex to target the mRNA cargo to distinct cellular compartments. This complex comprising BicD-Egl-dynein-dynactin is thought to associate with further proteins that regulate translation and stability of transported mRNA. During *Drosophila* development, the correct mRNA localization is crucial for specifying anteroposterior and dorsoventral axes of oocytes and embryos (Weil 2014). The identification of the mRNAs associated with BicD-Egl has been subject of investigation. *Clathrin heavy chain* (*Chc*) mRNA has been identified as a cargo of BicD. *Chc* mRNA requires BicD to be transported into the oocyte where is presumably needed to establish microtubule polarity, an endocytosis-independent role of *Chc* (Vazquez-Pianzola et al. 2014).

Even though, mRNA transport is an important process in mammals and BICD is highly conserved among metazoans, the role of BICD in mRNA transport during development in mammals has not been investigated. However, to study this possibility would be of special interest not only in the field of developmental science but also for the understanding of early developmental pathologies.

2.2.5. Lipid droplet transport

Lipid droplets are present at all developmental stages of *Drosophila* and are particularly important as energy sources (Kuhnlein 2012). It has been described that lipid droplets are moved by the dynein complex (Gross et al. 2000). Based on observations in the composition and motion of lipid droplets, it has been suggested that BicD binds to lipid droplets and recruits the dynein complex to transport those droplets in the forming embryo (Larsen et al. 2008).

In humans, increasing evidence suggests that disrupted lipid droplet function/localization may contribute to neurodegenerative disorders, for example Huntington's disease (Martinez-Vicente et al. 2010), Parkinson´s disease (Cole et al. 2002), and hereditary spastic paraplegias (Welte 2015). The role of BICD proteins in lipid droplet transport in mammals and defects in lipid metabolism has not been explored.

2.2.6. Bidirectional transport

A binding between BICD2 and the tail domain of kinesin-1 has been previously described (Grigoriev et al. 2007). However, this binding was suggested to be weaker than the association of BICD2 with dynein-dynactin complex. By using yeast two-hybrid system, the interaction between the middle part of BICD2 (Coiled coil 2 domain) and the tail of kinesin-1 (KIF5A) has been confirmed. However, this interaction seems to be strongly suppressed in the full-length BICD2, possibly by self inactivation due to interaction of the N-terminal domain of BICD2 (Coiled coil 1 domain) with its C-terminal domain (Coiled coil 3). Studies in *Drosophila* have suggested that BicD plays a role in plus end-directed microtubule transport, and in balancing plus end (kinesin mediated) versus minus end motion (dynein mediated) (Larsen et al. 2008). Recent studies have shown that dynein-dynactin complex and kinesin are opponents in a tug-war competition along microtubules, and BICD2 increases the force of dynein to successfully resist against kinesin (Belyy et al. 2016).

2.3. Importance of BICD2 in neuronal tissue development

2.3.1. Cerebellar development

In vivo studies in a homozygous *Bicd2* knockout mouse have provided further insights into BICD2 function (Jaarsma et al. 2014). *Bicd2*-deficient mice show cerebellar defects with severe hydrocephalus, and all died before postnatal day 30. Histological examination has revealed deficits in granule cell migration in the cerebellum. It is known that during cerebellar development, the granule cells (postmitotic neurons) migrate from the external granular layer (EGL) toward their final destination within the internal granular layer (IGL) (Figure 3) (Komuro and Yacubova 2003). The granule cells migrate along Bergmann glia cells, which determine their correct position from the molecular layer (ML) to the IGL (Komuro et al. 2001).

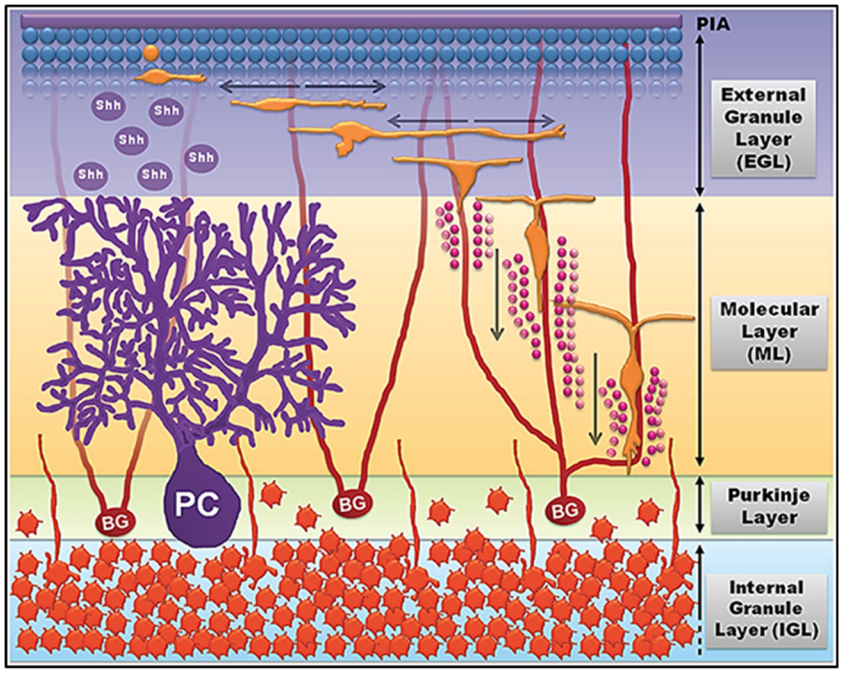

Figure 3. Cerebellar development and granule cell migration.
Precursor granule cells (blue spheres, top) proliferate in response to growth signals including sonic hedgehog (shh) released by Purkinje cells (PC, purple). Postmitotic granule cells (orange sphere) start to migrate and attach to Bergamnn glia cells (BG, red cells) to finally arrive at the internal granule layer. Source: (Tharmalingam and Hampson 2016).

Further histological studies have shown that BICD2 is highly expressed in Bergmann cells (Jaarsma et al. 2014). Moreover, conditional knockout mice, with *Bicd2* depleted in Bergmann cells, show decreased levels of Tenascin C, an extracellular matrix protein produced by Bergmann glia cells, possibly by defects in secretion due to the BICD2 deficiency. Tenascin C is known to mediate the attachment of neurons to Bergmann cells and stimulate migration (Bartsch et al. 1992, Chiquet-Ehrismann and Tucker 2011).

Importantly, besides the cerebellar defects, the homozygous *Bicd2* knockout mice do not show alterations in any tissue including spinal cord, and the heterozygous knockout mice do not display a pathological phenotype (Jaarsma et al. 2014). Individuals with SMALED2 do not show any pathological changes in the cerebellum. These differences in phenotypes between *Bicd2*-deficient mice and individuals with SMALED2 indicate that the missense BICD2 mutations exert gain-of-function effect rather than a loss-of-function effect.

2.3.2. Synaptic vesicle recycling

In *Drosophila*, the complete absence of BicD expression leads to decreased larval locomotion, and lethality is reported during pupa stage due to inability to eclose (Ran et al. 1994, Li et al. 2010). However, the transgenic expression of BicD in pan-neuronal tissue has been proven to fully rescue the larval locomotion and lethality, whereas

expression in the muscles does not (Li et al. 2010). These observations suggest that despite its widespread expression, BicD function is only obligatory in the nervous system during development.

Subsequent studies have shown that the C-terminal domain of BicD binds to Clathrin heavy chain (Chc), while the N-terminal domain recruits the dynein complex to transport clathrin-associated synaptic vesicles for their efficient recycling (Li et al. 2010). More studies seemed to be needed to investigate the extent of BicD participation in endocytosis and neuromuscular junction morphology.

3. Publications

The discovery of *BICD2* as the causing gene of SMALED2

Neveling, K.*, **Martinez-Carrera, L. A**.*, Holker, I., Heister, A., Verrips, A., Hosseini-Barkooie, S. M., Gilissen, C., Vermeer, S., Pennings, M., Meijer, R., Te Riele, M., Frijns, C. J., Suchowersky, O., Maclaren, L., Rudnik-Schoneborn, S., Sinke, R. J., Zerres, K., Lowry, R. B., Lemmink, H. H., Garbes, L., Veltman, J. A., Schelhaas, H. J., Scheffer, H. and Wirth, B. (2013). "Mutations in BICD2, which Encodes a Golgin and Important Motor Adaptor, Cause Congenital Autosomal-Dominant Spinal Muscular Atrophy." Am J Hum Genet 96(6): 946-954.
*These authors contributed equally to this work.

Synofzik, M., **Martinez-Carrera, L. A.**, Lindig, T., Schols, L. and Wirth, B. (2014). "Dominant spinal muscular atrophy due to BICD2: a novel mutation refines the phenotype." J Neurol Neurosurg Psychiatry 85(5): 590-592.

Martinez-Carrera, L. A. and Wirth, B. (2015). "Dominant spinal muscular atrophy is caused by mutations in BICD2, an important golgin protein." Front Neurosci 9: 401.

Functional analysis and disease model

Neveling, K.*, **Martinez-Carrera, L. A**.*, Holker, I., Heister, A., Verrips, A., Hosseini-Barkooie, S. M., Gilissen, C., Vermeer, S., Pennings, M., Meijer, R., Te Riele, M., Frijns, C. J., Suchowersky, O., Maclaren, L., Rudnik-Schoneborn, S., Sinke, R. J., Zerres, K., Lowry, R. B., Lemmink, H. H., Garbes, L., Veltman, J. A., Schelhaas, H. J., Scheffer, H. and Wirth, B. (2013). "Mutations in BICD2, which Encodes a Golgin and Important Motor Adaptor, Cause Congenital Autosomal-Dominant Spinal Muscular Atrophy." Am J Hum Genet 96(6): 946-954.
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Martinez Carrera, L. A., Gabriel, E., Donohoe, C., Hölker, I., Wason, A., Storbeck, M., Uhlirova, M., Gopalakrishnan, J. and Wirth, B. (2018). "Novel insights into SMALED2: BICD2 mutations increase microtubule stability and cause defects in axonal and NMJ development." Hum Mol Genet (Epub ahead of print).

Other disorders caused by variants in *BICD2*

Unger, A., Dekomien, G., Guttsches, A., Dreps, T., Kley, R., Tegenthoff, M., Ferbert, A., Weis, J., Heyer, C., Linke, W. A., **Martinez-Carrera, L.A.**, Storbeck, M., Wirth, B., Hoffjan, S. and Vorgerd, M. (2016). "Expanding the phenotype of BICD2 mutations toward skeletal muscle involvement." Neurology 87(21): 2235-2243.

