Muscle cell fate determination in embryonic and adult zebrafish muscle development

Bepaling van de cellulaire identiteit van spiercellen tijdens de embryonale ontwikkeling en in volwassen stadia in de zebravis
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 april 2010 des ochtends te 10.30 uur

door

Jin Ming Tee
geboren op 10 october 1981 te Kuala Lumpur, Maleisie

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Dedicated to my loving parents, who supported my dream of becoming a scientist.... you made it a reality!

The research described in this thesis was performed at Hubrecht Institute of the Royal Academy of Arts and Sciences (KNAW), within the Graduate School of Cancer Genomics and Developmental Biology in Utrecht, The Netherlands.

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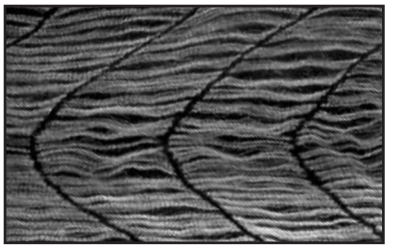

Cover: Mosaic representation of zebrafish skeletal muscle. As each living cell makes up the complexity of a functional muscle, each mosaic tile here represents each and every person who made this PhD research possible.

Contents

CHAPTER 1

General Introduction

Zebrafish as a model system

In the past years, zebrafish has earned its recognition in research of vertebrate development due to the powerful combination of embryology as well as forward and reverse genetics (Kimmel and Warga, 1988; Kimmel, 1989; Mullins et al., 1994; Solnica-Krezel et al., 1994). Several large and small scale mutagenesis screens yielded mutants revealing several aspects in pattern formation and organogenesis (Driever et al., 1996; Mullins et al., 1994; Solnica-Krezel et al., 1994). Furthermore, two groups have recently described the use of zinc finger technology to directly modify the genetic loci and generate targeted knockouts in zebrafish (Doyon et al., 2008; Meng et al., 2008). Zebrafish are relatively small, and easy to maintain. In addition to that, zebrafish are capable of producing many offsprings (approximately 200 per lay), and their embryos develop outside the body. The translucent embryos make zebrafish ideal for in vivo imaging and cell fate tracking. An important characteristic of the zebrafish is maternally contributed protein and mRNA, which often mediates normal development of a mutant zebrafish through gastrulation. During gastrulation, zygotic gene expression is switched on. Most zebrafish mutants from forward screens are recessive lethal zygotic-effect mutations.
In this thesis, the zebrafish is utilized as a model organism for vertebrate muscle development, and vertebrate adult muscle homeostasis.

Skeletal muscle development

Skeletal muscle development can be typically divided into three phases: (1) first myogenic phase (primary myogenesis), associated with early embryogenesis and specification of the muscle tissues; (2) second myogenic phase (secondary myogenesis), where an autonomous growth program occurs; and (3) adult muscle growth.
In general, the main skeletal muscle anatomy consists of the dermomyotome, myotome, and sclerotome (Figure 1), and is conserved throughout species. In mammals and avian, the sclerotome makes up a major part of the somite during development, and differentiates to form the vertebral body. However, in zebrafish and shark, the sclerotome is found only as a small population of cells (Mise et al., 2008) in the ventromedial region of the somite (Morin-Kensicki and Eisen, 1997). The dermomyotome is the source of the primary myotome, as well as contributing to the formation of the dermis, the endothelial and smooth muscle cells (Kardon et al., 2002). However, the

region of the dermomyotome responsible for generating these cells remains debatable. The dermomyotome is divided into epaxial and hypaxial domains, which gives rise to the epaxial muscles (deep muscle of the back) and hypaxial muscles (apendicular musculature, abdominal muscles, diaphragm, hypoglossal chords) respectively (Christ and Ordahl, 1995; Ordahl and Le Douarin, 1992).

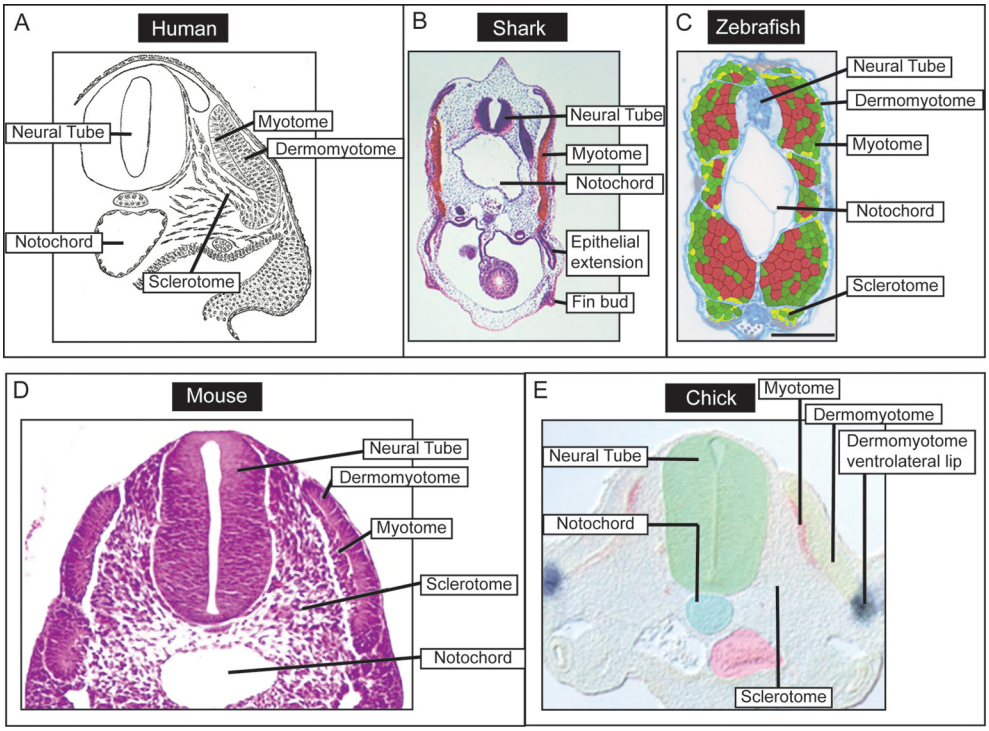

Figure 1. Comparison of vertebrate myotome. The anatomy of the vertebrate myotome is relatively conserved between human (A), shark (B), zebrafish (C), mouse (D), and chick (E). Adapted from Gray and Lewis, 1918; Bryson-Richardson, and Currie 2008 (B and E); Stellabotte et al., 2007 (C); Danilchik et al., 2006 (D).

Primary myogenesis

During primary myogenesis, cells located at the epithelial lips of the dermomyotome delaminate to lie between the dermomyotome and sclerotome, forming the primary myotome. The primary myotome consist of post-mitotic myocytes, which are aligned such that they span the somite length along the axis of the embryo (Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Gros et al., 2004; Kahane et al., 2002; Ordahl et al., 2001).

An early population of post-mitotic myocytes is derived from the dorsal medial portion of the somite prior to dermomyotome formation (Gros et al., 2004; Kahane et al., 1998). In the chick, these cells express myogenic transcription factors and consequently move directly underneath the forming dermomyotome. These cells subsequently differentiate to form the first fibers of the primary myotome in the rostral-caudal axis. These fibers have also been called pioneer muscle cells, a term that invokes comparison to a similarly named, early differentiating skeletal muscle cell population previously identified in zebrafish.

Maintenance of appropriate cell–cell contacts (Cadherin and α6β1 integrin) has been shown to be important for regulating the onset of myogenesis (Bajanca et al., 2006; Cinnamon et al., 2006), although the precise mechanism in which it occurs through is not fully understood. When blocking antibodies that target α6 integrin are added to cultured mouse explants, ectopic delaminations and premature myogenesis occurs (Bryson-Richardson and Currie, 2008). Contrastingly, loss of N-cadherin-mediated adhesion results in a loss of myogenic potential (Bryson-Richardson and Currie, 2008). However, it is somewhat unclear at what level these distinct cell-adhesion systems might operate.

Myogenic regulatory factors

Members of the basic helix-loop-helix domain containing family of myogenic regulatory factors (MRFs) are important for directing myogenesis. These transcriptional regulators include myogenic regulatory factor 5 (myf5), myogenic differentiation 1 (myod1 or more commonly known as myoD), myogenic regulatory factor 6 (myf6 or mrf4) and myogenin (myog). MRFs are responsible for directing the expression of genes required to generate the contractile properties of a mature skeletal muscle cell (Bryson-Richardson and Currie, 2008).

Myod1 itself can convert many cell types, including those of non-mesodermal origin, into myoblasts (Weintraub et al., 1991). It functions by directly binding to the promoters and enhancers of specific muscle genes and activates myogenic commitment and differentiation. Upregulation of Myf5 may compensate for knock-out of Myod1 gene function (Rudnicki et al., 1992), while a Myf5/Myod1 double knock-out makes no muscles at all, thus suggesting a functional redundancy or compensatory mechanism between these two MRFs (Rudnicki et al., 1993). Both Myf5 and Myod1 acts upstream of Myog. Myog mutants initiate myogenesis normally but possess

defects in the differentiation of myocytes and myofibers (Hasty et al., 1993; Nabeshima et al., 1993). It was initially believed that similar to Myog, Myf6 acts downstream of Myf5 and Myod1 (Patapoutian et al., 1995; Zhang et al., 1995). However, more recent studies suggested that Myf6 may have an earlier role in muscle determination (Kassar-Duchossoy et al., 2004).
Cross regulation amongst MRFs, as well as autoregulation to regulate transcription of Myod1 has been described (Kassar-Duchossoy et al., 2004; Lun et al., 1997; Maroto et al., 1997; Tajbakhsh et al., 1997; Thayer et al., 1989). However, patterning signals (e.g. Wnt, Notch, Shh and FGF) are the most well-studied form of MRF regulation, and will be discussed in more detail below. Notably, amongst these in the mouse are the up-regulation of Myod1 expression by Wnt signaling (Linker et al., 2003; Tajbakhsh et al., 1998) , and the downregulation of MyoD expression by Notch signaling (Hirsinger et al., 2001).

Signals regulating myogenesis in the somites

The genetic basis for muscle formation and the signaling pathways involved in patterning the myotome is similar in all vertebrates. Primary myogenesis is initiated by signals from the notochord (Shh) and neural tube (Wnt) that induce the expression of the MRFs (Figure 2). Activation of these signaling pathways determines the balance between determination, proliferation, survival and differentiation of muscle progenitors in the somite. Excision experiments on either the neural tube alone or neural tube and notochord together, resulted in loss of myotomal muscles (Christ et al., 1992). The signals are required to promote myogenesis only in the presomitic mesoderm and newly-formed somites. More mature somites do not need the presence of neighboring signaling tissues.

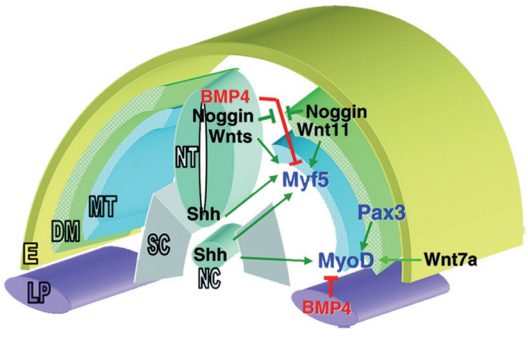

Figure 2. Signaling factors in embryonic skeletal muscle formation. Mesodermal somitic cells in the dorsal dermomyotome (DM) receive signals from surrounding tissues, which induce (Wnts, Shh, Noggin) or inhibit (BMP4) the expression of the primary MRFs (Myf5 and MyoD) and commitment to the myogenic lineage. Commited myoblasts migrate laterally to form the myotome (MT), which eventually forms the skeletal musculature. Pax3 promotes myogenesis in the lateral myotome. E, ectoderm; LP, lateral plate; SC, sclerotome; NC, notochord; NT, neural tube (Charge and Rudnicki).

Wnt signaling pathway

The Wnt signaling pathway plays a regulatory role in the core biological processes of proliferation, differentiation, and stem cell renewal. Deregulation of this pathway results in various forms of cancer and other diseases (Table 1). Since the discovery of the founding protein, WNT1, more than 60 proteins have been identified, many of which are conserved throughout evolution (Kusserow et al., 2005). Even the placozoans, which represents the simplest free-living animal of the primitive metazoan form, have a complete Wnt pathway (Srivastava et al., 2008).

Upon secretion, Wnt ligands bind to a number of distinct receptors on the cell membrane, and activate downstream canonical Wnt/β-catenin, non-canonical planar cell polarity (PCP) or the Wnt/Ca2+ pathways (van Amerongen et al., 2008). In general, Wnt1, Wnt3a and Wnt8 are considered to elicit 'canonical' signaling, whereas Wnt5a and Wnt11 are considered to be 'non-canonical' Wnt ligands. However, the subdivision of Wnts into the term 'canonical' and 'non-canonical' does not hold up to scrutiny, as the typically 'non-canonical' ligands, Wnt5a and Wnt11 has been shown to also activate β-catenin signaling cascade (He et al., 1997; Mikels and Nusse, 2006; Tao et al., 2005). Thus for the remainder of this thesis, the 'canonical' pathway will be referred to as Wnt/β-catenin pathway.

Briefly, Wnt/β-catenin signaling involves the binding of Wnt ligands to two recptors: Frizzled, through the receptor's cysteine-rich domain (CRD), and LRP5/6 (Bhanot et al., 1996; Wehrli et al., 2000; Yang-Snyder et al., 1996). In the absence of Wnt ligands, β-catenin is recruited to the destruction complex containing Axin as the scaffold protein, the tomour suppressor Adenomatous Polyposis Coli (Apc), and the kinases Casein Kinase I (CKIγ) and Glycogen Synthase Kinase 3β (GSK3β) (Figure 3). These kinases phosphorylate β-catenin, thus enabling its recognition by the E3 ubiquitin ligase complex, TrCP. The ubiquitinated β-catenin is then subsequently targeted for proteosomal degradation. This way, the cytosolic levels of β-catenin remains low, and nuclear accumulation of β-catenin, the hallmark of an activated Wnt/β-catenin pathway, is prohibited. The promoters of the Wnt target genes are occupied and repressed by the TCF/LEF family of transcription factors, together with its co-repressor Groucho (Brantjes et al., 2001).