Storbeck, M., Eriksen, B., Unger, A., Hölker, I., Aukrust, I., **Martinez-Carrera, L.A.**, Linke, W. A., Ferbert, A., Heller, R., Vorgerd, M., Houge, G. and Wirth, B. (2017). "Phenotypic extremes of BICD2-opathies: from lethal, congenital muscular atrophy with arthrogryposis to asymptomatic with subclinical features." Eur J Hum Genet 25(9): 1040-1048.

3.1. The discovery of *BICD2* as the causing gene of SMALED2

3.1.1. Publications

Neveling, K.*, **Martinez-Carrera, L. A**.*, Holker, I., Heister, A., Verrips, A., Hosseini-Barkooie, S. M., Gilissen, C., Vermeer, S., Pennings, M., Meijer, R., Te Riele, M., Frijns, C. J., Suchowersky, O., Maclaren, L., Rudnik-Schoneborn, S., Sinke, R. J., Zerres, K., Lowry, R. B., Lemmink, H. H., Garbes, L., Veltman, J. A., Schelhaas, H. J., Scheffer, H. and Wirth, B. (2013). "Mutations in BICD2, which Encodes a Golgin and Important Motor Adaptor, Cause Congenital Autosomal-Dominant Spinal Muscular Atrophy." Am J Hum Genet 96(6): 946-954.

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Synofzik, M., **Martinez-Carrera, L. A.**, Lindig, T., Schols, L. and Wirth, B. (2014). "Dominant spinal muscular atrophy due to BICD2: a novel mutation refines the phenotype." J Neurol Neurosurg Psychiatry 85(5): 590-592.

Martinez-Carrera, L. A. and Wirth, B. (2015). "Dominant spinal muscular atrophy is caused by mutations in BICD2, an important golgin protein." Front Neurosci 9: 401.

3.1.2. Description

Almost 20 years after Frijns and colleagues described family 1, and thanks to the advances in next generation sequencing, we were able to identify the genetic cause of the dominant non-5q SMA in family 1 (Neveling; Martinez-Carrera et al. 2013). To identify the chromosomal location of the disease-causing mutations, linkage analysis was performed in nine members of family 1, which identified a locus on chromosome 9 (chr9: 94,4440,951-104,432,543). Exome sequencing was performed in a single affected individual, and variants were filtered based on location on chromosome 9, nonsynonymous exonic and splice-sites. After excluding known SNPs and previously identified variants, just five variants in five different genes were found. Of those five genes, only *BICD2* was located within the linkage region. The heterozygous variant in *BICD2* (c.320C>T) results in the amino acid change p.Ser107Leu (Neveling; Martinez-Carrera et al. 2013).

To search for further *BICD2* variants that might cause dominant SMA, the seven exons of *BICD2* were analyzed by Sanger sequencing in twenty-three additional families with dominant non-5q-SMA. Further two heterozygous variants (c.563A>C [p.Asn188Thr] and c.2108C>T [p.Thr703Met]) were identified (Neveling; Martinez-Carrera et al. 2013). A rare SNV (c.269A>G [p.Lys90Arg]) was identified in two additional families. However, some affected individuals in these families did not carry this SNV, which discards this variant as disease-causing.

A fourth heterozygous variant (c.2239C>T [p.Arg747Cys]) was identified in all the affected individuals with dominant SMA, in a three-generation family (Synofzik et al. 2014).

Affected individuals with dominant SMA that carry *BICD2* variants, present weakness and atrophy predominantly affecting the proximal and distal muscles of the lower extremities. For this reason, the pathology was named spinal muscular atrophy, lower extremity predominant, autosomal dominant, type 2 (SMALED2 MIM 615290).

The symptoms are congenital or appear in early childhood, but few cases with late onset have been also described. The course of the disease is slowly progressive or nonprogressive. Tendon reflexes are reduced or absent. In the majority of the cases, the SMALED2 phenotype is very mild, and during clinical examination may be first-glance diagnosed as SMA type IV, the mildest form of the classical 5q-SMA. Delayed motor milestones, waddling gait, and chronic walking on toes are commonly reported. It seems very characteristic that many individuals with SMALED2 show evident wasting of the lower limbs and a very broad upper body, which resembles a bodybuilder-like shape. Another differential feature in many individuals with SMALED2 is the presence of contractures in knee, feet, and/or hip, which correlates with congenital onset, possibly due to decreased intrauterine movements (Neveling; Martinez-Carrera et al. 2013). In contrast, very few individuals with SMALED1 (Harms et al. 2010, Harms et al. 2012) and only very severe 5q-SMA cases present contractures (Rudnik-Schoneborn et al. 2008).

In this Section, I include a review about all the individuals with SMALED2 that have been reported (Martinez-Carrera and Wirth 2015). This review provides a summary of the clinical presentation including age of onset, presence of contractures, and pattern of muscular weakness/atrophy. Importantly, we show an overview about the location and possible molecular mechanism of the different mutations.

3.1.3. Own contributions

My contributions to (Neveling; Martinez-Carrera et al. 2013) are split into two parts. Here I include the part of the gene discovery and Section 3.2 includes the part of functional analysis for this paper.

In the gene discovery part of the study (Neveling; Martinez-Carrera et al. 2013), I designed, carried out and analyzed the exon sequencing of *BICD2* in all twenty three additional families. Subsequently to the variant identification, I performed the corresponding segregation analysis. For the manuscript, I contributed with the design of the figure 1, writing and reviewing.

In (Synofzik et al. 2014), I performed the Sanger sequencing, identified the variant in *BICD2*, and continued with segregation analysis. For the publication, I contributed with the preparation of the figure, writing my corresponding part and reviewing the draft.

I wrote the review (Martinez-Carrera and Wirth 2015).

3.2. Functional analyses and disease model

3.2.1. Publications

Neveling, K.*, **Martinez-Carrera, L. A**.*, Holker, I., Heister, A., Verrips, A., Hosseini-Barkooie, S. M., Gilissen, C., Vermeer, S., Pennings, M., Meijer, R., Te Riele, M., Frijns, C. J., Suchowersky, O., Maclaren, L., Rudnik-Schoneborn, S., Sinke, R. J., Zerres, K., Lowry, R. B., Lemmink, H. H., Garbes, L., Veltman, J. A., Schelhaas, H. J., Scheffer, H. and Wirth, B. (2013). "Mutations in BICD2, which Encodes a Golgin and Important Motor Adaptor, Cause Congenital Autosomal-Dominant Spinal Muscular Atrophy." Am J Hum Genet 96(6): 946-954.

*These authors contributed equally to this work.

Martinez Carrera, L. A., Gabriel, E., Donohoe, C., Hölker, I., Wason, A., Storbeck, M., Uhlirova, M., Gopalakrishnan, J. and Wirth, B. (2018). "Novel insights into SMALED2: BICD2 mutations increase microtubule stability and cause defects in axonal and NMJ development." Hum Mol Genet (Epub ahead of print).

3.2.2. Description of the studies

We proceeded to examine the effects of the four mutations described by us, on cellular processes involving BICD2 and that have been previously described as affected in neurodegenerative disorders, such as Golgi structure and interactions with dynein-dynactin complex and RAB6A.

In vitro overexpression experiments and studies of fibroblasts derived from individuals with SMALED2 showed that p.Thr703Met and p.Asn188Thr mutations cause Golgi fragmentation (Neveling; Martinez-Carrera et al. 2013).

Interaction studies revealed that two mutations change the binding of BICD2 with the dynein-dynactin complex. The p.Asn188Thr mutation slightly decreased the binding to dynein. The p.Arg747Cys mutation increases the interaction of BICD2 with the p150 subunit of dynactin and decreases the interaction with RAB6A (Martinez-Carrera et al. 2018).

We focused on the observations that some of the BICD2 mutations alter interactions with the dynein-dynactin complex and/or cause Golgi fragmentation. We investigated whether the BICD2 mutations impair microtubule organization. The fibroblast derived from individuals with SMALED2 exhibit longer and hyperstable microtubules, in comparison with controls. This effect was observed regardless of where the mutation is located, which may constitute a common cellular mechanism affecting microtubules (Martinez-Carrera et al. 2018).

The next step in our investigation was to determine the effect of BICD2 mutations on motor neurons, the disease relevant cell type in SMALED2. Motor neurons overexpressing BICD2 mutations developed axonal aberrations such as collateral branching and overgrowth, possibly by alterations in the microtubule array (arrangement) (Martinez-Carrera et al. 2018).

To study the *in vivo* consequences of BICD2 mutations, we generated a *Drosophila* model for SMALED2. The specific expression of BICD2 mutations in neuronal tissue, but not in muscle, led to impaired locomotion and reduced neuromuscular junction size (Martinez-Carrera et al. 2018).

3.2.3. Own contributions

In the functional analysis part of (Neveling; Martinez-Carrera et al. 2013), I performed the overexpression studies carrying out the site directed mutagenesis for each of the constructs used, high scale plasmid preparation of the constructs, transfection, imaging, analysis, and interpretation of the results. I conducted the expression analysis and Golgi studies in primary fibroblast cells derived from individuals with SMALED2. I wrote the corresponding part of functional analysis in the manuscript, and prepared the figures 3, 4 and S1.

In (Martinez-Carrera et al. 2018), I conducted the microtubule experiments in fibroblast cells, imaged them, analyzed and interpreted results. I cloned, purified and high scale produced the constructs for lentiviral transduction, isolated motor neurons and cultured them. I stained the transduced motor neurons and imaged them using confocal microscopy. I performed the quantification analyses, statistics, and interpretation. Regarding the part of *Drosophila melanogaster*, I designed and established the construct to be used for the generation of the transgenic flies, performed each cross to generate the SMALED2 flies and controls, validated them by PCR and western blot, carried out the locomotion tests. I dissected the NMJs from the larvae, stained, and imaged them using confocal microscope. I analyzed the data, applied statistics, and interpreted the

results. Regarding the interaction studies, I performed and analyzed all the experiments. For the expression analysis of BICD2 in murine tissues, I dissected the tissues, statistically analyzed and interpreted. I wrote the manuscript and prepared the figures 1, 2, 3, 4, 5, S1, S2, S3, S4, S5, S6, S7, S8, S9.

3.3. Other disorders caused by variants in *BICD2*

3.3.1. Publications

Unger, A., Dekomien, G., Guttsches, A., Dreps, T., Kley, R., Tegenthoff, M., Ferbert, A., Weis, J., Heyer, C., Linke, W. A., **Martinez-Carrera, L.A.**, Storbeck, M., Wirth, B., Hoffjan, S. and Vorgerd, M. (2016). "Expanding the phenotype of BICD2 mutations toward skeletal muscle involvement." Neurology 87(21): 2235-2243.

Storbeck, M., Eriksen, B., Unger, A., Hölker, I., Aukrust, I., **Martinez-Carrera, L.A.**, Linke, W. A., Ferbert, A., Heller, R., Vorgerd, M., Houge, G. and Wirth, B. (2017). "Phenotypic extremes of BICD2-opathies: from lethal, congenital muscular atrophy with arthrogryposis to asymptomatic with subclinical features." Eur J Hum Genet 25(9): 1040-1048

3.3.2. Description of the studies

3.3.2.1. Chronic myopathy

The p.Ser107Leu and p.Thr703Met mutations that we previously described in individuals with SMALED2, were also identified in two families where affected individuals present muscle weakness due to chronic myopathic alterations of skeletal muscle fibers with minor neurogenic changes (Unger et al. 2016). Muscle studies in affected individuals showed impairment of Golgi integrity, increased Golgi-derived vesicles in muscle fibers, and abnormal BICD2 localization. Two proteins, dysferlin and caveolin-3, were reported as strongly reduced at the sarcolemma (cell membrane of muscle fiber cell) in almost all muscle fibers. It is known that vesicular dysferlin is transported via kinesin in muscle cells and is important for muscle membrane repair, and caveolin-3 has been implicated in the trafficking of dysferlin to the plasma membrane (Cai et al. 2009, McDade and Michele 2014). Even though, the role of BICD2 in muscle has not been explored, it has been speculated that BICD2 mutations affect the transport of exocytic cargoes containing dysferlin and caveolin-3 leading to impaired muscle integrity (Unger et al. 2016).

Interestingly, segregation analysis of the individual with chronic myopathy that carries the p.Thr703Met showed that his unaffected mother carries the same heterozygous mutation. The mutant allele was confirmed to be expressed in the unaffected mother (Storbeck et al. 2017). The clinical features in SMALED2 are known to be variable, for example the presence of contractures and the onset of disease even in individuals harbouring the same mutation (Martinez-Carrera and Wirth 2015). However, it was not reported before an asymptomatic carrier, which suggest the existence of modifying elements (Storbeck et al. 2017).

3.3.2.2. Congenital arthrogryposis multiplex, respiratory failure and early lethality

This study identified three variants in *BICD2* (c.581A>G [p.Gln194Arg], c.1626C>G [p.Cys542Trp] and c.2080C>T [p.Arg694Cys]), in cases with an extremely severe form of congenital muscular atrophy with multiple joint contractures (arthrogryposis) affecting the whole body, respiratory insufficiency and death within four months after birth (Storbeck et al. 2017). Reduced fetal movement was reported. Two of the three mutations were confirmed to be *de novo*.

The variant c.581A>G (p.Arg694Cys) has been previously described in two additional families (Ravenscroft et al. 2016). In both cases the variant arose *de novo*, which suggested this is a recurrent *de novo* variation in *BICD2*. Affected individuals presented similar phenotypes as described by (Storbeck et al. 2017), but in addition, brain alterations including microcephaly and bilateral perisylvian polymicrogyria (Ravenscroft et al. 2016).

Muscle biopsy in individuals with congenital arthrogryposis multiplex revealed few muscle fibers, atrophy, and in some very little muscle tissue comprising mostly fat (Ravenscroft et al. 2016, Storbeck et al. 2017). Electromyography showed in two cases neurogenic pathology with signs of denervation (Storbeck et al. 2017).

Importantly, none of these three arthrogryposis multiplex-associated mutations has been found in individuals with SMALED2. In some cases of SMALED2 congenital contractures have been reported. However, the multiple contractures and the lethality in these severe cases, have never been seen before in typical cases of SMALED2.

Our study suggests that these mutations may have a more deleterious effect on BICD2 function, with an earlier *in utero* onset leading to reduced fetal movement and muscle shortening (contracture). However, the exact mechanism how these mutations affect so dramatically the early development of neurons and/or muscles is unknown.

These differences in the spectrum of disorders associated to BICD2 mutations, open the possibility to investigate further pathological consequences and perhaps to elucidate novel functions of BICD2.

3.3.3. Own contributions

In the paper about chronic myopathy (Unger et al. 2016), I contributed with the constructs used in the overexpression experiments performed in muscle. I contributed with the preparation and revision of the manuscript.

My contribution in (Storbeck et al. 2017) was to design and validate the PCR primers for RNA expression analysis. I provided the protocol and advised about the *BICD2* sequencing. For the manuscript, I prepared the table 1 to summarize the phenotypical features, and contributed with the writing and revision of the draft.

4. Unpublished findings

4.1. Impact of BICD2 mutations on endocytosis

The BICD2 disease-causing mutations, p.Thr703Met (Neveling; Martinez-Carrera et al. 2013) and p.Arg747Cys (Synofzik et al. 2014), localize to the C-terminal end of the BICD2 protein, which has been described in *Drosophila melanogaster* to interact with clathrin heavy chain (Li et al. 2010). The function of clathrin is important for the process of endocytosis and defects in endocytosis have emerged as a central pathological hallmark of autosomal recessive SMA (Hosseinibarkooie et al. 2016, Riessland et al. 2017). To test whether these BICD2 mutations have a direct impact on endocytosis, the cellular uptake of Fluorescein isothiocyanate (FITC)-labelled dextran was assayed in fibroblasts derived from affected individuals carrying the p.Thr703Met and p.Arg747Cys mutations. Fibroblasts from control individual and affected individuals were serum starved and subsequently treated with FITC-dextran for 15, 30, or 60 minutes (Figure 4A and 4B). FITC-dextran uptake was measured microscopically. The experiment was performed in triplicate.

Fibroblasts harboring the p.Thr703Met mutation displayed a significantly reduced FITC-dextran uptake at all assayed time points (Figure 4C). Even though mutant cells maintained dextran uptake over time, fluorescence levels were constantly lower as compared to control fibroblasts. This may indicate diminished endocytosis caused by this mutant BICD2 protein. However, cells carrying the p.Arg747Cys mutation showed only minor deviations from control fibroblasts (Figure 4D). This suggests that this mutation likely does not have any impact on the process of endocytosis in fibroblasts. Thus, this assay pointed out possible alterations in endocytosis caused by p.Thr703Met. However, FITC-dextran is in addition taken up by cells via pathways other than clathrin-mediated endocytosis, for example fluid-phase endocytosis (Pustylnikov et al. 2014).

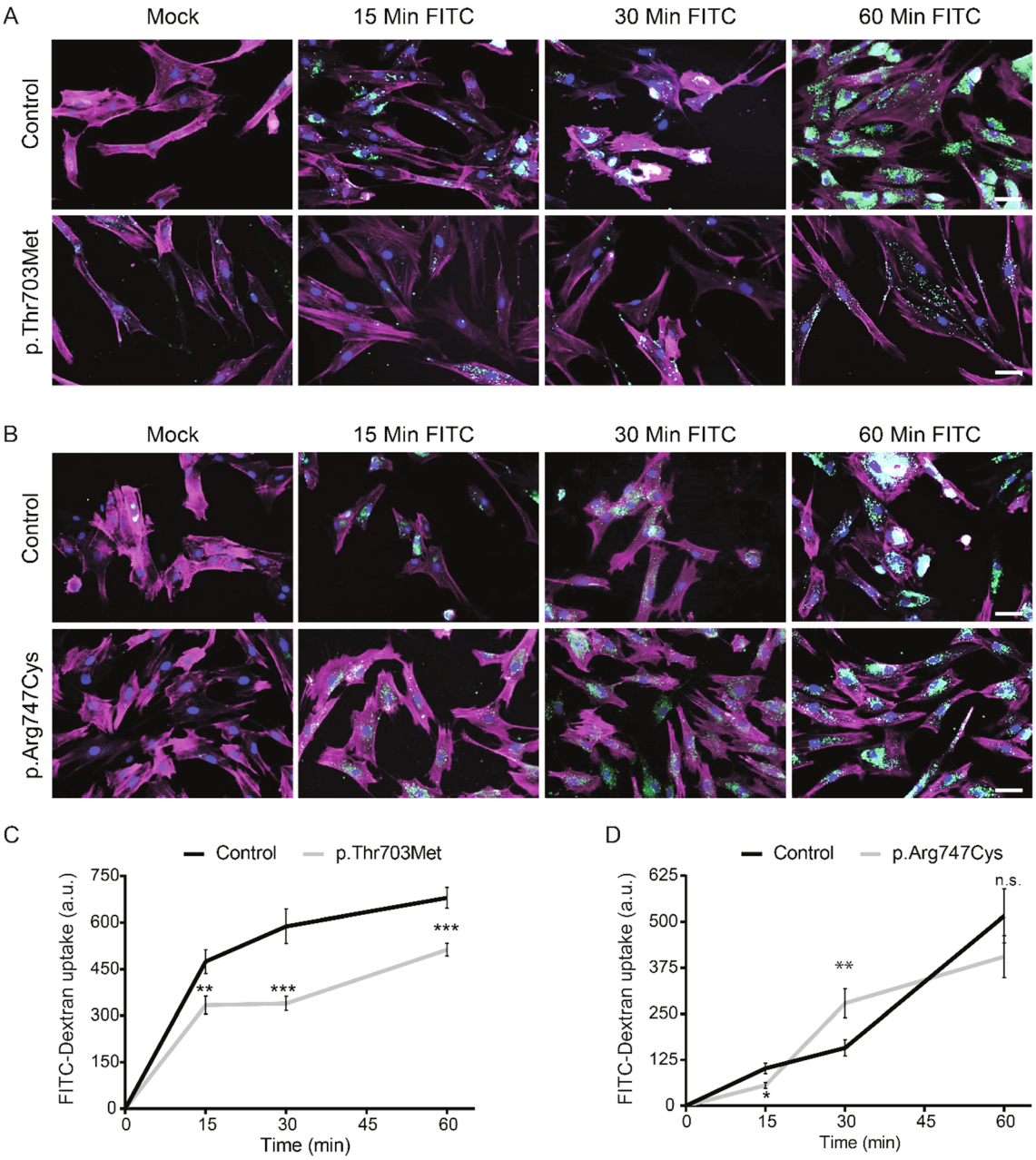

Figure 4. FITC-Dextran uptake in fibroblasts derived from affected individuals carrying the p.Thr703Met and p.Arg747Cys mutations in BICD2.
Fibroblasts were serum starved and treated with FITC-dextran for 15, 30 and 60 minutes. FITC-dextran uptake was analyzed microscopically. (A) and (B): Representative images of control and mutant fibroblasts at the indicated time points. Red channel: Phalloidin-AlexaFluor568; blue channel: DAPI; green channel: FITC-dextran. Scale bar corresponds to 50 µm. (C) and (D): Quantification of fluorescence intensity of FITC-dextran. The experiments were performed in triplicate. 50 cells were counted per time point. Error bars correspond to mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 in unpaired two-tailed *t*-test.