Upon binding of Wnt ligands to the Fz and LRP5/6 receptors, the cytoplasmic protein Dishevelled is escorted into the nucleus by its chaperones Pygopus and Legless (Townsley et al., 2004), which have been shown to function as

Table 1. Wnt Signaling Components in Human Genetic Diseases
(adapted from http://www.stanford.edu/~rnusse/wntwindow.html)

Gene	Disease	References
Wnt signaling ligands		
WNT3	Tetra-Amelia	(Niemann et al., 2004)
WNT4	SERKAL Syndrome	(Mandel et al., 2008)
	Mullerian-duct regression and virilization	(Biason-Lauber et al., 2004)
WNT5B	Type II Diabetes	(Kanazawa et al., 2004)
WNT7A	Fuhrmann Syndrome	(Woods et al., 2006)
WNT10A	Odonto-onycho-dermal dysplasia	(Adaimy et al., 2007)
WNT10B	Obesity	(Christodoulides et al., 2006)
	Split-hand/Foot malformation	(Ugur and Tolun, 2008)
Norrin	Familial Exudative Vitreoretinopathy	(Xu et al., 2004)
RSPO4	Autosomal recessive anonychia	(Bergmann et al., 2006; Blaydon et al., 2006)
Wnt signaling receptors		
LRP5	Bone density defects, Vascular defects in the eye (Osteoperosis- Pseudoglioma Syndrome)	(Boyden et al., 2002; Gong et al., 2001; Little et al., 2002)
	Familial Exudative Vitreoretinopathy	(Qin et al., 2005; Toomes et al., 2004)
LRP6	Early coronary disease	(Mani et al., 2007)
	Late onset Alzheimer's	(De Ferrari et al., 2007)
FZD4	Familial Exudative Vitreoretinopathy, retinal angiogenesis	(Qin et al., 2005; Robitaille et al., 2002)
VANGL	Neural tube defects	(Kibar et al., 2007)
Wnt/ß-catenin signaling Destruction complex		
AXIN1	Caudal duplication	(Oates et al., 2006)
AXIN2	Tooth Agenesis	(Lammi et al., 2004)
APC	Polyposis coli	(Kinzler et al., 1991; Nishisho et al., 1991)
Transcription Factor		
TCF7L2 (TCF4)	Type II Diabetes	(Florez et al., 2006; Grant et al., 2006; O'Rahilly and Wareham, 2006)
Regulators of Wnt signalling		
PORCN	Goltz Syndrome, Focal dermal hypoplasia	(Grzeschik et al., 2007; Wang et al., 2007)
WTX	WIlm's Tumour	(Major et al., 2007; Rivera et al., 2007)

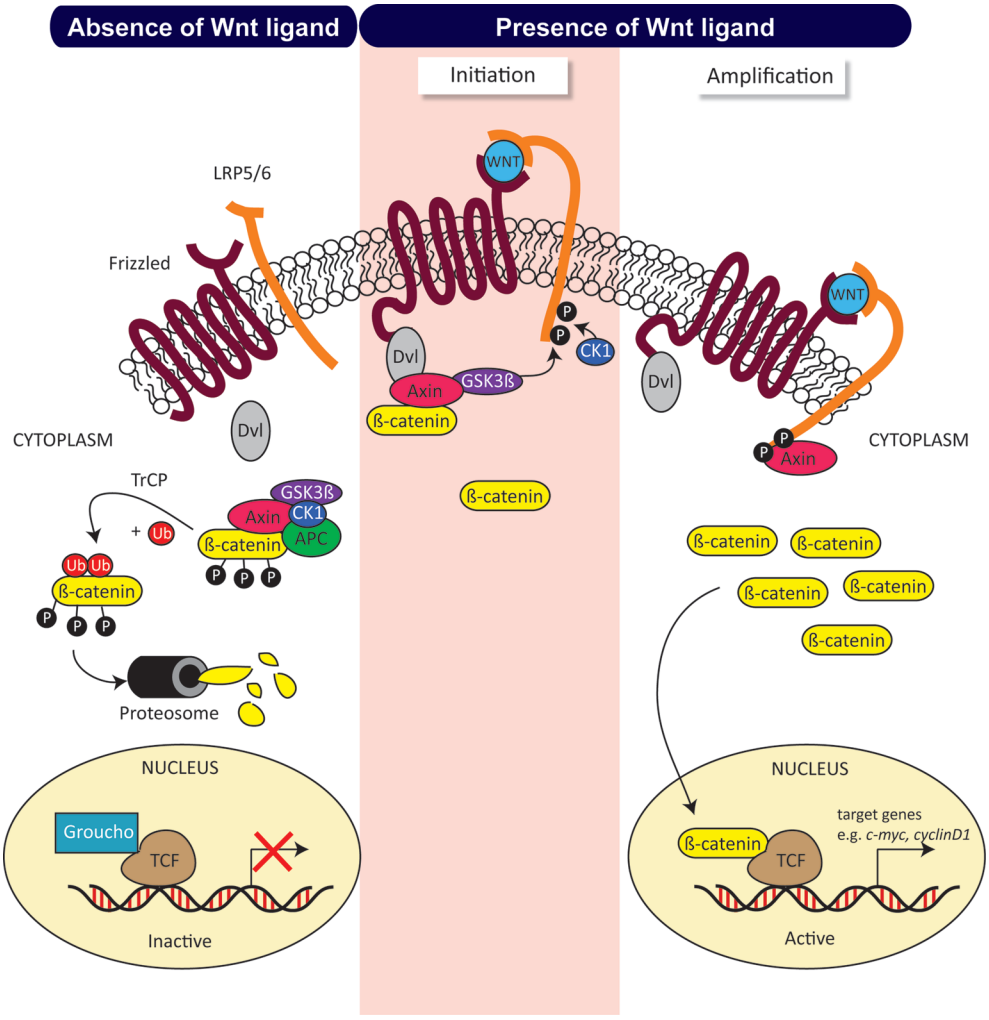

Figure 3. Model for the activation of the Wnt/β-catenin pathway. (A) In the absence of a Wnt ligand, β-catenin is phosphorylated and targeted for proteosomal degradation by a cytoplasmic destruction complex comprising of Axin, Apc, GSK3β and CKIγ. (B) In the presence of a Wnt ligand, which binds to the receptors Fz and LRP5/6, Dvl binds to Fz and recruits the destruction complex through interaction with Axin. Subsequently, GSK3β phosphorylates critical sites on LRP5/6, which together with residues phosphorylated by CKIγ, acts as docking sites for Axin. (C) Binding of Axin to LRP5/6 leads to inhibition of the destruction complex and stabilization of β-catenin. β-catenin accumulates and translocates into the nucleus, where it displaces Groucho repressors from TCF/LEF transcription factors, and activate downstream target genes (adapted from Fuerer et al., 2008).

essential co-activators. β-catenin overrules the Groucho-TCF/LEF repression, and replaces Groucho, thus converting the now β-catenin/TCF/LEF complex into a transcriptional activator (Gordon and Nusse, 2006). Remarkable progress has been made on this pathway, which has revealed the importance of Wnt/β-catenin signaling not only in metazoan development, but also in degenerative diseases and cancer (Clevers, 2006; de Longh et al., 2006; Fox and Dharmarajan, 2006; Krishnan et al., 2006; Polakis, 2000).
Many signaling pathways, including Wnt, FGF or Notch pathway, entail feedback loops to achieve better control of signal amplitude or duration (Freeman, 2000). Transcription of some components of the Wnt signaling pathway e.g. lef1, dkk1 and axin2 is mediated by β-catenin signaling, thus presenting feedback mechanisms. In case of negative feedback loops, oscillations can arise under certain conditions. During vertebrate somitogenesis, many genes show an oscillating expression pattern including Wnt, FGF and Notch pathway components (Dequeant et al., 2006). For the Wnt/β-catenin pathway, it has been shown that axin2 (Conductin/Axil), a close homolog to Axin with identical functions, is a direct target gene and a negative regulator of the pathway (Jho et al., 2002), and it has been suggested that this negative feedback loop causes the oscillation of the Wnt/β-catenin pathway during somitogenesis (Aulehla and Herrmann, 2004; Aulehla et al., 2003).

*Wnt/ β-catenin in myogenesis**
*Excerpt obtained from Bryson-Richardson and Currie, 2008.

Canonical Wnt signals from the neural tube and surface ectoderm are required for myogenic induction. In explants of mouse paraxial mesoderm, Wnt1, produced in the embryonic dorsal neural tube, induces myogenesis through the preferential activation of Myf5, whereas Wnt7A, produced in the dorsal ectoderm, preferentially activates MyoD1 (Tajbakhsh et al., 1998). Wnt signals seem to be transduced directly in muscle progenitors by Frizzled receptors that activate the canonical Wnt/β-catenin pathway within the epaxial dermomyotome. Activated β-catenin has been shown to directly induce Myf5 expression, although which Wnt ligand specifically triggers myogenesis *in vivo* remains to be genetically defined (Borello et al., 2006). A number of new effectors of Wnt signaling have been recently uncovered that act during myogenic induction. In *Xenopus laevis*, R-spondin 2 (rspo2) which is co-expressed with and positively regulated by Wnt signals, synergizes with Wnts to activate β-catenin during myogenesis. In embryos depleted of rspo2,

myod1 and myf5 are not activated, disrupting muscle development. In the mouse, myogenic induction by Wnt has been shown to require adenylyl cyclase signaling by protein kinase A (PKA) through its phosphorylation of its target transcription factor, cAMP response element binding (CREB) protein (Chen et al., 2005). There is currently no evidence for the direct binding of CREB to the regulatory regions of myogenic factor encoding genes, but as proposed by (Chen et al., 2005), the presence of CRE sites in the Myf5 and Pax3 enhancer region is highly suggestive of Wnt activation of Pax3 and Myf5 through direct CREB binding.

Non-canonical Wnt signaling has also been implicated in aspects of early myogenesis in the chick embryo. Non-canonical Wnt11 is an epithelialization signal that acts on the medial dermomyotome, and together with Wnt6 secreted from the surface ectoderm, maintains the essential epithelial nature of the dorsomedial and ventrolateral lips of the dermomyotome (Geetha-Loganathan et al., 2006; Linker et al., 2005). Recently, somewhat contradictory results have been suggested, suggesting a role for non-canonical, β-catenin independent Wnt signaling in direct induction of myogenesis within mouse explants of presomitic mesoderm (Brunelli et al., 2007). Therefore, the exact role of Wnt signaling pathways in early myotome formation remains to be defined.

Myogenesis in the developing zebrafish

Embryonic myogenesis

Embryonic myogenesis in zebrafish has several unique features. For example, cells are specified to the muscle lineage much earlier in zebrafish and other teleosts compared to amniotes during mid-gastrulation (at 70-75% epiboly) (Weinberg et al., 1996), which probably reflects the early requirement to generate body movements imposed by external fertilization. The slow-twitch and fast-twitch muscle fiber are anatomically separate entities, and this trait is maintained throughout adult stages. In addition, zebrafish somites develop an external cell layer that has some conserved molecular and cellular characteristics of the amniote dermomyotome (Hollway et al., 2007), which supplies myogenic precursors for post-embryonic growth (Gros et al., 2005).

Similar to other vertebrates, primary myogenesis in zebrafish is characterized by the formation of an early differentiation or primary myogenic phase, in which post-mitotic fibers come to span the somite in a rostral-caudal wave.

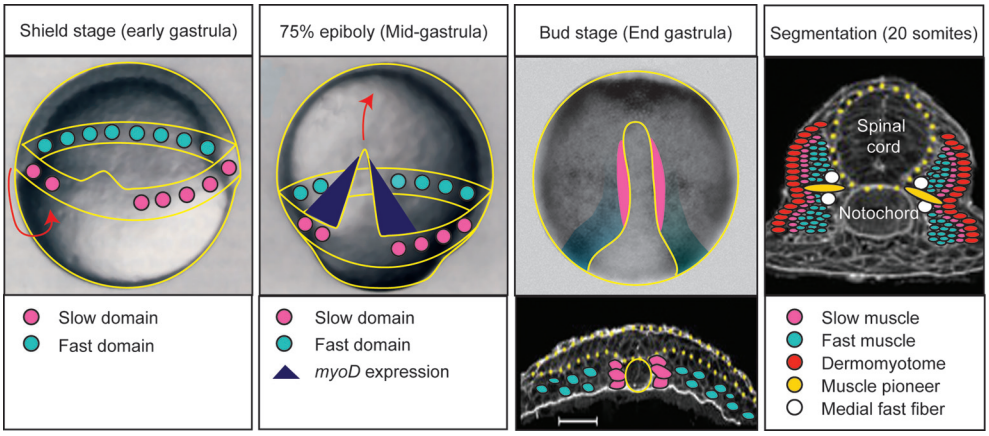

Figure 4. Schematic illustrations of zebrafish muscle development. (a) Slow and fast muscle precursors occupy a distinct region in the marginal zone at the gastrula stage. Pink dots indicate the locations of slow muscle or muscle pioneer cell precursors. Green dots indicate the locations of fast muscle cell precursor. Muscle precursor cells are not yet committed at this stage. Muscle precursor cells in the marginal zone undergo involution (arrow). (b) Myogenic gene expression starts during the mid-gastrulation stages. Black stripe indicates myod expression. Pink dots indicate the location of slow muscle and muscle pioneer cell precursors. Green dots indicate the location of fast muscle cell precursors. Presumptive notochord (N) and muscle precursors extend anteriorly (arrow) due to convergence extension movements. (c) Slow muscle precursors are specified by signaling from the notochord. PInk indicates the location of adaxial cells, precursors of muscle pioneer and other slow muscle cells. Green indicates the location of fast muscle precursors. Upper panel shows the dorsal view, lower panel shows a cross-section. (d) Four types of zebrafish muscle cells towards the end of the segmentation period (24 hpf). A cross-section through the trunk of a late segmentation stage embryo. Adaxial cells migrate to the lateral surface of the somite where they differentiate into slow muscle cells (pink). A subset of the adaxial cells remains deep within the somite and differentiates into muscle pioneer cells (yellow). Fast muscle cells differentiate in the central part of the somite (green). A subset of Eng positive fast muscle cells, medial fast muscle cells, differentiate next to the notochord (white). The external cell layer, dermomyotome, appears during the late segmentation stages (red). (Adapted from Ochi and Westerfield, 2007)

Prior to segmentation, two distinct cell types can already be distinguished morphologically and by their gene expression patterns: the slow-twitch muscle (red muscle) domain and the fast-twitch muscle (white muscle) domain (Figure 4). The slow and fast muscle fibers have distinct physiological and biochemical properties. The slow muscle fibers are located superficially, just underneath the skin, while the fast muscle fibers are located deeper in the myotome (Devoto et al., 1996). A group of cuboidal cells of the presomitic mesoderm termed adaxial cells, reside adjacent to the notochord. The adaxial

cells are able to initate myogenesis, and differentiate into a single stack of twenty elongated cells that migrate radially through the somite to form a superficial layer of slow muscle fibers (Devoto et al., 1996). This migration of adaxial cells is driven by differential cell adhesion through the actions of Cadherin transmembrane proteins (Cortes et al., 2003). Undifferentiated adaxial cells express myod1 and myf5 (Coutelle et al., 2001; Weinberg et al., 1996), and their specification is regulated by Hedgehog morphogens in a dose-dependent manner (Blagden et al., 1997; Ingham and Kim, 2005). The adaxial cells do not arise from the dermomyotomal-like compartment of the zebrafish somite, but in terms of the timing of muscle differentiation, could be thought to be the zebrafish equivalent of the dorsomedial lip of the amniote somite (Bryson-Richardson and Currie, 2008). Adaxial myoblasts are able to intiate myogenesis prior to segmentation (Devoto et al., 1996; Weinberg et al., 1996). A subset of the adaxial cells forms the muscle pioneer cells, which are the first differentiated muscle fibers. The muscle pioneer cell population remains in a medial position in the somite and express engrailed genes (Devoto et al., 1996; Weinberg et al., 1996). The muscle pioneers are thought to serve as intermediate targets for the growth cones of the earliest motor axons, because ablation of muscle pioneers affects growth of the Cap and Mip primary motoneuron axons (Melancon et al., 1997). It is unclear if muscle pioneer cells persist or have a later role in mature zebrafish.