4.2. Impact of p.Thr703Met mutation on centrosomes and cell cycle

Previous analyses have described a G2-phase-specific role of BICD2 in the regulation of centrosome positioning prior mitosis (Splinter et al. 2010). To initiate mitosis, centrosomes split at the end of G2-phase to later allow spindle formation (Wang et al. 2014) (Figure 5A, left panel). The fibroblasts derived from an individual with SMALED2 that carries the p.Thr703Met mutation showed particularly slow proliferation rates in cell culture as compared to control and other BICD2 mutant cells. Mutant and control cells were initially synchronized to G0 phase by serum starvation for 48 hours (Figure 5A, right panel). Cell cycle was resumed by addition of serum. After 1 h of serum addition, the cells are expected to be in G1/S phase transition and at 24 h to complete the cell cycle (Gabriel et al. 2016). At the indicated time points, the cells were fixed and centrosomes were immunostained using an antibody against γ-tubulin and confocal imaged (Figure 5B). Joined centrosomes are visible as two nearby localized dots close to the nucleus. Split centrosome are visible as apart dots.

Quantification of mutant vs. control cells showed only a slight increase of mutant cells with split centrosomes directly after serum starvation and 1 hour after re-entry of cell cycle (Figure 5C). Strikingly, almost 60 % of fibroblasts harboring the p.Thr703Met mutation showed split centrosomes 24 hours after cycle re-entry, while only single control cells displayed this phenomenon. This strongly suggests that almost all control cells have completed cell cycle after 24 hours, but a large fraction of mutant cells failed to enter mitosis. Evaluating the sole centrosome status without considering other indicators, implies that many mutant cells are retained in G2-phase. The slow proliferation might be attributable to aberrant cell cycle progression and/or pre-mitotic cell cycle arrest.

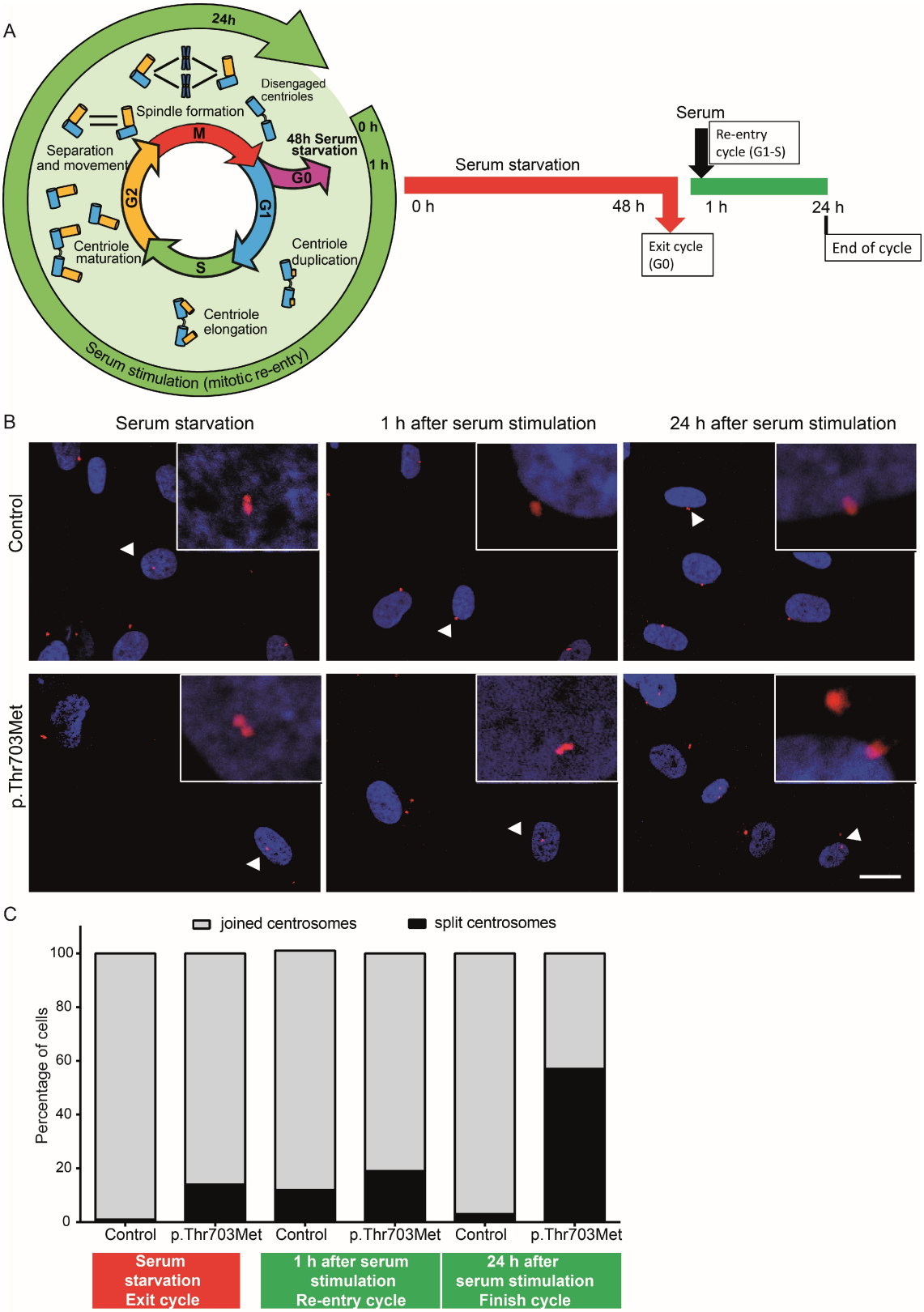

Figure 5. Centrosome splitting in primary fibroblasts carrying the p.Thr703Met mutation.
(A) Cell cycle and centrosome status. Left: scheme was modified based on the review by Wang et al. (Wang et al. 2014). Right: experimental plan. (B) Representative confocal images of synchronized control fibroblasts and fibroblasts harboring the p.Thr703Met mutation at 0, 1, and 24 hours after cell cycle re-entry. Red channel: γ-tubulin (centrosomes); blue channel: DAPI (nuclei). Arrowheads point to areas that are magnified in the squares. Scale bar corresponds to 20 µm. (C) Quantification of cells with joined and split centrosomes (N= 150). The experiment was performed once.

4.3. Implications of p.Arg747Cys mutation in protein structure and aggregation

To functionally characterize the mutation p.Arg747Cys, the BICD2-tGFP construct carrying the respective variant was generated as described in Section 4.5. HeLa cells were transfected with wild type BICD2-tGFP and the mutant p.Arg747Cys construct using lipofectamine. The cells transfected with the mutant, but not wild type construct, showed cytoplasmic protein accumulations (Figure 6A). This phenomenon was observed only for this particular mutant BICD2 construct and protein accumulation has never been observed in cells transfected with other BICD2-tGFP constructs (Neveling; Martinez-Carrera et al. 2013). To test whether these accumulations comprised insoluble and aggregated protein, cell lysates of transfected HEK293T cells were fractionated into soluble and insoluble fraction and analyzed by western blotting (Figure 6B). BICD2 was normalized to β-actin and the soluble/insoluble ratio was calculated as the mean of three independent transfection experiments. The BICD2-tGFP construct harboring the p.Arg747Cys mutation significantly displayed a 1.8-fold decrease of soluble/insoluble ratio as compared to wild type BICD2-tGFP. This suggests that BICD2 p.Arg747Cys has a propensity to become insoluble, which might be due to aberrant protein conformation.

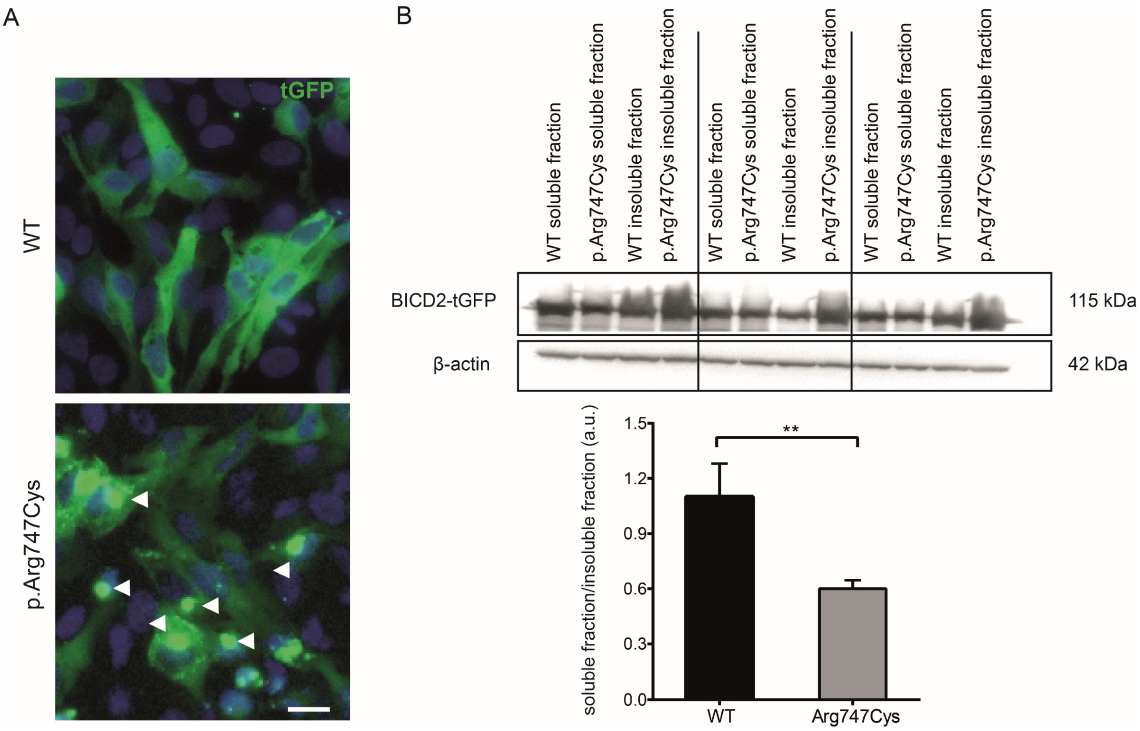

Figure 6. The BICD2 p.Arg747Cys mutation forms insoluble aggregates.
(A) Representative images of HeLa cells after 48h of transfection with p.CMV-BICD2-tGFP constructs (wild type and mutant p.Arg747Cys). Note that the mutant p.Arg747Cys forms aggregates, which are indicated by arrows. (B) Upper panel: western blot of soluble and insoluble fractions from lysate of HEK293T cells transfected with control and mutant constructs. For BICD2-tGFP detection, antibody against BICD2 was used. All BICD2 bands were normalized to β-actin expression. Lower panel: western blot quantification. The ratio of soluble/insoluble BICD2 was calculated for each transfection experiment. Bars represent the mean ratios of 3 transfection experiments. Error bars denote standard deviation. ** $p<0.01$ in unpaired two-tailed *t*-test.