The cells of the lateral pre-somitic mesoderm of zebrafish were shown to differentiate into the embryonic fast muscle fibers (Devoto et al., 1996), and two main sub-populations have since been characterized. A population in the posterior-lateral epithelial somite requires Fibroblast growth factor 8 (fgf8) signaling for myod1 expression and terminal differentiation (Groves et al., 2005). The other population arises medially within the somite, and like the slow muscle pioneers, expresses engrailed gene, in response to Hh signaling (Wolff et al., 2003). Interestingly, in fgf8 mutants, a residual medial fast fiber population was identified that was not dependent on Hh signaling and presumably is regulated through some other midline signal (Groves et al., 2005). Hh and Fgf8 signaling suppress Pax3/7 and promote the expression of myf5 and myod1 in specific muscle progenitor cell populations (Feng et al., 2006; Hammond et al., 2007). A combination of vital dye staining and lineage-tracking techniques in zebrafish were used to show that somite sub-domains are generated through a whole-somite rotation of 90˚ from the starting position, which begins during mid-somitogenesis and is complete by the end of the segmentation period (Hollway et al., 2007). Somite-rotation has been shown to require Sdf cytokine signalling (Hollway et al., 2007).

Coincident with migration of the slow-muscle cells, the posterior half of the somite differentiates to form fast-muscle fibres medial to the lateral, post-migratory slow-muscle population. The anterior component of the somite forms the external cell layer of Pax3/7 expressing cells (Hollway et al., 2007; Stellabotte et al., 2007). A subset of these cells migrate from the external cell layer to form the fast muscle fibers in the late embryo and larval stages, whilst others remain in the external cell layer and are thought to be a source of myogenic progenitor cells for later stages of post-embryonic growth (Hollway et al., 2007; Stellabotte et al., 2007). The external cell layer also provides progenitors used in the growth of the dermis and pectoral and dorsal fin muscles, suggesting it has a functional role equivalent to the amniote dermomyotome (Hollway et al., 2007).

Post embryonic and adult myogenesis

A second phase of myogenesis begins in the late embryo and early larval stages, contributing to the increase in size of the somite/myotome. Primary embryonic and fast muscle fibers are supplemented by fibers from discrete germinal zones, situation mainly at the dorsal and ventral regions of the myotome. This process has been termed stratified hyperplasia (Rowlerson and Vegetti, 2001). Growth of the myotome also involves an increase in the length and diameter of fibers, a process that requires the absorption of myogenic progenitor cells to provide additional nuclei (Johnston et al., 2006). Using zebrafish mutants deficient in midline Hedgehog signaling, it was shown that the first wave of stratified hyperplasia does not require a scaffold of embryonic slow muscle fibers, and proceeds independently of Shh (Barresi et al., 2001). The origin of the myogenic precursors that fuel later phases of stratified hyperplasia remains to be established, but could also be the external cell layer which persists in adult stages (Hollway et al., 2007; Stellabotte et al., 2007). Expansion in the number of fast muscle fibers with growth also preceeds via an initial phase of stratified hyperplasia (Rowlerson and Vegetti, 2001). Fate mapping studies have shown that a Pax7 expressing sub-set of the external cell population migrate through the embryonic slow muscle and give rise to fast muscle fibres in larvae (Hollway et al., 2007; Stellabotte et al., 2007).

The second and main mechanism for increasing fast fiber number is mosaic hyperplasia. It involves myogenic progenitor cells distributed throughout the myotome and results in successive waves of myotube production producing a characteristic mosaic pattern of fiber diameters. Mosaic hyperplasia

involves myogenic progenitor cells distributed throughout the myotome and results in successive waves of myotube production producing a characteristic mosaic pattern of fibre diameters. The origin of myogenic precursor cells required for mosaic hyperplasia is still unknown, although it is suggested that the undifferentiated Pax7 expressing muscle precursor cells located at the external cell layer of the zebrafish could be a possible source.

Although the idea of quiescent adult muscle stem cells is commonly accepted in vertebrates, there is not much known regarding its role in zebrafish. Cells that express a number of satellite-specific markers such as Pax7 and met proto-oncogene (Met) have been identified in juvenile and adult zebrafish muscle (Hollway et al., 2007). Lineage analysis further suggests that dermomyotomal-like origins for these cells, implying that dermomyotomally derived satellite-like cells are present in distantly-related vertebrates (Hollway et al., 2007). It has been shown that satellite cells undergo asymmetric cell division in vivo and in vitro, and that they segregate the asymmetric fate determinants Delta and Numb as well as myf5 into one or other Pax7-expressing daughter cell; this process is a hallmark of self-renewal in other stem cell populations (Bryson-Richardson and Currie, 2008; Kuang et al., 2007; Shinin et al., 2006) (Figure 5).

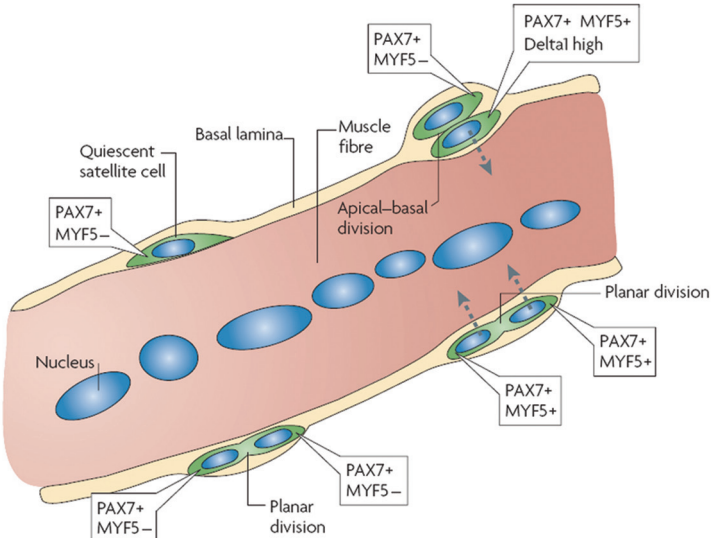

Figure 5. Adult myogenesis. Quiescent satellite cells are marked by the expression of paired box gene 7 (Pax7), met proto-oncogene (Met), M-cadherin and other satellite cell markers, and differ from cells that are commiting to the muscle progenitor fate, which begin to express myogenic factor 5 (Myf5). More recently, analysis of cultured fibers has identified that the plane of satellite cells (green) carrying out planar division generally giving rise to daughter cells with symmetric Myf5 expression. Cells dividing along an apical-basal plane generally have asymmetric expression of Myf5. In this way, a satellite cell can divide and give rise to committeed progenitors (Myf5 positive) as well as maintaining a pool of uncommitted satellite cells (Myf5 negative) (Bryson-Richardson and Currie, 2008).

Outline of this thesis

Realizing that the zebrafish opens up unique possibilities to understand fundamental principles of muscle cell development I decided to employ this model system to obtain more insight into molecular mechanisms governing myogenesis, focusing on the signal transduction mechanisms important here. I decided to explore the important aspects of muscle development, primary myogenesis, secondary myogenesis and adult muscle regeneration. For secondary myogenesis, I focused on the Wnt/ß-catenin pathway, an important developmental pathway but unexplored in the context of muscle fiber development. With respect to the muscle precursor maintenance and adult muscle regeneration, I decided to concentrate on the role of d-Asb11. This protein was established as a principal regulator of early neurogenesis but its role in other lineages remained unexplored. Its expression pattern (at least of its human homologues) suggested high expression in muscle tissue, warranting research in this area.

In **Chapter 3**, we report that timely and dosage regulated restriction of the Wnt/β-catenin pathway is required for safeguarding myofibrillogenesis and regulating muscle growth. We provided evidence that this may work through genetic interaction with Myostatin.
In **Chapter 4**, we report that the Cullin box domain in d-Asb11 is necessary for proper Notch activation in vivo. We show that there may also be a Cullin box independent role in her4::gfp Notch activation.
In **Chapter 5**, we report that d-Asb11 is a principle regulator of both embryonic as well as regenerative vertebrate myogenesis. Disruption of d-Asb11 resulted in perturbed early myogenesis, i.e. myogenic compartmental size, and less efficient regeneration.
In **Chapter 6**, we report that d-Asb11 regulates myogenesis via regulation of CKM. We speculate that this works via a direct interaction with CKM, as downstream modulation of Notch signaling does not rescue the phenotype. However, further experimentation is required to prove this.
In **Chapter 7**, we report the feasibility of using Evans Blue Dye as a marker of muscle injury in zebrafish. We provide a detailed protocol with video showing the procedures.
This thesis is concluded in **Chapter 8**, and discussions pertaining to the research chapters are further elaborated here.

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CHAPTER 2

Anchoring skeletal muscle development and disease: The role of ankyrin repeat domain containing proteins in muscle physiology

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Submitted

The protein family of Ankyrin repeat-containing proteins derives its name from the Ankyrin polypeptides that serve as anchor proteins and thus constitute a critical structural component in the erythrocyte membrane (Bennett, 1978). Since the discovery of this protein more than 30 years ago, Ankyrin has emerged as a multifunctional protein, present in a variety of tissues and cell types, including skeletal and cardiac myocytes (Ayalon et al., 2008; Hashemi et al., 2009), neurons, photoreceptors (Kizhatil et al., 2009a; Kizhatil et al., 2009b), and epithelial cells (Bennett and Baines, 2001; Kizhatil et al., 2007a; Kizhatil et al., 2007b). Although Ankyrins fulfill important functions in many cell types, anchoring cytoskeletal components to the intracellular machinery in muscle tissues is especially important. Ankyrins are especially prominent in contractile tissues and genetic knock-out experiments convincingly demonstrate the importance of Ankyrins in this respect (Borzok et al., 2007; Mohler et al., 2004; Mohler et al., 2003). This point is further highlighted by the high expression of Ankyrins in the muscle types of non-vertebrates (Chen et al., 2001), showing the strong evolutionary pressure that exists on the presence of Ankyrin proteins in this type of tissue.

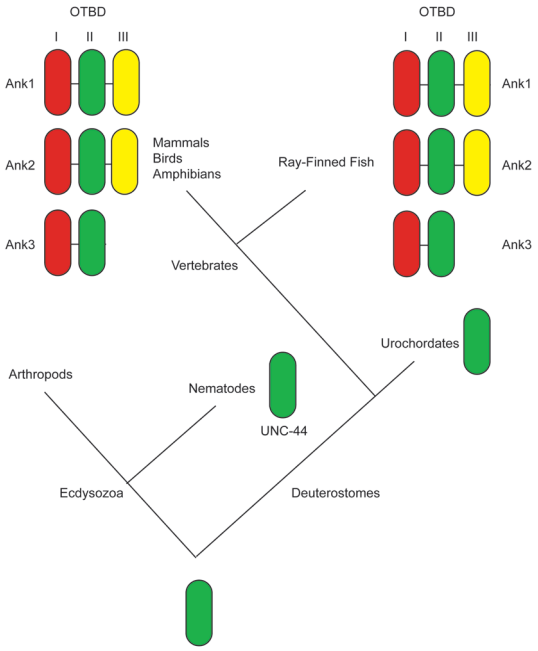

Figure 1. Proposed model of evolutionary events leading to Obscurin-Titin-binding domain (OTBD) in present-day ankyrins. In vertebrates, successive duplications led to 3 different modules I, II and III. Ank1 and Ank2 has all three modules, while Ank3 has only modules I and II. Adapted from Hopitzan et al. (Hopitzan et al., 2006).

In higher vertebrates, there are three canonical ankyrin genes: ANK1 (Ankyrin-R polypeptides) (Lux et al., 1990), ANK2 (Ankyrin-B polypeptides)(Otto et al., 1991) and ANK3 (Ankyrin-G polypeptides)(Kordeli et al., 1995), with only ANK1 (Lambert and Bennett, 1993; Lux et al., 1990) and ANK3 (Kordeli et al., 1995; Kordeli et al., 1998; Mohler et al., 2004; Peters et al., 1995; Thevananther et al., 1998) being expressed in the skeletal muscle. The presence of three ankyrin genes

is likely due to genome duplications in vertebrates. The nematode C. elegans and urochordate C. intestinalis possess only a single ankyrin gene, while the genome of arthropoda such as D. melanogaster contains two ankyrin genes. One view of Ankyrin evolution is that they are a solution to the problems of independent motility in Metazoans by contributing to membrane resilience to the forces of muscle contraction (Bennett and Baines, 2001; Hopitzan et al., 2006). Based on the obscurin-titin binding domain (OTBD), at the C-terminal domain of ankyrins, the Kordeli group described a proposed evolutionary event leading to present day ankyrins (Figure 1) (Hopitzan et al., 2006). Interestingly, a vertebrate-specific module of the OTBD is expressed exclusively in muscle tissues, after the divergence from Urochordates. Following the discovery and resolution of the primary sequence of Ankyrin proper, it soon emerged that a variety of other proteins contained one or more repeats of a motif that bear structural resemblance to a stretch of 33 amino acid residues present in the original Ankyrin protein, and was thus named Ankyrin repeat (Sedgwick and Smerdon, 1999). Such Ankyrin repeats were first identified in the sequence of yeast Swi6p, Chc10p and Drosophila Notch (Breeden and Nasmyth, 1987), and was later named after the cytoskeletal protein Ankyrin as it consists of 22 tandem 33 amino acid Ankyrin repeats (Lux et al., 1990). As Ankyrin repeats are present abundantly in a multitude of proteins in all branches of eukaryotic life, the ankyrin repeat as a motif almost certainly predates the ancestral eukaryote living approximately 2.3 billion years ago. The potential of Ankyrin repeat proteins to interact strongly with itself has made this motif exquisitely suitable for functioning as part of a membrane anchor in muscle tissue, explaining the importance of Ankyrins for muscle contraction. In addition, it has emerged that the ankyrin motif is present in many other genes expressed in muscle. In the present review, we aim to explore the various functions of the ankyrin repeat domain for skeletal muscle physiology and come to the conclusion that the ankyrin repeat domain is unusually important for the biochemistry of contractile tissue.