4.4. Summary of results obtained from functional characterization of BICD2 mutations

Table 2. Overview of functionally assessed consequences of disease-causing BICD2 mutations.

Mutation	p.Ser107Leu	p.Asn188Thr	p.Thr703Met	p.Arg747Cys
Interaction studies	no alterations	↓ Dynein intermediate chain (DIC)	no alterations	↑ Dynactin p150 ↓ RAB6A
Overexpression in HeLa cells	Golgi fragmentation	Golgi fragmentation	Golgi fragmentation	Insoluble aggregates
Fibroblast cells	no alterations	slight Golgi fragmentation	severe Golgi fragmentation, centrosome splitting, G2-arrest, ↓ endocytosis	no alterations
Microtubule (MT) dynamics (fibroblasts)	↑ MT stability	↑ MT stability	↑ MT stability	↑ MT stability
MN transduction	↑ axonal branching	↑ axonal branching	↑ axonal branching, extreme long axons	↑ axonal branching, extreme long axons, altered localization (at growth cones, concentrates dynactin)
Fly model system	NMJ size ↓ Locomotion ↓	N/A	NMJ size ↓ Locomotion ↓	N/A

4.5. Methods (unpublished data)

4.5.1. Eukaryotic cell culture

Primary human fibroblasts, HEK293T (human embryonic kidney), and HeLa (cervical cancer) cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) complemented with 10 % fetal bovine serum, penicillin, streptomycin and amphotericin B. Cells were maintained in 25 cm² or 75 cm² tissue culture flasks under sterile conditions at 37°C, 5 % CO2 and 95 % relative humidity. When reaching confluence, cells were split using Trypsin-EDTA to detach cells from growing surface.

4.5.2. Endocytosis assay using FITC-dextran

To assess possible impact of mutated BICD2 protein on endocytosis, FITC-Dextran uptake (fluorescein isothiocyanate coupled to Dextran) was assayed in primary fibroblasts, as previously described (Hosseinibarkooie et al. 2016). In brief, prior to treatment, cells were starved for 10 minutes in starvation media (transparent DMEM with 2 % fetal bovine serum). Cells were incubated with starvation medium containing 5 mg/ml FITC-Dextran (Sigma-Aldrich 46945) for 15, 30 and 60 minutes, respectively. Untreated cells after starvation were used for fluorescence background subtraction. Subsequently, cells were washed in ice cold PBS and fixed in PBS containing 4% PFA for 10 minutes. After washing in PBS, cells were immunostained with AlexaFluor568-phalloidin (1:100, Life Technologies A12380) and DAPI (1 µg/ml, Sigma Aldrich D9542), followed by 3 washes with PBS for 15 min. Cells were rinsed with water and mounted using Mowiol. The FITC-Dextran uptake (green channel intensity) was microscopically analysed using a Zeiss microscope (Axio Imager.M2).

4.5.3. Analysis of centrosomes in primary fibroblasts

Primary fibroblasts were initially synchronized by serum starvation as previously described (Gabriel et al. 2016). Upon reaching desired confluency, cells were incubated in DMEM containing 0.5% fetal bovine serum for 48 hours. Starvation media was replaced by DMEM containing 10 % fetal bovine serum and cells were incubated for 24 hours. Cells were washed in warm serum-free medium and fixed for 15 minutes in warm PBS containing 4% PFA. After removal of fixation solution, cells were washed three times for 3 minutes with PBS without $MgCl_2$ containing 30 mM glycine. Cells were permeabilized for 10 minutes at room temperature in PBS containing 0.5 % Triton X 100 and repeatedly washed three times for three minutes in PBS/glycine. Blocking was carried out for 2 hours at room temperature or overnight at 4°C in PBS containing 0.5 % fish-gelatin. Subsequently, cells were incubated with primary antibody (mouse anti

γ-tubulin 1:500, Sigma-Aldrich T6557) in blocking solution at 4°C overnight followed by 2x3 minute washing steps with blocking solution. Incubation with secondary antibody (Alexa Fluor 568 donkey anti-mouse 1:1000, Life Technologies A10037) and DAPI (1 µg/ml, Sigma Aldrich D9542) was performed for 1 h at room temperature, followed by 2x3 minute washing steps and rising with water prior mounting with Mowiol.

Confocal images were acquired using an Olympus Fluoview FV 1000 scanning confocal microscope and processed using Adobe Photoshop.

4.5.4. Generation of a mutant BICD2 p.Arg747Cys expression vector

A pCMV6 vector containing the wildtype *BICD2* cDNA was acquired from Origene (Cat. No. RG209960). The vector encodes human wild type BICD2 protein that is C-terminally tagged with turbo-GFP (tGFP). The BICD2 variant c.2239C>T (p.Arg747Cys) was introduced by site-directed mutagenesis using the Agilent QuikChange II XL kit (200521) according to the manufacturer's instructions. Oligonucleotides for mutagenesis were 5'-CACCTTCTCCTCGCTGTGTGCTATGTTTGCCAC-3' (sense) and 5'-GTGGCAAACATAGCACACAGCGAGGAGAAGGTG-3' (antisense). The cDNA sequence obtained upon site-directed mutagenesis was validated by Sanger sequencing of the insert. In preparation for transfection experiments, recombinant plasmids were produced in large scale and isolated using the EndoFree Plasmid Maxi Kit (Qiagen 12362) according to the manufacturer's instructions.

4.5.5. Solubility assay for mutant BICD2 protein

To express mutant BICD2 protein, HEK293T cells were transiently transfected with BICD2 expression vectors (wild type and harboring the p.Arg747Cys mutation). Cells were transfected in 6-well plates at a confluence of approx. 80% using the Lipofectamine2000 transfection reagent (Life Technologies 11668019). 3 µg of plasmid DNA and 10 µl of transfection reagent were each mixed with 250 µl of OptiMEM transfection medium according to the manufacturer's instructions. Cells were cultivated for 48 hours after transfection to allow expression of recombinant protein. Cells were washed in PBS and centrifuged at 4°C, 2000 x g for 15 seconds. The cell pellet was resuspended in 100 µl PBS containing protease inhibitors, sonicated 3 times for 30 seconds and incubated for 10 minutes on ice. The lysate was centrifuged for 20 minutes at 16,000 x g and 4°C to sediment insoluble debris. The supernatant was collected as the soluble fraction. The pellet was resuspended in RIPA buffer complemented with 2% SDS, 2 mM DTT and protease inhibitors. The lysate was incubated at room temperature and vortexed 3 times in a 15 minute interval. The sample was centrifuged at 16,000 x g to sediment insoluble debris. The supernatant was collected as the insoluble fraction of

proteins. Protein fractions were analyzed by SDS-PAGE with subsequent western blotting. Briefly, proteins were resolved in 12% acrylamide gels and transferred to PVDF membranes that were previously activated with methanol. Blocking was performed using TBS 6% milk powder for 2 h at room temperature. The membrane was then incubated at 4°C overnight with the primary antibody rabbit anti BICD2 (1:1000, Sigma-Aldrich HPA023013) and 1 h at room temperature with mouse anti β-actin (1:20,000, Proteintech 60008). After washing 5 times for 5 minutes with TBS-tween, the HRP-conjugated secondary antibodies against mouse IgG (1:10,000, Dianova 115035000) and against rabbit IgG (1:5,000, Cell Signaling 7074) were added and incubated for 1 h at room temperature. The blot was washed 5 times for 5 minutes with TBS-tween. For band detection, Super Signal West Pico Chemiluminescent substrates (Thermo Scientific 34078) were used following the manufacturer´s instructions.

4.5.6. Statistical analyses

Unpaired two-tailed ***t***-test was calculated using GraphPad Prism version 6.0d.

5. Discussion

Autosomal dominant spinal muscular atrophies are considered highly heterogeneous due to the variability in clinical presentation and diversity of causing genes.

In a combinatorial strategy of using next-generation sequencing (NGS) technologies and Sanger sequencing, the data presented in this PhD thesis identified heterozygous missense variants in *BICD2* in affected individuals with spinal muscular atrophy, lower extremity predominant, autosomal dominant inherited (SMALED). The identification of *BICD2* as SMALED-causing gene, allowed the characterization of the second type of SMALED (SMALED2 MIM 615290).

Simultaneously to us, other two research groups described variants in *BICD2* as the genetic cause of SMALED2 (Oates et al. 2013, Peeters et al. 2013). Almost 50 cases with SMALED2 have been reported to be caused by variants in *BICD2* (Martinez-Carrera and Wirth 2015). This number of cases is expected to increase overtime, thanks to the inclusion of *BICD2* in several gene panels, which are being more widely and frequently used in genetic diagnostics.

In general, the clinical presentation of SMALED2 is mild. However, the onset and presence of contractures are variable in affected individuals. Of the four variants identified in this work, two variants (c.320C>T [p.Ser107Leu] and c.2108C>T [p.Thr703Met]) were found in cases with congenital onset and feet contractures (Neveling; Martinez-Carrera et al. 2013). The other two variants (c.563A>C [p.Asn188Thr] and c.2239C>T [p.Arg747Cys]) were identified in individuals with a late onset in adulthood and without contractures, reasons why these variants are thought to be the mildest (Neveling; Martinez-Carrera et al. 2013, Synofzik et al. 2014).

This doctoral thesis further focused on the uncovering and understanding of the pathological consequences of the BICD2 mutations mentioned above. The functional analysis of the BICD2 mutations showed alterations in different processes, where BICD2 has an important function.

5.1. Alterations exerted by mutations in BICD2

c.320C>T (p.Ser107Leu)

The variant c.320C>T has been frequently described (in 29 of 49 cases) and is considered a hotspot mutation (Rossor et al. 2015). This variant is located within a cytosine-guanine (CpG) dinucleotide. Cytosine is subject of methylation nevertheless, spontaneous deamination of 5-methylcytosine may occur, yielding thymine instead (Shen et al. 1994).

Previously, this variant was reported to increase the interaction with dynein (Oates et al. 2013, Peeters et al. 2013). However, our interacting studies showed no difference in interaction in comparison with wild type (Martinez-Carrera et al. 2018). The discrepancies might be due to differences in cell types used for the co-immunoprecipitation or due to differences in interpretation/analysis of the band intensities of immunoprecipitants and inputs.

Golgi fragmentation was observed upon overexpression of this mutant in HeLa cells (Neveling; Martinez-Carrera et al. 2013). This observation was also reported by another group (Peeters et al. 2013). Golgi fragmentation has been associated with inhibition of dynein function (Burkhardt et al. 1997, Harada et al. 1998, Quintyne et al. 1999). It is possible that even when the interaction between BICD2 and dynein is not altered, the motility and/or function of dynein can be affected. However, to explore this possibility, the analysis of function and activity of dynein-dynactin complex is necessary.

c.563A>C (p.Asn188Thr)

This variant decreases the interaction of BICD2 with the intermediate chain of dynein (Martinez-Carrera et al. 2018). Upon overexpression of this mutation, HeLa cells showed Golgi fragmentation which was also observed in fibroblast derived from individuals with SMALED2 carrying this variant (Neveling; Martinez-Carrera et al. 2013).

c.2108C>T (p.Thr703Met)

This mutation is localized in the BICD2 domain that interacts with RAB6A, a small GTPase that coordinates Golgi-ER transport (Hoogenraad et al. 2001). Nevertheless, no change in the interaction with RAB6A was observed (Martinez-Carrera et al. 2018).

However, our experimental evidence suggests that this mutation exerts alterations in Golgi structure (Neveling; Martinez-Carrera et al. 2013), endocytosis and centrosome splitting (Section 4, unpublished findings).

Fibroblast cells derived from an affected individual that carries this mutation, showed extremely diffused (interpreted as fragmented) Golgi apparatus (Neveling; Martinez-Carrera et al. 2013). The grade of fragmentation was more prominent for this mutation in comparison with the fibroblast harboring the p.Asn188Thr mutation, possibly correlating with the severity of the disease.

Further experiments suggested that this mutation decreased endocytosis (Section 4.1, unpublished findings). Studies in *Drosophila* have shown that BicD interacts with clathrin heavy chain and recruits the dynein complex to promote endocytosis (Li et al. 2010). However, the interaction with clathrin was not assessed in the present study. It is also possible that the impaired endocytosis might be due to incorrect recruitment of dynein complex to mediate vesicle transport.

Additionally, the fibroblasts carrying the p.Thr703Met mutation showed centrosome splitting, which is compatible to G2 phase of the cell cycle. Even after stimulation for a period of time (24 h), in which it is expected that the cells complete a cell cycle, the mutant cells continued with their centrosomes in split status suggesting an arrest in G2 phase. It is known that in the late G2 phase, prior to entry mitosis, BICD2 contributes to tether the centrosomes together through regulation of dynein and kinesin-1 (Splinter et al. 2010). The p.Thr703Met mutation might alter this regulation failing to maintain the centrosomes together impeding the entry to mitosis. However, the implications of altered centrosome splitting specifically in postmitotic motor neurons and how this may affect motor neuron function and maintenance are unclear.

c.2239C>T (p.Arg747Cys)

The p.Arg747Cys mutation is localized in the C-terminal domain of BICD2, and decreased the interaction of BICD2 with RAB6A. Unexpectedly, this mutation increased the amount of coprecipitated p150 subunit of dynactin (Martinez-Carrera et al. 2018). The p150 subunit is part of the dynactin complex, together with the dynactin subunit 2 (DCTN2) (Echeverri et al. 1996, Holzbaur and Tokito 1996). Previous studies suggested that the C-terminal domain of BICD2 interacts with DCTN2 (Matanis et al. 2002). Even though, the direct interaction with DCTN2 was not analyzed in the current study, the

increased coprecipitated amount of p150 suggests that this mutation recruits more dynactin into the complex.

Overexpression in HeLa cells from a CMV-BICD2-tGFP vector carrying this mutation, using transfection via Lipofectamine, showed aggregate formation (Section 4.3, unpublished findings). Analysis of soluble and insoluble fractions confirmed that this protein displayed more insolubility in comparison with control. However, this mutant did not show aggregate formation in lentiviral transduced motor neurons, which is possibly due to weaker gene expression of lentiviral transduction as compared to transfection of CMV vectors by lipofection (Martinez-Carrera et al. 2018). Thus, it cannot be stated with certainty whether protein aggregation of mutant BICD2 protein occurs in motor neurons under physiological conditions.

5.2. Pathological consequences of alterations in different cellular processes due to BICD2 mutations

Our results showed that SMALED2-associated BICD2 mutations exert alterations in different cellular processes, which are in general very important for all the cells. However, alterations in any of those processes may contribute to the impairment of motor neurons, the cell type that seems to be primarily affected in SMALED2.

Golgi fragmentation was observed in association with some of the mutations (Martinez-Carrera and Wirth 2015). Previous studies have shown that in undifferentiated neurons the coordinated position and activity of centrosomes, Golgi apparatus and endosomes is necessary to undergo polarization (de Anda et al. 2005). We cannot discard the possibility that BICD2 mutations may affect neuronal polarization. However, to address this question it would be necessary to establish an experimental set up in very early developmental stages using tools, such as cell reprogramming.

We found that fibroblasts from an affected individual with SMALED2 presented decreased endocytosis (Section 4.1, unpublished findings). Fibroblast cells and other cell types share mechanisms of endocytosis. For the development and maintenance of neurons, endocytosis is crucial (Cosker and Segal 2014). Studies in *Drosophila* have shown that BicD is required to promote synaptic vesicle recycling (Li et al. 2010). The effect of decreased endocytosis observed in fibroblast might be of greater impact in the synapse with potential consequences specifically present in neurons.

Some of the mutations alter the interaction with dynein-dynactin complex (Martinez-Carrera et al. 2018). Dynein-dynactin complex is the major motor complex driving minus end-directed microtubule transport. In neurons, intracellular transport is fundamental for

morphogenesis, function and survival (Hirokawa et al. 2010). Multiple studies have shown that BICD2 functions as cargo adaptor and regulator of dynein (Hoogenraad et al. 2001, Matanis et al. 2002, Splinter et al. 2010, Splinter et al. 2012). The mutations in BICD2 that change interaction with the dynein-dynactin complex may impair the function of this complex, transport and/or targeting of cargos that are necessary for development and maintenance of neurons. Regarding the mutations that do not display any alteration in interactions, we cannot discard the possibility that these mutations might also impair dynein movement by changing the stability of the complex (Hoang et al. 2017).

5.3. SMALED2-associated BICD2 mutations stabilize microtubules in fibroblast cells

Further studies performed in the fibroblast cells derived from the affected individuals with SMALED2 revealed that the microtubules in the mutant cells are more stable and resistant to depolymerization in comparison to control (Martinez-Carrera et al. 2018). The most significant and strongest effect was observed in the cells carrying the mutation p.Thr703Met, which also showed a high disruption of the Golgi apparatus (Neveling; Martinez-Carrera et al. 2013). Previous studies have shown that the close association between the Golgi apparatus and the microtubule dynamics plays and important role in Golgi structure and function (Burkhardt 1998).

These results strongly suggest that BICD2 mutations stabilize microtubules. However, it has never been reported before whether BICD2 participates in the formation and/or stabilization of microtubules.

5.4. BICD2 mutations share a common effect on motor neurons

Motor neurons that express the mutations located in the C-terminal domain of BICD2, p.Thr703Leu and p.Arg747Cys, showed extreme long axons in comparison with control (Martinez-Carrera et al. 2018). Strikingly, the four BICD2 mutations cause aberrations in axonal morphology. The axons developed extreme long branches, which might have as pathological consequence, misguidance and failure to innervate the targeted muscle, a phenomenon previously reported in regeneration studies (Brushart 1988, Al-Majed et al. 2000, Guntinas-Lichius et al. 2001, Guntinas-Lichius et al. 2002).

Similarly, increased axonal branching and outgrowth have been reported in studies performed in neurons upon kinesin-5 depletion or dynein overexpression (Ahmad et al. 2006, Myers and Baas 2007).

It is important to mention that the microtubule network (array) in the axon is assembled differently. The so called neuronal microtubules are nucleated at the centrosome located in the cell body, and then severed into short and long fragments by proteins such as spastin and katanin (Yu et al. 1993, Ahmad et al. 1999) Those microtubule fragments are then transported by dynein from the centrosome along the axon and aligned at the plus-end tip (Baas et al. 2006). Those fragments act as nucleating elements for the assembly of other microtubules. However, this process is dynamic and requires kinesin to promote retraction, acting as a growth brake by antagonize the forces of dynein (Myers and Baas 2007).

In this manner, the balance between dynein and kinesin is crucial to assure the correct assembly of the axonal microtubule array. BICD2 interacts also with kinesin and has an important function in the regulation of force balances between dynein and kinesin (Belyy et al. 2016). Even though changes by BICD2 mutations in the interaction with kinesin were not addressed in our interacting studies, it is of consideration that alterations on one motor protein can affect the function of the other.

We hypothesize that BICD2 is involved in the assembly of microtubules in motor neuronal axons. This possible function of BICD2 has never been described and might constitute a key clue to understand the direct pathomechanism of BICD2 mutations.