Ankyrin Repeat Proteins

Following the recognition that a pan-eukaryotic ankyrin repeat motif existed, further investigations have identified a multitude of sometimes very different proteins that display such ankyrin repeats in their primary structure. Often these proteins as a whole, as well as the ankyrin repeats in particular, exhibit strong evolutionary conservation, which is testimony to the versatile action

ankyrin repeats can have in cellular function. Indeed, established functions for ankyrin repeat-containing proteins are diverse and include regulation of transcription, cell cycle, cell fate determination, cytoskeletal integrity, cellular mechanosensation, and endocytosis (Mosavi et al., 2004). The suitability of ankyrin repeat proteins to act in many diverse physiological settings is dependent on its capacity to interact with other polypeptides, especially with other ankyrin repeats. Furthermore, it is unique in its capacity to be stable both in the highly different redox potential settings of the intracellular and extracellular compartments (Michaely and Bennett, 1992; Sedgwick and Smerdon, 1999). Many investigators have speculated on the importance of this interaction to allow development of complicated multilcellular life forms (Marcotte et al., 1999). Ankyrin repeat proteins typically function in mediating specific protein-protein interactions, although recently it has been shown to be required for an enzymatic function as well (Rider and Zhu, 2009). A literature search on the cellular roles of ankyrin repeats reveals a strikingly high proportion of muscle-specific publications (12% against e.g. < 4 % for PH or SH2 domains), which may be related to the unusual strong nature of ankyrin repeat interactions which can easily survive the mechanical strains of changes in cell shape and the changes in pH and oxidative status that characterizes the muscle cell. An exhaustive screen of the available literature on ankyrin repeat containing proteins is given in Table 1, and subdivided with respect to subclass within the repeat superfamily of proteins.

ANK1, sANK1 and ANK3 are members of the ankyrin superfamily, which is composed of proteins that are ubiquitously expressed and typically found within the membrane associated cytoskeleton. ANKG112, a small cytoplasmic ankyrin isoform, is also important for vesicle transport. Less is known regarding the roles of Asb family of proteins in muscle development, although various Asb proteins are found to be expressed in the skeletal muscle. The most well-studied are the muscle ankyrin repeat proteins, which are generally important for stress response. While 50% homology is relatively high, the tissue distribution of the three MARPs is different (CARP highest in cardiac muscle, Ankrd2 and DARP in skeletal), and there is no upregulation or compensation of the remaining MARP when one or more are removed (Barash et al., 2007). The question of the functional homology of the three genes, therefore, remains unclear. The possible functions and importance for muscle-expressed members of the superfamily of ankyrin repeat domain-containing proteins (which also include the Notch protein) will be the subject of this review, the order of the proteins described following the course of their expression during myogenesis.

Table 1. Ankyrin repeat proteins expressed in skeletal muscle

Protein	Number of repeats	Organism	Function	Partners	References
Canonical Ankyrins					
sANK1	2		Linker between sarcomere and sarcomeric reticulum (SR)	Obscurin	(Borzok et al., 2007)
ANK3/ANKG	24		SR and post-synaptic membrane organization		(Kordeli et al., 1998)
ANKG119	13		Cell membrane organization and vesicle transport	BIΣ-spectrin	(Devarajan et al., 1996)
Ankyrin and SOCS box containing proteins					
ASB2β	11	Mouse	Differentiation	FLNb	(Bello et al., 2009)
ASB5	6	Rabbit, Mouse	Not known		(Boengler et al., 2003; Seale et al., 2004)
ASB8	4	Human	Not known		(Liu et al., 2003)
ASB11	6	Zebrafish	Proliferation and maintenance of muscle progenitor compartment	Ckm?	Unpublished data (Chapter 5)
ASB15	10, 7	Mouse, Human	Protein synthesis, Differentiation	Akt	(McDaneld et al., 2006; McDaneld and Spurlock, 2008; Yoshida, 2005)

Muscle Ankyrin repeats					
Ankrd2	4		Stress response	Titin YB1	(Miller et al., 2003)
CARP	4		Stress response	Titin Myopalladin	(Bang et al., 2001)
DARP	4		Stress response, energy metabolism	Titin Myopalladin	(Ikeda et al., 2003)
Other Ankyrin repeat proteins					
Myotrophin	3	Rat	Intitiation of muscle hypertrophy	Actin capping protein; NFKB	(Furukawa et al., 2003)
Notch	7		Muscle differentiation	SKIP	(Zhou et al., 2000)
βCAP73	6	Bovine	Cell motility		(Welch and Herman, 2002)
Tankyrase2	24	Human	Cytoplasmic signal transduction	Grb14	(Lyons et al., 2001)

Skeletal muscle progenitor cells arise from the paraxial mesoderm which gives rise to the somites. Somites are formed sequentially as segments of the paraxial mesoderm on each side of the neural tube, from anterior to posterior, at regular time intervals. Somites are transient structures that later differentiate into different types of tissues that will give rise to several trunk structures: sclerotome (precursor of the bones, cartilages and tendons), myotome (precursor of muscle) and dermatome (precursor of the dermis) (Brand-Saberi and Christ, 2000) (Figure 2). The primary myotome is formed as the first differentiated muscle from the dermomyotome between E11.5 and E15.5 in the mouse. There, some myoblasts irreversibly exit the cell cycle, align with each other, and fuse, forming multinucleated myotubes. After primary myogenesis, secondary myoblasts in the dermomyotome use the primary myotome as a scaffold to attach to and fuse with each other, giving rise to secondary myotubes (Bryson-Richardson and Currie, 2008). A similar molecular process of myogenesis occurs postnatally, to recruit adult muscle precursors into forming new myofibers during skeletal muscle damage.

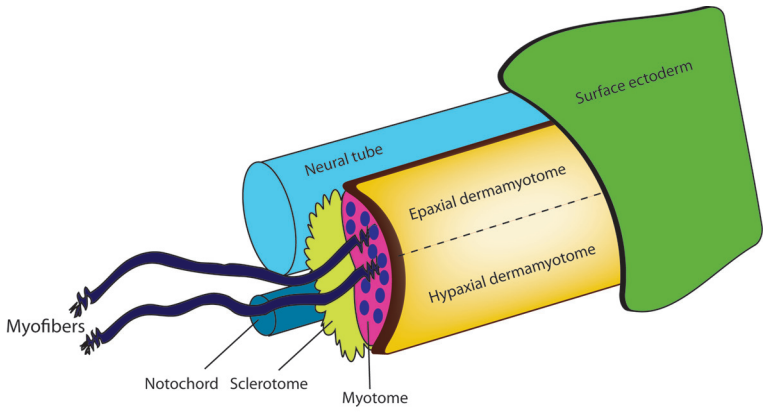

Figure 2. Caricature showing the structures in the skeletal muscle.

The genetic basis for muscle formation and the signaling pathways involved in patterning the myotome is similar in all vertebrates. Primary myogenesis is initiated by signals from the notochord (SHH), neural tube (WNT) and overlying surface ectoderm (FGF) that induce the expression of the basic-helix-loop-helix myogenic regulatory factors: myogenic factor 5 (Myf5), myogenic factor 6 (Myf6, also known as Mrf4), myogenic differentiation (MyoD), and myogenin (Myog). Several ankyrin repeat proteins have been shown to negatively regulate these myogenic regulatory factors, and will be described below.

Notch ICD

Notch is emerging as an important molecule in organogenis and very broadly, it can be stated that it acts to stimulate proliferation in progenitor compartments whilst simultaneously inhibiting/delaying differentiation. Notch inhibits myogenesis caused by Myf5 or MyoD (Kopan et al., 1994) or inhibits DNA binding by mef2c and its cooperation with myod and myogenin (Wilson-Rawls et al., 1999). The ankyrin repeat located in the ICD of notch plays a significant role in these inhibitory actions for myogenesis (Kopan et al., 1994; Wilson-Rawls et al., 1999). Thus Notch ICD is a crucial determinant of compartment size in pregestational muscle.
It is important to note that proper Notch-induced gene expression in many cases involves a process called lateral inhibition. Once Notch signaling is initiated by its ligands of the Delta family, Delta is downregulated in the Notch signaling cell, in turn this causes diminished Notch signaling in the neighboring cells, which react to upregulate Delta, amplifying differences between adjacent cells. Powerful negative feedback mechanisms, however, act on this lateral inhibition and a substantial original bias is essential for Notch signaling to ensue. Recent work showed that in the developing nervous system especially, the subfamily of six ankyrin repeat domain containing ASB proteins is important for creating the original bias that allows lateral inhibition to develop (Diks et al., 2008). We discuss below that ASB proteins are also important in later stages of muscle development, by controlling important steps of muscle cell differentiation, probably at least partly though control of Notch signaling.

Ankyrin Repeat and SOCS Box Containing Proteins (ASB)

Ankyrin repeats and SOCS box (ASB) family of proteins contain two functional domains: an ankyrin repeat region where specific protein-protein interactions occur, and a SOCS box region, which serves as a generic adaptor directing the degradation of proteins targeted by the ankyrin repeat region (Kile et al., 2002). To date, 18 ASB proteins have been identified. The 18 ASB proteins have varying forms and number of ankyrin repeats and other novel regions, suggesting they bind differing target proteins (Li et al., 2007). Human ASB3 and ASB8 proteins are strongly expressed in the skeletal muscle (Chung et al., 2005; Liu et al., 2003), while ASB6, ASB7 and ASB9 proteins are weakly expressed in the skeletal muscle (Human Protein Atlas). In mice, Asb2, Asb5, Asb8, and Asb10 proteins are strongly expressed in the skeletal muscle (Kile

et al., 2001; Kile et al., 2000). Interestingly, Asb5 was found to be expressed in both quiescent and activated satellite cells (Boengler et al., 2003; Seale et al., 2004), as well as 3 days after differentiation (Seale et al., 2004). Although ASB proteins are known to be expressed in skeletal muscle for some time now, the important functional role of ASBs in skeletal myogenesis has only recently received recognition. Different ASBs, however, have markedly different actions in muscle development, maybe as a consequence of the different number of ankyrin repeat these proteins contain.

Asb-5 is expressed in the earliest phase of muscle development following somitogenesis in the embryo, as it is present in the MyoD positive myogenic cells. Asb-5 forms together with Asb-9, Asb-11, Asb-13 and Asb-15, a specific subfamily of the ASB protein family, with the amino acid bases being highly similar, and are different from the other ASBs as they contain 6 ankyrin repeats. The functional importance of Asb-5 expression is not known, but the homologous Asb-11 is essential for canonical Notch signaling by allowing lateral inhibition (a process that leads to the formation of complementary expression of Notch and Delta in early embryogenesis) and Notch signaling is an established driver of further muscle cell differentiation at this stage of development. Furthermore, in C2C12 cells, a cellular model for certain aspects of myogenesis, showed that forced expression is sufficient to drive transactivation of a Notch reporter (Peppelenbosch, 2004). Thus, it is tempting to speculate that Asb-5 expression helps myogenesis at this stage. Our group identified that zebrafish Asb11 (which is evolutionary equivalent both to mammalian ASB9 [which is expressed in muscle] and mammalian ASB11 [which is not readily detected in muscle]) is expressed in and important for maintenance of the muscle precursor pool (Tee, 2010) (unpublished data). Furthermore, adult zebrafish with a mutation resulting in homozygous loss of Asb11 was found to be less efficient in muscle regeneration post-injury (Tee, 2010) (unpublished data). Thus, sequential expression of the homologous Asb5 and Asb-9 driving Notch signaling may be an important determinant of muscle development.

Asb2β protein, containing 11 ankyrin repeats, was identified in chick embryonic as well as adult skeletal muscle and regulates muscle differentiation by targeting actin /myoblasts fusion and myotube formation, and thus this protein seems important for this later phase of muscle development (Bello et al., 2009).

Finally, Asb15 is expressed in fully differentiated muscle and has emerged as a regulator of protein synthesis, probably via activation of mTor and MAPK pathways and subsequent activation of p90Rsk and p70S6 kinase (McDaneld

and Spurlock, 2008). As protein synthesis is an important adaptive response towards strain and exercise, this ankyrin repeat containing protein seems important in a specific phase of myogenesis. So, in toto a picture emerges in which different ASB proteins govern the transition of one specific phase of muscle development to the next (Figure 3).

Myotrophin/V-1 Ankyrin Repeat Containing Proteins

Myotrophin is a ubiquitously expressed 12kDa cytoplamic protein (Sivasubramanian et al., 1996a) that was first isolated from the hearts of spontaneously hypertensive rats (Sen et al., 1990), and 2 years later in the rat cerebellum, where it was named V-1 protein (Taoka et al., 1992). Protein sequencing (Taoka et al., 1992) and cDNA cloning (Taoka et al., 1994) revealed that myotrophin/V-1 was composed of 117 amino acids and 78% of the entire molecule was occupied by two and a half internal 33 amino acid ankyrin repeats (alternatively cdc10/SW16 motif) arranged in tandem.
Myotrophin/V-1 is evolutionarily conserved and is expressed at low basal levels in every mammalian organ and cell type (Anderson et al., 1999; Sivasubramanian et al., 1996b) with the least expression in skeletal muscle (Sivasubramanian et al., 1996b). The levels of Myotrophin/V-1 were found to be elevated in tissues of failing human hearts (Sil et al., 1993), although the levels of these proteins gradually decreased in human plasma during the progression of heart failure (O'Brien et al., 2003). Myotrophin/V-1 has been shown to stimulate protein synthesis in cardiomyocytes leading to hypertrophy, as well as the expression of a number of cardiac genes (e.g. beta myosin heavy chain and atrial natriuretic peptide) and proto-oncogenes (e.g. c-myc, c-fos and c-jun) (Gupta et al., 2002; Gupta and Sen, 2002; Hayashi et al., 2001; Knuefermann et al., 2002; Mosavi et al., 2002; Mukherjee et al., 1993; Sivasubramanian et al., 1996a) and hence seems to play an important role in muscle adaptation to increased load. How this relates to its functions in non-muscle tissue, however, remains unclear.
Myotrophin/V-1 resembles a truncated form of I-KBα protein without the signal response domain, nuclear localization signal masking domain and PEST degradation domain (Knuefermann et al., 2006). The ankyrin repeats in Myotrophin/V-1 are capable of interacting with the rel domain of NF-Kβ protein, which is also an ankyrin repeat containing protein itself (Knuefermann et al., 2002). Several studies have proposed that Myotrophin/V-1 is an extracellular growth factor, which functions to initiate cell surface signal transduction events leading to cardiac hypertrophy (Sen et al., 1990; Sil et

al., 1998). Contrasting studies however shows that extracellular expression of Myotrophin/V-1 does not provoke hypertrophy (Pennica et al., 1995; Yamakuni et al., 2002), and that its function is mainly intranuclear, acting as a modifier of NFKβ in the nucleus, possibly by promoting the formation of Rel family homodimers over heterodimers. As NF-KB activation is a predicted response to challenging muscle load, it is easy to envision how such a nuclear function could be implicated in the regulation hypertrophic response. Also, the presence of a nuclear localization signal and the absence of a clear secretion signal (like is present in IGF-1, to which in the original publications on the extracellular functioning of Myotrpohin/V-1 the protein was compared), we strongly favor the nuclear hypothesis, although definitive experiments that include the introduction of Myotrophin/V1 variants that lack the nuclear localization domain could help provide the definitive answer here (Gupta et al., 2002; Knuefermann et al., 2006).

Although an action for myotrophin/V-1 as a hypertrophic molecule in cardiac muscle is fairly well established, there is less known regarding the role of Myotrophin/V-1 in skeletal muscle. A study showed that exogenous application of Myotrophin/V-1 to skeletal muscle cells has hypertrophic effects, suggesting that the protein has at least the potential to act as hypertrophic molecules in such tissue. However, whether it also functions as such in practice is still a very open question (Hayashi et al., 1998). Expression of myotrophin/V-1 in myoblasts decreases during the process of muscle differentiation, reaching an undetectable level in mature skeletal muscle, suggesting that it does not have a major physiological role in this context. In contrast, the expression of myotrophin/V-1 is markedly increased in regenerating muscles of Duchenne muscular dystrophy and of its animal model, mdx mouse (Furukawa et al., 2003). Thus, further work is necessary to address this issue.

Muscle Ankyrin Repeat Proteins (MARPs)

There are three identified proteins in the family of muscle ankyrin repeat proteins (MARPs): CARP/MARP, Ankrd2/Arpp, and DARP. All three molecules were identified previously by their cytokine-like induction following cardiac injury and muscle denervation (CARP/MARP) (Aihara et al., 2000; Baumeister et al., 1997; Kuo et al., 1999), skeletal muscle stretch (Ankrd2/Arpp) (Kemp et al., 2000), or during recovery after metabolic challenge (DARP) (Ikeda et al., 2003). These three isoforms share in their C-terminal region, a minimal structure composed of four ankyrin repeats involved in protein-

protein interaction, PEST motifs characteristic of proteins targeted for rapid degradation protein, and at the N-terminal region, a putative nuclear localization signal (Lydie et al., 2009; Miller et al., 2003). The members of this nuclear as well as cytoplasmic family of proteins (Ishiguro et al., 2002; Tsukamoto et al., 2002; Zou et al., 1997) are found in the central I-band of the sarcomeres, where they bind to the N2A region of titin (Miller et al., 2003), and the amino terminus of nebulin anchoring protein myopalladin (Bang et al., 2001). The titin-binding domain is located in the second ankyrin repeat in all three proteins (Miller et al., 2003). Their induction following strain and muscle injury and their capacity to reinforce muscle structure through their interaction with structural elements of contractile machinery by introducing the highly robust pH- and redox-insensitive ankyrin bonds as a responsive to excessive demand to the muscle tissue, supports their function as a resource of last resort to maintain muscle function despite high demands. In agreement with this notion, mice lacking all three MARP proteins show a relatively mild phenotype, with a trend towards a slow fiber type distribution, but without differences in muscle fiber size (Barash et al., 2007) Thus expression of this family of ankyrin repeat domain-containing proteins seems a way of muscle cells to deal with excessive mechanical load. CARP also known as C-193, was originally isolated as a cytokine responsive gene in fibroblasts (Chu et al., 1995), but its main action seems to lie in the heart, where it helps in controlling hypertrophic reponses by providing negative feedback to the genomic cardiac hypertrophic response. However, as a cytoplasmic structural protein, it reinforces the cardiac contractile machinery, also a response which acts to limit the consequences of excise demand on the heart pump function. Support for this view comes from the observation that it is naturally upregulated during hypertrophy and downregulated during atrophy and that aberrant upregulation of this protein can actually drive atrophy under certain conditions (Baumeister et al., 1997; Stevenson et al., 2003; Yang et al., 2005). CARP is expressed throughout all the heart chambers. Furthermore, the protein is also present albeit much more weakly expressed in skeletal muscle (Ishiguro et al., 2002; Tsukamoto et al., 2002), where it probably serves similar functions, although this has been less well investigated. Its possible beneficial role as a cardiac anti-hypertrophic mediator has over the past 15 years prompted a significant research effort into this protein. As to be expected from such an anti-hypertrophic gene, CARP inhibits cardiac-specific gene expression and hence its expression is differentially regulated between embryonic and adult heart (Baumeister et al., 1997; Jeyaseelan et al., 1997; Kuo et al.,

1999; Zou et al., 1997), as to allow proper cardiogenesis. The protein has both a nuclear and a cytoplasmic action, its role in the negative feedback on cardiac hypertrophic genomic responses dependent on the former form of the protein (Jeyaseelan et al., 1997; Zou et al., 1997), although not all mechanistic details as to how nuclear CARP influences gene expression have been elucidated. Overexpression in cardiomyocytes results in suppression of cardiac troponin C and atrial natriuretic factor transcription (Jeyaseelan et al., 1997). CARP interacts with integral components of the muscle such as desmin and titin (Mikhailov and Torrado, 2008). CARP also interacts with the transcription factor YB1 and inhibits the synthesis of the ventricular specific myosin light chain 2v (MLC-2v) (Zou et al., 1997).

As stated, the molecular function of CARP in skeletal muscle is less known, but also there it seems mainly to act in limiting the consequences of excessive load. Recently, it has been suggested that CARP is important for sarcomere length stability and muscle stiffness, as well as having an inhibitory role in regenerative response of muscle tissue (Barash et al., 2007). CARP overexpression induces a switch towards fast-twitch fibers (Lydie et al., 2009). Interestingly, CARP was found to be expressed exclusively in small regenerating myofibers in muscular dystrophy patients (Nakada et al., 2003b) as well as significantly upregulated in numerous muscular dystrophy models and denervation induced atrophy (Lydie et al., 2009). In vascular smooth muscle cells, increased CARP expression has been demonstrated to be associated with upregulation of the protein p21WAF1/CIP1, and inhibitor of the cell cycle (Kanai et al., 2001), which might also be seen as a protective responsive. Thus CARP as protein involved in limiting damage to muscle overactivation does not show an absolute restriction towards the skeletal muscle lineage.

Like CARP, Ankrd2 acts to limit damage following excessive demand on muscle and accordingly it was first identified as a stretch responsive gene product upregulated in stretched muscle (Kemp et al., 2000). Ankrd2 expression is not easily induced, upregulation only seen under eccentric contractions, while most other muscle proteins such as myoD, myogenin, muscle LIM protein and CARP are sensitive to mechanical strain under both isometric and eccentric contractions (Barash et al., 2004; Hentzen et al., 2006). Thus also Ankrd2 induction seems to be a protection mechanism of last resort. Ankrd2 shows a destinctive preference for expression in slow skeletal fibers and cardiac atria (Kojic et al., 2004; Pallavicini et al., 2001). Ankrd2 interacts with transcription factors YB-1, PML and p53 (Kojic et al., 2004; Pallavicini et al., 2001), and is localized to PML bodies in proliferating myoblasts where

it modulates their transcriptional activity. Ankrd2 accumulates in the nuclei of myofibers located adjacent to severely damaged myofibers after muscle injury. It translocates from the I-band to the nucleus after muscle injury, and may participate in regulation of gene expression (Tsukamoto et al., 2008). Hence, different from CARP, it only acts on the transcriptional level and thus does not serve as a structural component, maybe because other ankyrin repeat-containing proteins are induced at lower levels of muscle stress and occupy the available binding sites for such proteins in the contractile machinery.

The least studied MARP is DARP, which is expressed in both heart (low expression) and skeletal muscle (high expression). It was identified by its upregulation in Type 2 diabetes and insulin-resistant animals. Thus, DARP has been implicated with a potential role in energy metabolism (Ikeda et al., 2003). Similar to CARP, DARP interacts with titin-N2A and myopalladin (Miller et al., 2003).

Application of Ankyrin Repeat Proteins in Muscle Disease

As evident from the above, the different stages of muscle development and their phenotypic reaction to strain and exercise are under the control of different ankyrin repeat domain containing proteins and accordingly, their expression at different stages of muscle development seems to be tightly regulated. This offers the obvious possibility that manipulating such expression may be useful for dealing with muscle diseases. These hopes are especially fostered now that adeno-associated virus gene therapy introduced in human muscle has proven both save and useful for the treatment of LPL deficiency (Mingozzi et al., 2009) and thus introduction of specific ankyrin repeat containing proteins into patients is certainly technically and ethically feasible.

Muscle diseases such as muscular dystrophies or inherited myopathies have a general characteristic of progressive muscle weakness and degeneration. In the past decade, great advances have been made to the clinical studies on muscle disease. The most recent advances in studies on clinical as well as experimental muscle disease, such as muscular dystrophies and related myopathies, as well as the state of our present knowledge on these diseases have been recently reviewed in (Cardamone et al., 2008) and (Willmann et al., 2009). In a recent gene expression profiling of patients in the pre-symptomatic phase of Duchenne muscular dystrophy (DMD), altered expression of more than 30 ankyrin repeat proteins was identified (Pescatori

et al., 2007), which makes sense in view of the importance this family of proteins has in dealing with stress and damage to muscle tissue. Of special interest is the recent discovery that ankyrin proteins (ankyrinB and ankyrinG) bind to dystrophin and dystroglycan respectively, and are required for the retention of these proteins at the costameres (Ayalon et al., 2008), further highlighting the importance of the ankyrin repeat domain in the context of the demands muscle physiology makes on protein-protein interactions. This point is especially vividly illustrated by a Becker muscular dystrophy mutation, which reduces ankyrinB binding, and impairs sarcolemmal localization of dystrophin-Dp71 (Aartsma-Rus et al., 2006; Ayalon et al., 2008) causing disease and thus demonstrates the deleterious muscle-specific consequences of failure of ankyrin bonding to occur. In line with the role of ankyrin repeat domain proteins as a last line of defense against excessive muscle load is the increase in CARP expression with an array of muscle pathologies: DMD, spinal muscular atrophy, facio-scapulo-humeral muscular dystrophy, amyotrophic lateral sclerosis, and peroxisome proliferator-activated receptor induced myopathy (Casey et al., 2008; Nakada et al., 2003a), as well as the mdx, Swiss Jim Lambert (SJL) and muscular dystrophy with myotitis (MDM) animal models, deficient respectively in dystrophin, dysferlin and titin (Bakay et al., 2002; Nakada et al., 2003a; Nakada et al., 2003b; Nakamura et al., 2002; Porter et al., 2002; Suzuki et al., 2005; Witt et al., 2004). Expression of MARP is reduced in dystrophic muscle (Pallavicini et al., 2001) but increased following denervation (Tsukamoto et al., 2002), in a mouse model of muscular dystrophy with myositis due to titin N2A deletion and in heart failure (Zolk et al., 2002). Thus human muscle disease highlights the special importance of the ankyrin bond for muscle physiology.