5.5. SMALED2 *Drosophila* model: BICD2 mutations cause locomotion impairment with defects in NMJ development

The p.Ser107Leu and p.Thr703Met mutations, associated with congenital onset of SMALED2 and contractures were studied *in vivo*. The generated mutant and wild type *Drosophila* lines carry in one allele the transgenic vector and in the other the endogenous *BicD*, in attempt to mimic the heterozygous dominant state of SMALED2 (Martinez-Carrera et al. 2018). We were able to control the expression of the mutations in a tissue specific manner by using the UAS-GAL4 system. The two mutants when expressed in neuronal tissue, showed impaired locomotion in adults and reduced NMJ size in the third larval stage. Upon expression only in muscles, the mutants did not show any locomotion impairment. These findings confirm the pathogenicity of BICD2 mutations *in vivo* and support that neurons are specifically affected. As mentioned in previous sections, studies in *Drosophila* have shown that indeed BicD plays an important role in NMJ development due to synaptic vesicle recycling (Li et al. 2010). Further analyses are needed to clarify whether the BICD2 mutations alter the NMJ development via impairing dynein transport at the synapse, effects on microtubules or clathrin mediated endocytosis.

5.6. BICD2 mutations are associated with other disorders besides SMALED2

5.6.1. Chronic myopathy

We consider that the p.Ser107Leu and p.Thr703Met mutations cause a severe SMALED2 presentation due to the congenital onset and presence of contractures. In addition, these mutations were clearly confirmed as pathological with neuron specificity. However, the same mutations were found in individuals with chronic myopathy, affecting primarily muscles and with only minor neurogenic changes (Unger et al. 2016). Further analysis in muscle showed striking impairment of Golgi integrity and vesicle pathology. In addition, overexpression studies evidenced abnormal localization of both mutants. A specific BICD2 function in muscle has not been described. However, these clinical and functional findings point to possible effects of this BICD2 mutations on muscle that may be evident only in more complex organisms but not in *Drosophila*.

We have also demonstrated that BICD2 expression levels in mouse muscles increase over time, a phenomenon that is more evident observed in gastrocnemius, the most commonly affected muscle in SMALED2 and chronic myopathy (Martinez-Carrera et al. 2018).

The mother of the affected individual with chronic myopathy associated with the p.Thr703Met mutation, also carries the same mutation but is unaffected (Storbeck et al. 2017).

It is unclear how these individuals with chronic myopathy did not develop major neurogenic changes as in SMALED2 individuals or shown in our SMALED2 animal model. We are convinced that these mutations have the potential to impair motor neurons. However, other factors such as genetic modifiers may contribute to protect from potential alterations in motor neurons, and even in the case of the unaffected carriers, to avoid any pathological feature.

5.6.2. Congenital arthrogryposis multiplex, respiratory failure and early lethality

The recent identification of three *BICD2* variants in lethal cases of congenital arthrogryposis multiplex and respiratory failure, expand the spectrum to a severe extreme of BICD2-pathies (Storbeck et al. 2017). This striking phenotype points that those BICD2 mutations may exert different pathological effects than those observed in the current work. The causes of congenital arthrogryposis are often attributed to a very

early *in utero* onset of muscular atrophy that leads to reduced fetal movement. Further analyses are needed to investigate the mechanism of how these particular mutations cause such pathological effects in early development. It is important to consider that BICD2 might have other cargos or functions still not known, and that alterations in these processes or pathways may be responsible of devastating consequences.

5.7. Synopsis of alterations to due to SMALED2-causing BICD2 mutations

We include an overview of the different defects in cellular processes, exerted by BICD2 mutations (Figure 7). Despite each mutation seems to exert different consequences, all cause microtubule hypestability and aberrations in axonal morphology. This strongly suggests a shared pathomechanism, while the disease severity may be defined by individual effects of a respective mutation. Two BICD2 mutations present not only pathological consequences in axon development but also at NMJ level. All together, we provided substantial experimental evidence of the pathological effects due to BICD2 mutations leading to motor neuronal defects causing SMALED2.

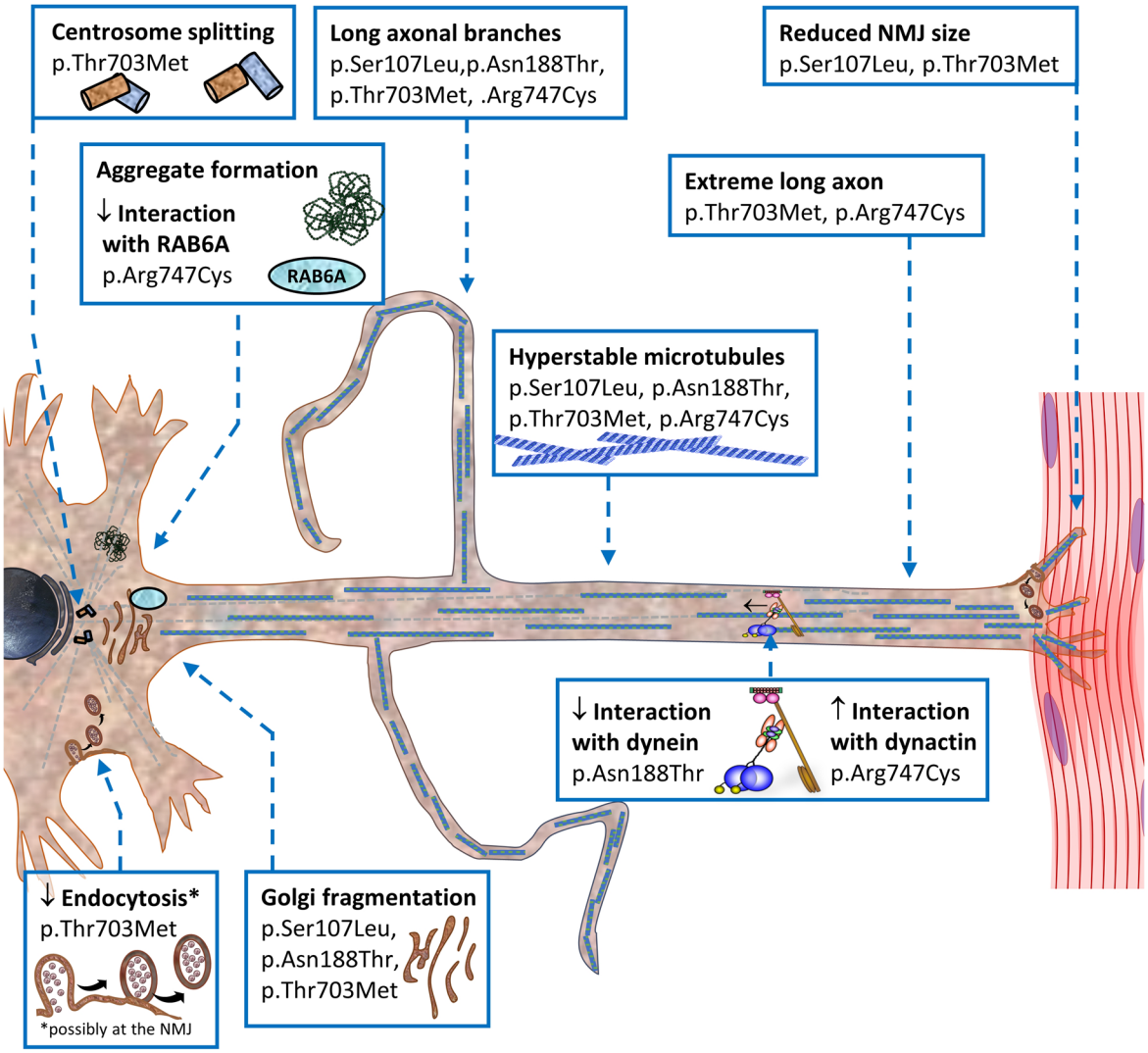

Figure 7. Schematic synopsis of alterations due to SMALED2- causing BICD2 mutations.
Each of the cellular processes where BICD2 mutations exert an alteration is shown. The rectangles comprise the magnification representation of the alteration, and the corresponding mutation(s) that are implicated.

6. Summary

Autosomal dominant spinal muscular atrophy is considered rare in comparison with the recessive 5q-linked form. However, for the majority of autosomal dominant SMA, the genetic causes are unknown. In this respect, the present doctoral thesis focused on the identification and functional characterization of the disease-causing gene of autosomal dominant spinal muscular atrophy, lower extremity-predominant, type 2 (SMALED2 MIM 615290).

By applying next generation sequencing and Sanger sequencing, we were able to identify in affected individuals with SMALED2, four heterozygous missense variants (c.320C>T [p.Ser107Leu], c.563A>C [p.Asn188Thr], c.2108C>T [p.Thr703Met] and c.2239C>T [p.Arg747Cys]) in the *Bicaudal-D2* gene (*BICD2* MIM 609797). Moreover, to gain insights into the pathogenesis of SMALED2, the molecular consequences of these protein alterations were further investigated using *in vitro* and *in vivo* approaches.

BICD2 is a highly conserved protein and interacts with several important players involved in axonal transport (dynein-dynactin complex [DTCN2] and kinesin [KIF5A]), endocytosis (clathrin heavy chain), vesicle transport (RAB6A) and others. BICD2 has been implicated in retrograde dynein-mediated axonal transport along the microtubules, in the vesicle transport from the Golgi apparatus to the endoplasmic reticulum and in the centrosome positioning.

Our *in vitro* studies revealed that each of the SMALED2-associated mutations exerts different consequences in the cell, including Golgi fragmentation, changes in interaction with dynein-dynactin complex and RAB6A, decreased endocytosis, centrosome splitting and G2 phase arrest, and aggregate formation. Studies performed in fibroblast cell lines derived from individuals with SMALED2 showed that all the mutations cause microtubule hyperstability.

Clinical findings of affected individuals suggest that the motor neuron is the disease-relevant cell type in SMALED2. Transduced motor neurons that express the SMALED2-associated mutations displayed axonal aberrations such as increased branching and overgrowth. Both findings, microtubule hyperstability and axonal aberrations during development, strongly suggest an involvement of BICD2 either in formation, stability or assembly of microtubules. Thus, our data points to a novel function of BICD2 in microtubule regulation, that when altered constitutes a shared pathomechanism of SMALED2-associated mutations, while the disease severity may be defined by individual effects of a respective mutation.