In apparent agreement with such a role, ankyrin repeat containing proteins on skeletal muscle is not limited to muscle degenerative diseases, Ankrd2 was detected in approximately 90% of rhabdomyosarcoma tissues but only when accompanied by morphological evidence of skeletal muscle differentiation of tumour cells (Ishiguro et al., 2005), suggesting that in the context of neoplastic dedifferentiation and functionality expression of ankyrin repeat proteins correlates with functionality. It would be interesting to investigate to which extent expression of such protein is sufficient to counteract dedifferentiation. In any case, the fact that all these muscle abnormalities are associated with expression of specific ankyrin repeat domain proteins fits well with notion that developmental programs are controlled (and structural elements also partially effected) by expression of specific ankyrin repeat domain proteins. On a related note, the high binding affinity of ankyrins in

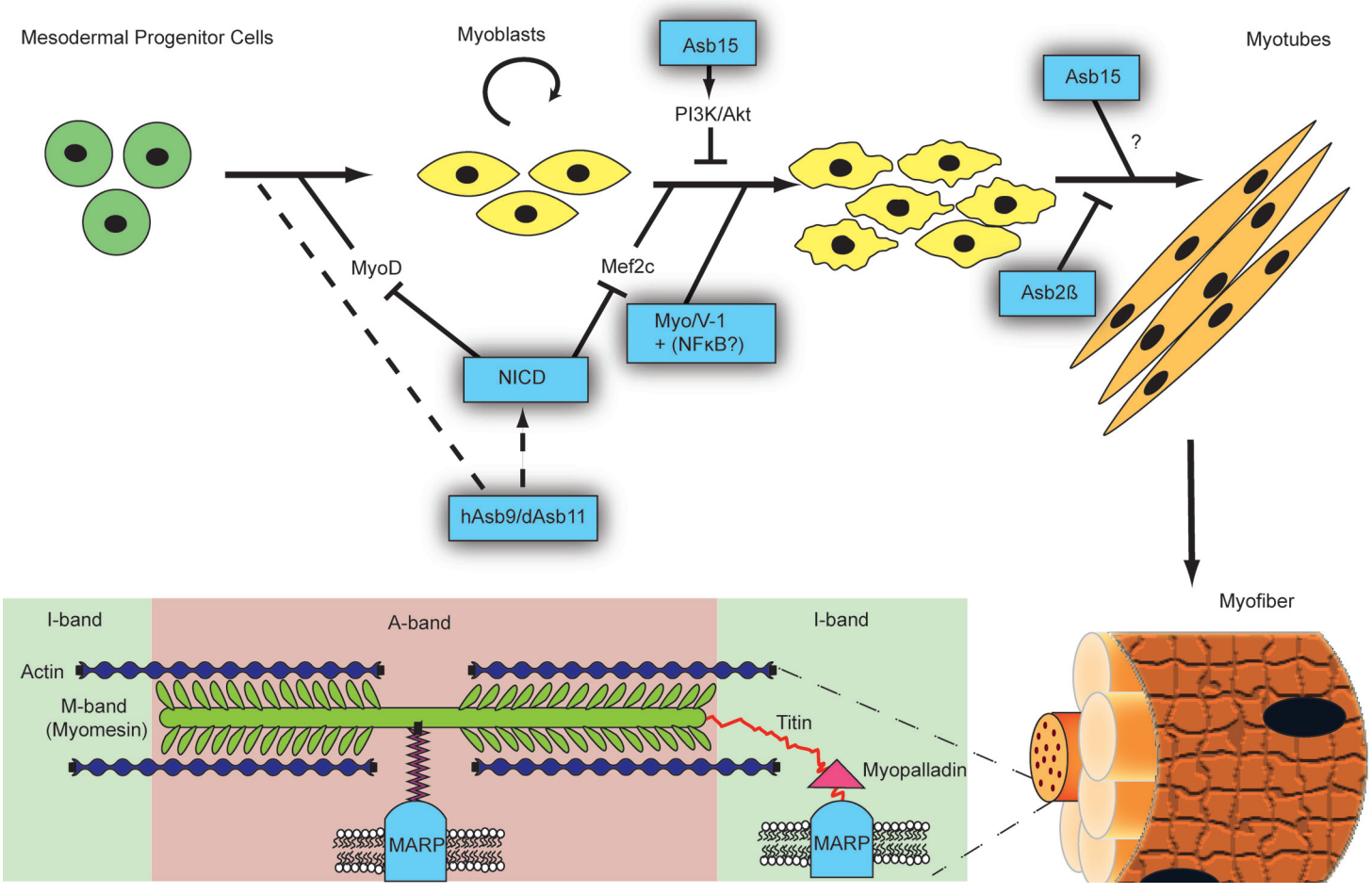

Figure 3. Summary of the ankyrin repeat proteins in muscle biology.

strengthening and maintaining the skeletal muscle structure suggests that the induction of ankyrin repeat containing proteins in skeletal myopathies may play a role in the survival of the diseased muscle fibers. Many clinical conditions such as heart failure, inflammatory myopathies, chronic arthritis, and aging are associated with muscle wasting and weakness. Furthermore, elderly or bed-ridden patients and space travelers, undergoing long periods of muscle disuse often show signs of muscle weakness and atrophy. While myostatin (Sharma et al., 2001; Zimmers et al., 2002) and glucocorticoids (Tischler, 1994) have been studied for a role in atrophy, and both can induce atrophy in normal muscle, neither is required for disuse atrophy in vivo (McMahon et al., 2003; Tischler, 1994). Kadarian and Hunter has recently shown in vivo that inhibition of the ankyrin repeat containing proteins Bcl-3 and NfKb1prevents muscle atrophy (Hunter and Kandarian, 2004). Now that the clinical tools are coming available that allow temporary expression of proteins in humans, it is envisionable that patients after long bed rest and muscle disuse might be treated by expression of muscle strengthening ankyrin repeat domain proteins in the most important skeletal muscles to aid revalidation.

Concluding remarks

Although the ankyrin bond has a general importance for vertebrate cellular biochemistry and physiology, its specific properties have led for it to acquire specific functions in muscle biology (Figure 3). The specific expression of different ankyrin repeat domain containing proteins during the various phases of muscle development allows this module to mediate specialized functions during muscle development. Obviously this allows for a high level of regulation, but also offers the opportunity for clinical use during muscle specific disease. We predict that further research will further reveal unique functions for ankyrin repeat domain containing superfamily members in muscle cell physiology.

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Declaration of Interest

The authors report no declaration of interests.

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CHAPTER 3

Regulation of slow and fast muscle myofibrillogenesis by Wnt/ß-catenin and Myostatin signaling

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Introduction

Understanding muscle development is crucial for generating novel regenerative therapies for muscle diseases and treating muscle injuries. Extensive research has contributed to the current understanding of various aspects of somitogenesis and myogenesis. The periodicity of rostral-caudal somite formation [1] as well as their differentiation into the axial skeleton, skeletal muscle and dorsal dermis are similar in all vertebrates [2]. Furthermore, the zebrafish determinate muscle growth by hyperplasia, the increase in muscle fiber number, and by hypertrophy, the increase in muscle fiber size, are comparable to mammalian muscle growth making it a suitable model system to study myofibrillogenesis and various myopathies [3-5].

Wnt/β-catenin pathway plays a crucial role in early somitogenesis and myogenesis in birds [6,7], mice [8-10] as well as in zebrafish [11] by affecting skeletal muscle development at several levels, including mesodermal patterning, segmentation clock and myoblast differentiation [12,13]. The Wnt/β-catenin signaling regulates Lef/Tcf –mediated transcription of downstream target genes via the transcriptional coactivator β-catenin [14]. In the absence of Wnt ligand, β-catenin is targeted for proteosomal degradation by a "destruction complex" comprising of CK1, GSK3β, Axin1 and Apc1. The fine balance between proliferation and differentiation required for proper development and growth of the myotome depends on signaling cues originating from tissues surrounding the somites [15,16], including Wnt ligands. Cumulative evidence implicates Hedgehog and Fgf8 signaling in specification and differentiation of slow and fast twitch muscle fibers respectively, during the first wave of myogenesis [17-20]. Although recent work has shown the role of Hedgehog signaling in differentiation of a subset of secondary slow twitch muscle fibers [19], the precise molecular mechanism underlying specification and maintenance of secondary fast twitch muscle fibers as well as the Hedgehog independent slow twitch muscle fibers remains to be elucidated.

This study shows that upward deviation from the tightly controlled physiological level of Wnt/β-catenin activity by genetic and chemical intervention in zebrafish embryos leads to compromised growth and maintenance of slow and fast muscle fibers. This phenotype derives from hyperproliferation of the Pax3/7+ premyogenic precursors. Hence, misexpression of p21CIP/WAF or mstn in the embryos with gain-of-Wnt/β-catenin function restores the integrity as well as morphology of the fast muscle fibers. We further discuss the possibility that this tight and opposing regulation of myofibrillogenesis

by Wnt/β-catenin and Myostatin could operate through their genetic interaction.

Results

Wnt/β-catenin hyperactivity causes loss of somites and aberrant muscle fibers

Wnt/β-catenin gradient has been shown to be important for somite segmentation [9]. Importantly, it has been suggested that Wnt/β-catenin is downregulated in the somite following skeletal muscle differentiation [21]. We investigated the expression of Wnt/β-catenin reporter TOPdGFP [22] during post-segmentation corresponding to the second wave of myogenesis. Consistent with previous studies [21], we observed only faint expression in the trunk and tail of wild-types at 28 hours post-fertilization (hpf) (Fig. 1A). Next, by employing the homozygous compound zebrafish mutants of axin1 [23,24] and apc1 [25] (hereafter referred to as axin1/apc1), we investigated whether Wnt/β-catenin is hyperactivated in the somites. Indeed, there was strong ectopic expression of the TOPdGFP reporter in axin1/apc1 mutants in a rostro-caudally rising gradient (Fig. 1A). This corresponded to enhanced expression of the Wnt transcription factor and its direct target gene lef1 [26] throughout the somites (Fig. 1B), showing that Wnt pathway is overactivated in the somites of axin1/apc1 mutants.

At completion of segmentation and the first wave of myogenesis at 24 hpf, axin1/apc1 embryos had a normal number of somites, size of somites (Fig. S1), as well as normal muscle fiber formation (data not shown). The earliest clear somite phenotype in axin1/apc1 mutants was at 36 hpf, with a slight decrease in somite number from approximately 31 to 29 (Fig. 1, C and D). Strikingly, at 54 hpf, there was a severe tail truncation due to loss of approximately 10 somites (Fig. 1, C and D). The formation of normal somites at 24 hpf (Fig. S1) eliminates somite fusion and abnormal initiation of segmentation as an underlying cause of somite loss. Hence, this late and gradual somite-loss strongly suggests that the underlying mechanism does not entail a defect in somite induction and/or patterning.

Next, we examined whether upregulation of the Wnt/β-catenin signaling would affect the fast- and slow-twitch muscles that make up the myotome. The slow muscle fibers appeared to be hypertrophic, as well as hyperplastic with an additional 2-4 fibers per somite (n=4) (Fig. 2A). Strikingly, the fast muscle fibers were disorganized, with some muscle fibers detaching from the

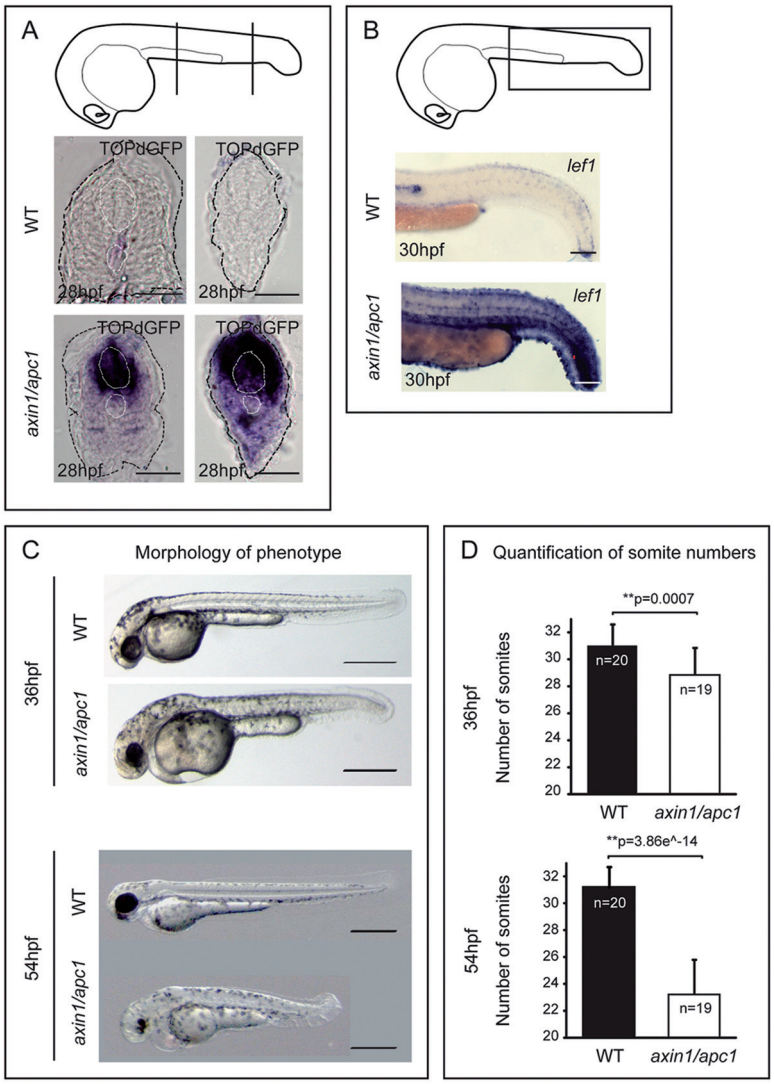

Figure 1. Hyperactivation of Wnt/β-catenin pathway leads to late somite-loss. (A) TOPdGFP transgenic embryos report activated Wnt/β-catenin signaling, i.e. TOPdGFP transcripts. Cartoon depicts the level of vibratome sectioning i.e. left panel at the yolk extension and right panel caudal to the yolk extension. Scale bar, 50 µm. (B) Hyperactivation of a target of Wnt/β-catenin pathway lef1, as shown with WISH, in axin1/apc1 mutants matches the expression of TOPdGFP. Scale bar, 250 µm. (C) The axin1/apc1 embryos are slightly shorter compared to wild-type embryos at 36 hpf, top panels. At 54 hpf, the difference becomes striking, bottom panels. Scale bar, 500 µm. (D) Somite counts at 36 hpf and 54 hpf, corresponding to embryos depicted in (C) with error bars showing the standard deviation.

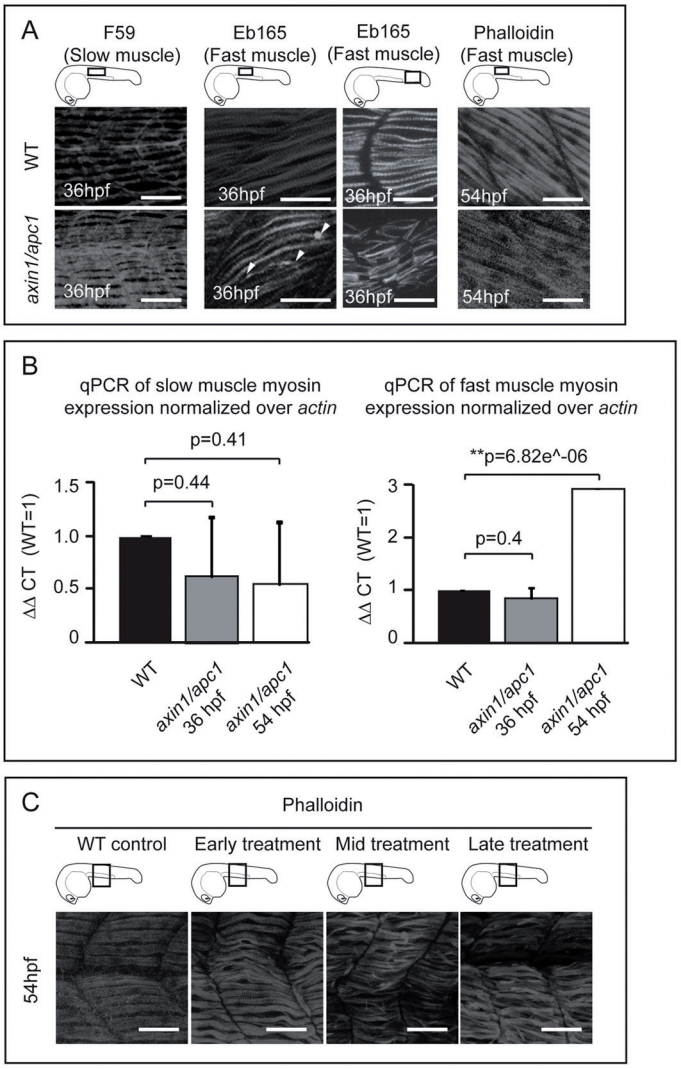

Figure 2. Late hyperactivation of Wnt/β-catenin pathway leads to aberrant myofibrillogenesis. (A) axin/apc1 embryos at 36 hpf have thickened slow muscle fibers, left panels. The fast muscle fibers at 36 hpf are detached from the vertical myoseptum, forming lesions (white arrow heads) and are disorganized, middle panels. At 54 hpf, fast muscle fibers are thickened (hypertrophic), right panels. All embryos were imaged at the level of the yolk extension, except for the third panels from the left where images were taken more caudally, as depicted in cartoons . Images for F59 and Eb165 at 36 hpf are cumulative z-stacks. Images for Phalloidin at 54 hpf are single z-plane at the level of fast muscle fibers. Scale bar, 25 µm. (B) Quantitative real-time PCR (qRT-PCR) of myhz2 (fast muscle specific) and myhz5 (slow muscle specific) mRNA expression normalized to actin. Total RNA was isolated from 36 hpf and 54 hpf wild-type and axin1/apc1 embryos. Graphs show that the quantity of of myhz5 is not significantly different in axin1/apc1 embryos, failing to identify subtle difference as shown in Fig. 2A. The quantity of myhz2 is upregulated in axin1/apc1 embryos at 54hpf. (C) LiCl treatment during various time intervals. Early treatment = tailbud stage and 16 hpf for 40 minutes each on the same clutch of embryos, mid treatment = at 16 hpf and 24 hpf and late treatment = at 24 hpf and 30 hpf. Embryos were stained with Phalloidin for visualization of all muscle fibers. All embryos were imaged at the level of the yolk extension, as depicted in cartoon. Images are cumulative z-stacks. Scale bar, 25 µm.

vertical myoseptum, forming small lesions, while becoming hypertrophic only at 54 hpf (Fig. 2A). Confirming the distinct effects of hyperactive Wnt/β-catenin on slow versus fast muscle fibers, quantification by RT-qPCR of myosin heavy chain specific for slow or fast twitch muscle fibers revealed an increase in fast muscle myosin at 54hpf (Fig. 2B). However, there is no significant difference observed for slow muscle myosin. We confirmed the specific role of Wnt/β-catenin by employing a chemical activator of the Wnt/β-catenin pathway, lithium chloride (LiCl). The wild-type embryos treated with LiCl prior to completion of somitogenesis, at tailbud and mid-somitogenesis were truncated and curled albeit no detached muscle fibers were present (Fig. 2C,

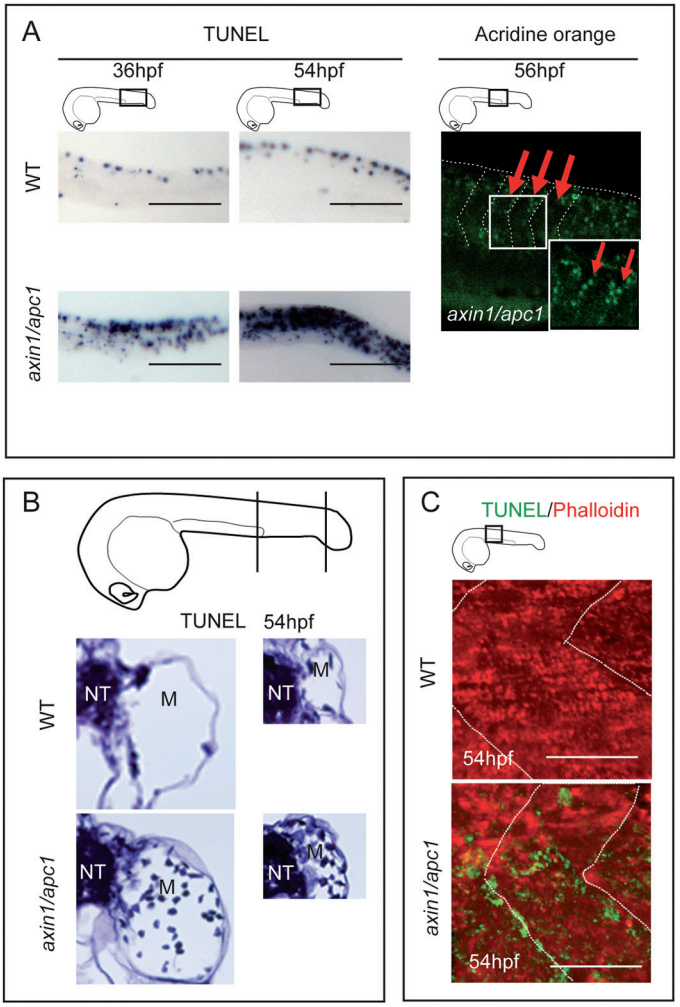

Figure 3. Apoptosis at somite boundaries and muscle fibers. (A) From 36 hpf onwards, axin1/apc1 embryos show an increase in apoptosis as labeled by TUNEL. Insets show the imaged area. Scale bar, 100 µm. Apoptotic cells labeled with acridine orange lining up at the somites boundaries, right panel. Arrows and lines mark the somite boundaries. Scale bar, 25 µm. (B) TUNEL labeling at 54hpf show an increase in apoptotic cells in the myotome. Cartoon depicts the level of sectioning i.e. left panel at the posterior end of the yolk extension and right panel posterior end of the tail. NT-neural tube; M-myotome. (C) Co-labeling of fluorescent TUNEL assay (apoptotic cells) and phalloidin labeling (muscle fibers), left and middle panel. This CLSM image was taken caudal to the yolk extension, at a single z-plane of 5µm of fast muscle fibers.

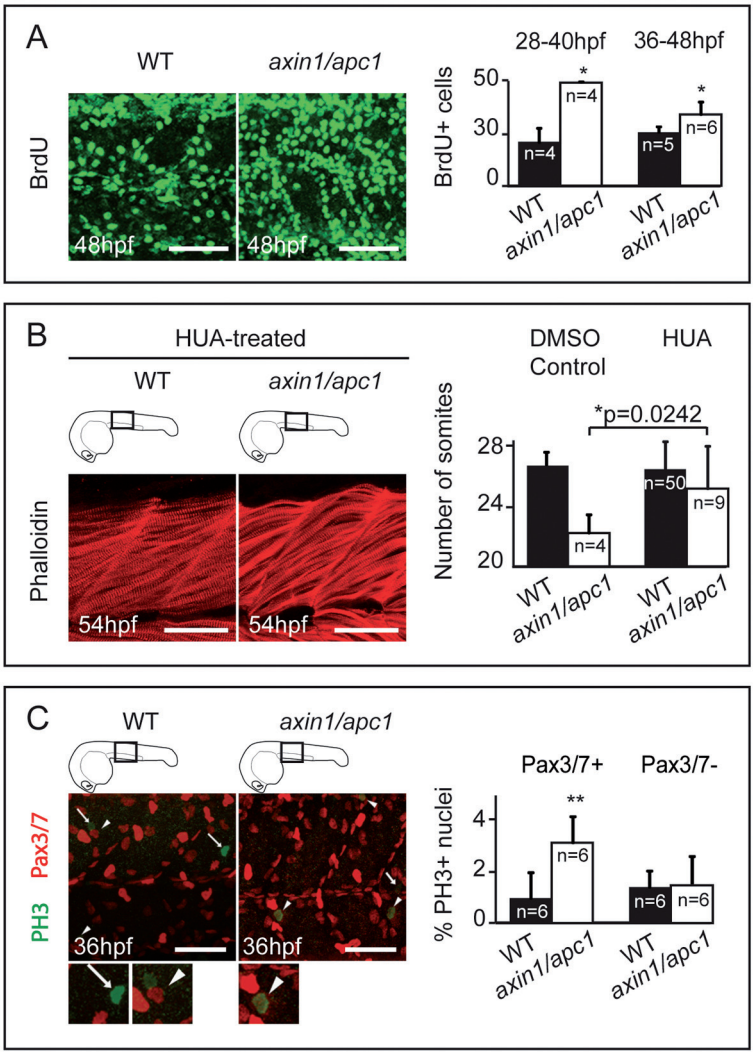

Figure 4. Myotome hyperproliferation and sustained differentiation in axin/apc1 embryos. (A) BrdU pulse was performed at 36 hpf, chased for 12 hours, and imaged at 48 hpf. Embryos were imaged at the level of the yolk extension. Scale bar, 25 µm. BrdU+ pulse was performed at 28 hpf or 36 hpf, and quantification of number of BrdU+ proliferating cells per somite was done 12 hours later at 40 hpf or 48 hpf, respectively. (C) HUA treatment of embryos from 24 hpf until fixation at 54 hpf. Inhibition of proliferation with HUA from 24 hpf results in rescue of muscle hypertrophy. Muscle fibers were stained with Phalloidin, and imaged at 54 hpf at the level of the yolk extension. Compare with untreated wild-types in Fig. 2A (right panels). Scale bar, 25 µm. Quantification of number of somites is increased in axin1/apc1 mutants upon HUA treatment. (E) Colocalization of Pax3/7+ and PH3+ cells shows proliferating muscle progenitors. Quantification of proliferating Pax3/7+ and Pax3/7- cells in the wild-types vs. axin1/apc1 mutant embryos shows significantly more proliferating Pax3/7+ cells in the mutants. Scale bar, 50 µm.

early treatment). In contrast, the embryos treated after 24 hpf showed severe muscle fiber detachment and hypertrophy (Fig. 2C, mid-treatment and late-treatment), resembling axin1/apc1 mutants. Hence, the muscle fiber defect in the axin1/apc1 mutants is likely also caused by late Wnt/β-catenin hyperactivation.
The fast muscle fiber degeneration beginning at 36 hpf, corresponded with gradual increase in apoptosis (Fig. 3A), with apoptosis occurring within the myotome (Fig. 3B) and along the vertical myoseptum (Fig. 3, A and C).

Hyperactive Wnt/β-catenin drives muscle progenitors into unscheduled proliferation

The ability of Wnts to enhance proliferation in the dermomyotome [27] led us to hypothesize that unscheduled proliferation in the somites might lead to muscle hypertrophy in axin1/apc1 mutants. While at 16 hpf there was no difference in proliferation between mutants and wildtypes (data not shown), from 28 hpf onwards, BrdU pulse experiments identified a sharp increase in number of cells in S-phase (Fig. 4A), which was confirmed by increased labeling of phosphohistone H3 (PH3)+ mitotic cells (data not shown) and their quantification by FACS (Fig. S2B). To investigate whether this unscheduled increased proliferation caused muscle hypertrophy, we partially inhibited cell division with a combination of aphidicolin [28] and hydroxyurea (HUA) [29] from 24 hpf until fixation at 54 hpf. Strikingly, the fast muscle hypertrophy (Fig. 4B; compare to Fig. 2A, right panels) and degeneration (Fig. S3A) as well as the number of somites were partially rescued (Fig. 4B) confirming that hyperproliferation leads to the fast muscle hypertrophy and degeneration.
Pre-myogenic embryonic muscle progenitor cells expressing Pax3/7 transcription factors give rise to myoD+ myoblasts, myogenin+ myocytes and myotubes, that terminally differentiate into muscle fibers [30]. To determine whether these Pax3/7+ progenitors were stimulated by Wnt/β-catenin to hyper-proliferate, we performed co-labeling of PH3 and Pax3/7 in the axin1/apc1 mutants. There was a significant increase in co-localization of PH3+ mitotic nuclei and Pax3/7+ nuclei in the axin1/apc1 mutants (Fig. 4C) as compared to wild-types, suggesting that Wnt/β-catenin drives unscheduled proliferation of pre-myogenic progenitors. Surprisingly, the absolute number of Pax3/7+ cells was unaltered in axin1/apc1 mutants (data not shown), suggesting that the newly born progeny of dividing Pax3/7+ cells was not maintained in a Pax3/7+ precursor state, but instead was instructed to differentiate. Hence, we examined myogenic differentiation in the mutants.

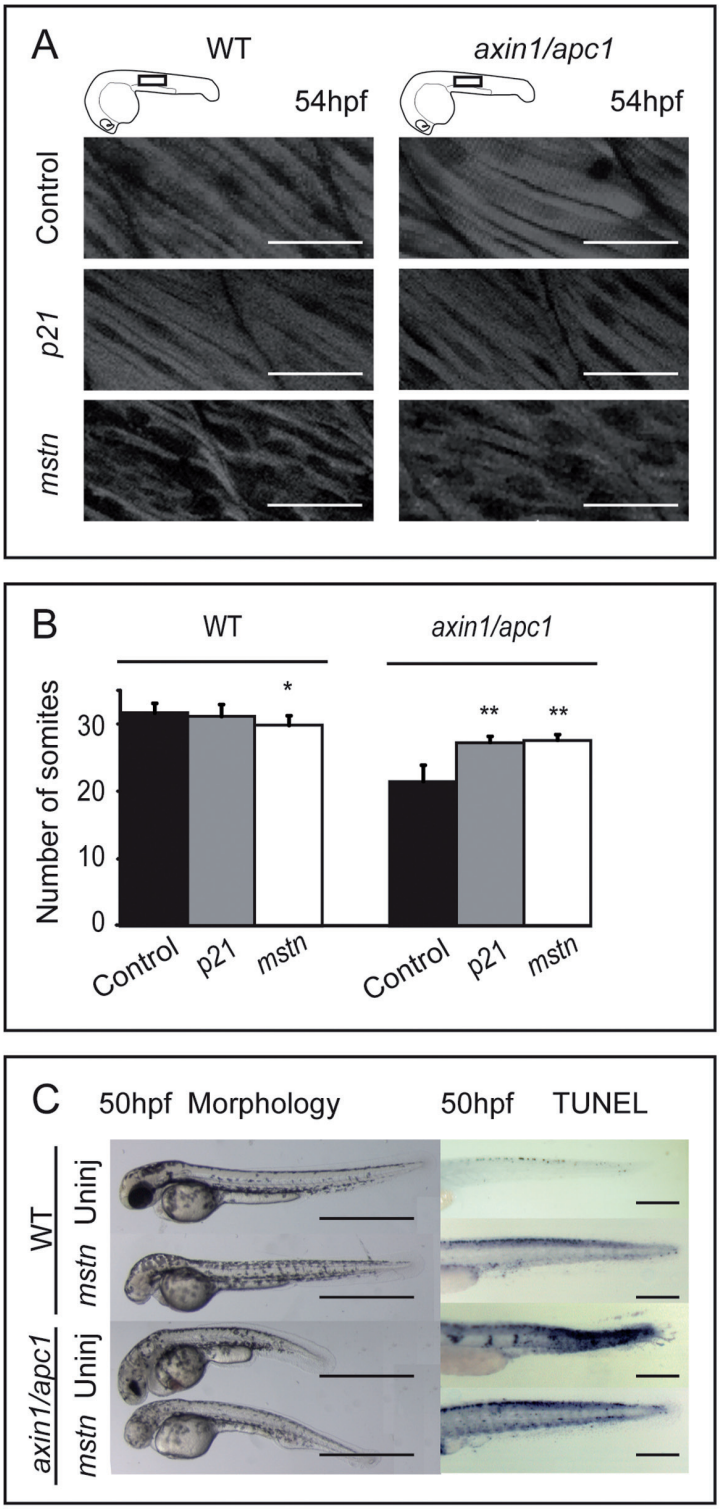

Figure 5. Misexpression of mstn rescues axin1/apc1 embryos. (A) Injection of 5 pg p21CIP/WAF or mstn mRNA into 1 cell stage embryos, and phenotype assessment at 54 hpf. Slightly hypotrophic muscle fibers are observed in p21 as well as mstn-injected wild-type embryos confirming efficiency of misexpression. Muscle hypertrophy in mstn-injected axin1/apc1 embryos is partially restored to normal. (B) Quantification of the somite number of uninjected and p21CIP/WAF or mstn mRNA (5pg) injected embryos. (C) Misexpression of mstn partially restores the truncated somite phenotype, as well as cell survival in axin1/apc1. Scale bar, 0.5 mm.