We generated and characterized the first *in vivo* model for SMALED2. We made use of the bioengineered tools of *Drosophila* melanogaster, a widely used animal system in studies of neurodevelopmental disorders. The *Drosophila* lines that expressed in neuronal tissue the mutant BICD2 constructs showed markedly impaired locomotion during early adulthood with reduced neuromuscular junction size during development. No pathological effect was observed when expressing the mutations in muscle, which supports a primarily neurogenic involvement, as reported by clinicians in SMALED2 cases. The mutations studied in the *Drosophila* model for SMALED2, were identified in individuals with chronic myopathy. This finding opened up a controversial debate of whether those mutations affect primarily neurons or muscles. Histological and functional studies revealed that these BICD2 mutations alter Golgi structure in muscle and possibly exocytosis. In addition, one asymptomatic carrier was identified suggesting variable expressivity or incomplete penetrance, possibly due to the presence of protective genetic modifiers.

Unexpectedly, our study unraveled an early lethal form of congenital arthrogryposis multiplex with respiratory failure associated to *de novo* variants in *BICD2*. The extreme pathological consequences due to these mutations may lead to the uncovering of a likely different mechanism as the observed in SMALED2.

Thus, the investigations carried out during this PhD project led to the discovery of *BICD2* as the genetic cause of SMALED2, provided substantial experimental evidence of the pathological effects due to BICD2 mutations, and contributed to the identification of other disorders associated to *BICD2*.

7. Zusammenfassung

Autosomal dominante spinale Muskelatrophie tritt im Vergleich zu 5q-assoziierter SMA selten auf, doch für die meisten dominanten Formen ist die genetische Ursache nicht bekannt. Daher konzentriert sich die vorliegende Doktorarbeit auf die Identifizierung und funktionelle Charakterisierung des krankheitsverursachenden Gens für autosomal dominante spinale Muskelatrophie mit vorwiegender Beinbeteiligung Typ 2 (SMALED2, MIM615290).

Durch die Anwendung von Hochdurchsatz-Sequenziertechnologien (NGS) und Sanger Sequenzierung konnten wir die vier folgenden heterozygoten missense-Varianten im *Bicaudal D2* (*BICD2*) Gen (MIM 609797) bei Patienten mit SMALED2 identifizieren: c.320C>T [p.Ser107Leu], c.563A>C [p.Asn188Thr], c.2108C>T [p.Thr703Met] and c.2239C>T [p.Arg747Cys]. Des Weiteren wurden die molekularen Konsequenzen dieser Veränderungen in der Proteinsequenz *in vitro* und *in vivo* analysiert, um tiefere Einblicke in die Entstehung der Pathogenität von SMALED2 zu ermöglichen.

BICD2 ist ein hoch konserviertes Protein, das mit Proteinen, die im axonalen Transport (Dynein-Dynactin Komplex [DTCN2] und Kinesin [KIF5A]), Endozytose (clathrin heavy chain), vesikulärer Transport (RAB6A) und anderen, interagiert. BICD2 spielt eine wichtige Rolle im retrograden Dynein-abhängigen axonalen Transport entlang der Mikrotubuli, im vesikulären Transport vom Golgi zum endoplasmatischen Reticulum und im Zentrosom.

Unsere *in vitro* Studien zeigten, dass jede der mit SMALED2 assoziierten Mutationen verschiedene zelluläre Konsequenzen nach sich zieht. Diese umfassen Fragmentierung des Golgiapparats, Veränderungen der molekularen Interaktion mit Dynein-Dynactin sowie mit RAB6A (ein Regulator von Transportprozessen zwischen endoplasmatischem Reticulum und Golgiapparat), verminderte Endocytose, Aufspaltung der Zentrosomen sowie Zellzyklusunterbrechung in der G2-Phase, und letztlich die Bildung von Protein Aggregaten. Experimente an Fibroblasten, die von an SMALED2 erkranken Individuen generiert wurden, deuteten auf eine gesteigerte Stabilität der Mikrotubuli hin. Dieses Verhalten war allen getesteten BICD2 Mutationen gemein.

Klinische Befunde der betroffenen Individuen lassen vermuten, dass es sich bei SMALED2 um eine primäre Erkrankung der unteren motorischen Neuronen handelt. Murine motorische Neuronen, die mit SMALED2 assoziierten mutierten BICD2 Konstrukten transduziert wurden, zeigten axonale Störungen wie z.B. die häufige Bildung von Verzweigungen und verstärktes axonales Längenwachstum. Letzteres und die erhöhte Stabilität der Mikrotubuli lassen vermuten, dass BICD2 entweder in die

Bildung, die Stabilität, oder den Zusammen- und Umbau größerer Mikrotubulifragmente involviert ist. Unsere Daten lassen auf eine neue Funktion von BICD2 bei der Regulation von Mikrotubuli schließen, die – falls beeinträchtigt – einen gemeinsamen Pathomechanismus SMALED2-assoziierter Mutationen darstellt, wobei der Schweregrad der Erkrankung durch individuelle Effekte der jeweiligen Mutation definiert sein könnte.

Im Rahmen der durchgeführten *in vivo* Studien, haben wir das erste Tiermodel für SMALED2 generiert und charakterisiert. Die Taufliege *Drosophila melanogaster* ist genetisch leicht zu manipulieren und ist ein häufig genutzter Modelorganismus zur Untersuchung neuronaler Erkrankungen. Die Expression mutierter BICD2 Konstrukte in neuronalem Gewebe in *Drosophila* führte zu deutlichen motorischen Defiziten der jungen, adulten Fliegen und zu verminderter Größe der neuromuskulären Endplatten während der Entwicklung. Verkleinerte motorische Endplatten oder motorische Defizite waren nicht nachweisbar, wenn mutierte BICD2 Konstrukte in muskulärem Gewebe exprimiert wurden. Dies deutet auf ein primär neurogenes Geschehen hin, so wie es an Hand klinischer Daten zu vermuten wäre. Die Identifizierung einiger BICD2 Mutationen in Individuen mit chronischer Myopathie eröffnete eine kontroverse Diskussion über die entweder neuronale oder muskuläre Ätiologie von SMALED2. Histologische und funktionelle Untersuchungen zeigten, dass Myopathie-assoziierte BICD2 Mutationen die Struktur des Golgiapparats verändern und möglicherweise die Exozytose beeinflussen. Außerdem wurde ein klinisch asymptomatischer Träger einer krankheitsverursachenden BICD2 Mutation identifiziert, was auf eine variable Expressivität und ggf. reduzierte Penetranz in Anwesenheit genetisch modifizierender Faktoren hindeutet.

Unerwarteter Weise konnten wir eine frühletale Form multipler kongenitaler Arthrogrypose mit respiratorischer Insuffizienz mit *de novo* Mutationen in BICD2 in Verbindung bringen. Basierend auf diesen Mutationen könnte eine derart schwerwiegende klinische Ausprägung zur Identifizierung weiterer BICD2 bezogener Krankheitsmechanismen führen, die über die von SMALED2 bekannten Mechanismen hinausgehen.

Zusammenfassend führten die im Rahmen dieser Doktorarbeit durchgeführten Studien zur Identifizierung von *BICD2* als genetische Ursache von SMALED2 und unterstützen durch grundlegende experimentelle Beweisführung die pathologische Kausalität der BICD2 Mutationen. Sie leisten außerdem einen Beitrag zur Identifizierung weiterer BICD2-assoziierter Erkrankungen.

8. Additional investigations during the PhD thesis

8.1. Publication

Komlosi, K., Hadzsiev, K., Garbes, L., **Martinez Carrera, L. A.**, Pal, E., Sigurethsson, J. H., Magnusson, O., Melegh, B. and Wirth, B. (2014). "Exome sequencing identifies Laing distal myopathy MYH7 mutation in a Roma family previously diagnosed with distal neuronopathy." Neuromuscul Disord 24(2): 156-161.

8.2. Abstract of the publication[1]:

We described a Hungarian Roma family, originally investigated for autosomal dominant distal muscular atrophy. The mother started toe walking at 3 years and lost ambulation at age 27. Her three daughters presented with early steppage gait and showed variable progression. Muscle biopsies were nonspecific showing myogenic lesions in the mother and lesions resembling neurogenic atrophy in the two siblings. To identify the causative abnormality whole exome sequencing was performed in two affected girls and their unaffected father, unexpectedly revealing the MYH7 mutation c.4849_4851delAAG (p.K1617del) in both girls, reported to be causative for Laing distal myopathy. Sanger sequencing confirmed the mutation in the affected mother and third affected daughter. In line with variable severity in Laing distal myopathy our patients presented a more severe phenotype. Our case is the first demonstration of Laing distal myopathy in Roma and the successful use of whole exome sequencing in obtaining a definitive diagnosis in ambiguous cases.

8.3. Own contributions

I contributed with the primer design, establishment, performance, and analysis the sequencing of the *DYNC1H1* and *BICD2* for all the individuals included in this study.

[1] The abstract is extracted from the original publication

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Appendix

Publications

I. **Neveling, K.*, Martinez-Carrera, L. A.* et al. (2013). "Mutations in BICD2, which Encodes a Golgin and Important Motor Adaptor, Cause Congenital Autosomal-Dominant Spinal Muscular Atrophy." Am J Hum Genet 96(6): 946-954. (*These authors contributed equally to this work)**

II. **Synofzik, M., Martinez-Carrera, L. A. et al. (2014). "Dominant spinal muscular atrophy due to BICD2: a novel mutation refines the phenotype." J Neurol Neurosurg Psychiatry 85(5): 590-592.**

III. **Martinez-Carrera, L. A. and Wirth, B. (2015). "Dominant spinal muscular atrophy is caused by mutations in BICD2, an important golgin protein." Front Neurosci 9: 401.**

IV. **Martinez Carrera, L. A., Gabriel, E. et al. (2018). "Novel insights into SMALED2: BICD2 mutations increase microtubule stability and cause defects in axonal and NMJ development." Hum Mol Genet (Epub ahead of print).**

V. **Unger, A., Dekomien, G. et al. (2016). "Expanding the phenotype of BICD2 mutations toward skeletal muscle involvement." Neurology 87(21): 2235-2243.**

VI. **Storbeck, M., Eriksen, B. et al. (2017). "Phenotypic extremes of BICD2-opathies: from lethal, congenital muscular atrophy with arthrogryposis to asymptomatic with subclinical features." Eur J Hum Genet 25(9): 1040-1048**

VII. **Komlosi, K., Hadzsiev, K. et al. (2014). “Exome sequencing identifies Laing distal myopathy MYH7 mutation in a Roma family previously diagnosed with distal neuronopathy.” Neuromuscul Disord. 24(2): 156-61**

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