Consistent with the unperturbed Pax3/7+ muscle progenitors at 16 hpf, myoD was unaltered in axin1/apc1 mutants during initial myogenesis (data not shown). Importantly, later in development, its timely downregulation failed and its expression was sustained (Fig. S3B). Consistent with prolonged myoD expression in mutants, myogenin expression was also extended in axin1/apc1 (Fig. S3C). As myoD+ myoblasts are known to proliferate it is possible that the ectopic and extended myoblast maintenance also contributes to the hyperproliferation in the mutants. To test this, we performed co-labelling of anti-MyoD- with anti-BrdU antibody. We observed substantial increase in MyoD+ cells in axin1/apc1 embryos (Fig. S3D), confirming the increased myoD RNA expression in the mutants. However, we have not observed an increase in proliferating (BrdU+) myoD+ cells (data not shown). The data is consistent with a positive role of Wnt/β-catenin signaling in driving myogenic differentiation [13].

Myotomal proliferation and hypertrophy in axin/apc mutants are counteracted by misexpression of Mstn and its downstream target p21CIP/WAF

Reportedly, Wnt/β-catenin through its direct target c-myc, can downregulate p21CIP/WAF (also known as cyclin-dependent kinase inhibitor 1A) [31]. We hypothesized that sustained myotomal proliferation in axin1/apc1 mutants works through Wnt/β-catenin-mediated inhibition of p21CIP/WAF. We tested the hypothesis by examining the capacity of p21CIP/WAF mRNA injected into axin1/apc1 mutant to rescue muscle fiber phenotype. Employing misexpression with p21CIP/WAF mRNA concentration that only subtly affected the wildtypes, muscle fiber hypertrophy was rescued in injected axin1/apc1 embryos (Fig. 5A), suggesting that muscle fiber degeneration is due to hyperproliferation caused by failure of timely p21CIP/WAF-dependent cell cycle exit. However, we cannot exclude the possibility that forced cell cycle exit mediated by p21CIP/WAF misexpression in itself, and independently of its postulated positioning downstream of the Wnt pathway, may have brought about the rescue.
p21CIP/WAF has been implicated in muscle differentiation as the downstream target of Mstn, a TGF-β family member. Mstn is a key negative regulator of muscle growth that promotes terminal differentiation of embryonic muscle progenitors through the activation of p21CIP/WAF [32]. Decreased levels of Mstn, context dependently lead to muscle hypertrophy [32]. Based on the hypertrophic muscle phenotype in the compound mutants and upon late LiCl treatment of wildtype embryos, we hypothesize that p21CIP/WAF

may be epistatic to Mstn and that overactive Wnt pathway may through inhibition of Mstn downregulate p21CIP/WAF. We tested this possibility by performing rescue of muscle fiber degeneration in the axin1/apc1 mutants via misexpression of mstn mRNA. While at this particular concentration of mstn, wild-types showed slightly hypotrophic muscle fibers, the axin1/apc1 embryos showed a partial rescue of the hypertrophic muscle fibers (Fig. 5A). Consistently, misexpression of mstn rescued the number of somites (n=8) and length of mutant embryos (Fig. 5, B and C). Importantly, cell survival in axin1/apc1 mutants was also rescued (Fig. 5C, right panels).

To further explore opposing effects of Wnt/β-catenin and Myostatin on phenotypic aspects of myofibrillogenesis we asked whether morpholino (MO)-mediated knock-down of Mstn would result in a similar hypertrophic phenotype as hyperactive Wnt/β-catenin in zebrafish embryos. Injection of 5ng mstn MO resulted in hypertrophic muscle fibers, while injection of 2ng Lef1 MO resulted in hypotrophic muscle fibers (Fig. 6). We further co-injected 2ng Lef1 MO with 10ng Mstn MO, and asked whether muscle fibers would be hypertrophic or hypotrophic. The slow and fast muscle fibers appear hypertrophic (Fig. 6). As a loss of Mstn signaling would be expected to lead to hyperproliferation, and loss of Wnt/β-catenin signaling to reduction of proliferation of premyogenic precursors the data suggests that loss of Mstn is dominant over the loss of Wnt/β-catenin signaling.

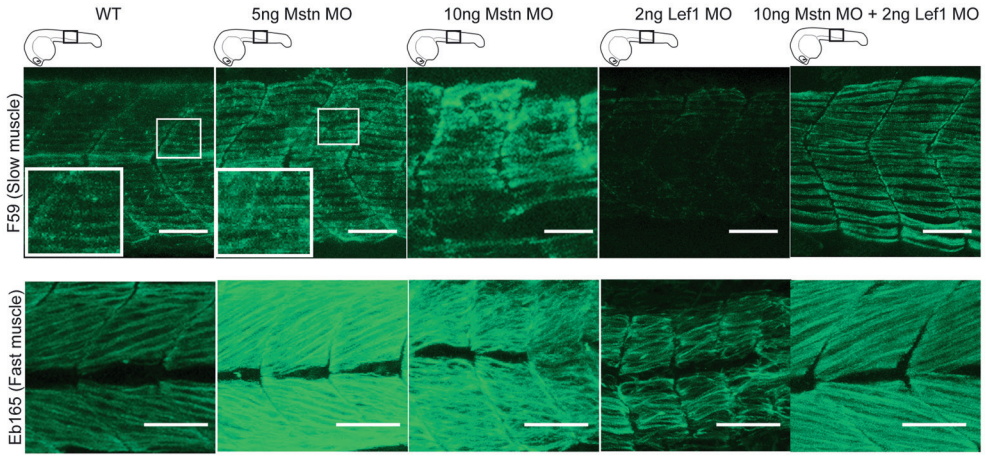

Figure 6. Mstn is dominant over Wnt/β-catenin in myofibrillogenesis. Representative images of injection of 5 (n=30)or 10ng (n=30)Mstn MO results in muscle hypertrophy, whereas injection of 2ng Lef1 MO (n=30)results in hypotrophic muscle fibers. Co-injection of 10ng Mstn MO with 2ng Lef1 MO (n=10) results in hypertrophic muscle fibers. Images of all embryos are cumulative z-stacks and taken at the level of the yolk extension, as depicted in cartoons. Scale bar, 50 μm.

As expression profiling in Mstn loss-of-function (LOF) identified modulation of Wnt- pathway components [33], we examined for possible genetic interaction between the two pathways, by using gain and loss of Wnt/β-catenin signaling. To establish a suitable genetic means for analysis of mstn mRNA upon loss of Wnt/β-catenin function, we first tested whether morpholino (MO)-mediated knock-down of Lef1 [22], which is upregulated in axin1/apc1 mutants (Fig. 1B) would rescue their aberrant somitogenesis. Knockdown of Lef1 with 2 ng MO in wild-types resulted in loss of a number of somites (59%, n=54) at 54 hpf (Fig. 7A), suggesting that Lef1 is required for normal somitogenesis. Notably, in 50% of Lef1-MO-injected axin1/apc1 mutants, the normal number of somites was restored, establishing a mechanistic link between Lef1 hyperactivity and somite loss (Fig. 7A).

To test whether GOF and LOF of Wnt/β-catenin signalling, affected the levels of mstn RNA we employed quantitative real-time PCR to quantify expression of mstn mRNA in axin1/apc1 mutants versus Lef1-morphants at different developmental times. The data showed that reduced Wnt/β-catenin signaling in Lef1 morphants resulted in upregulation of mstn mRNA (Fig. 7B, C), whereas hyperactivity of the pathway in the axin1/apc1 mutants led to a slight mstn downregulation at 36hpf (Fig. 7B), and a more significant downregulation at 54hpf (Fig. 7C). We then analyzed the level of Myostatin protein at 54hpf. Predictably and in agreement with the qPCR data the level of processed mature monomeric Myostatin protein was significantly induced in Lef1-morphants suggesting that reduced Wnt signalling through lef1 led to de-repression of Myostatin. Surprisingly, in axin1/apc1 embryos level of Myostatin protein is similar to that wild-type embryos suggesting that subtle increase of myostatin mRNA in the Wnt/β-catenin GOF context, does not translate into an increase in the stable protein (Fig. 7D). Why and how this mechanistically takes place need further investigation. As lef1 was upregulated in the somites of axin/apc mutants at 30 hpf, we tested whether misexpression of mstn would alter this Wnt/β-catenin downstream target gene. Misexpression of mstn downregulated ectopic lef1 in the mutants as well as slightly downregulating lef1 expression in wildtype siblings (Fig. 7B) showing genetic interation between Mstn and Wnt/β-catenin signalling and probably underlying the mechanism of muscle fiber rescue. To corroborate possible interaction between the two pathways we performed in silico analysis to identify putative TCF-binding elements (TBE) in myostatin promoter, as have been identified in the promoters of Wnt target genes [34]. Indeed, we found 3 putative TBE (NNCAAAG) within a 2.8kb sequence upstream of the myostatin gene at positions -2790, -2389 and -1578 (data

not shown), opening up the possibility of a direct interaction between Wnt and Myostatin.

Together, these data suggest an existence of a genetic interaction between Wnt/β-catenin and Mstn in myofibrillogenesis possibly existing as a negative

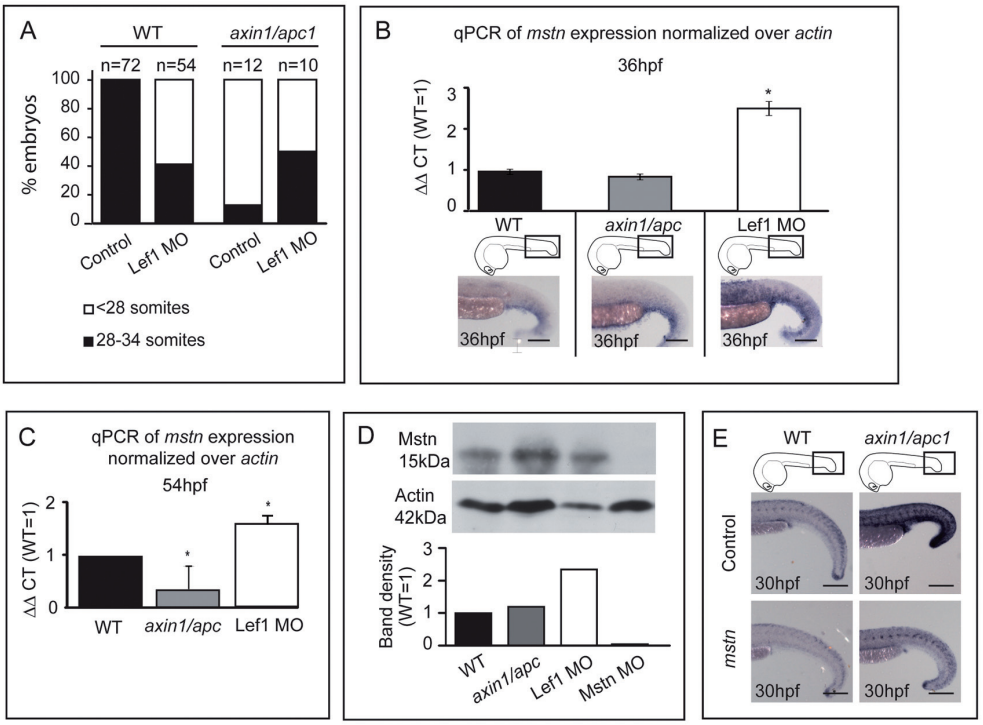

Figure 7. mstn is upregulated in LOF Wnt/β-catenin. (A) 2ng Lef1 MO was injected at 1-cell stage into wild-type or axin1/apc1 embryos, and the number of somites was counted at 54 hpf. Two independent clutches of axin1/apc1 heterozygous incross were analyzed (Total n=64), in which 10 were genotyped as axin1/apc1 homozygous. (B) Quantitative real-time PCR (qRT-PCR) of myostatin mRNA expression normalized to actin. Total RNA was isolated from 36 hpf wild-type, axin1/apc1 and Lef1 morphant embryos. Graphs show that expression of mstn is upregulated in Lef1 morphants, corresponding to the in situ hybridization with mstn probe in bottom panels. Scale bar, 100 µm. (C) Quantitative real-time PCR (qRT-PCR) of myostatin mRNA expression normalized to actin. Total RNA was isolated from 54 hpf wild-type, axin1/apc1 and Lef1 morphant embryos. Graphs show that expression of mstn is downregulated in axin1/apc1 embryos and upregulated in Lef1 morphants (D) Western blot on lysates collected from 54hpf. (E) Misexpression of mstn mRNA downregulates the Wnt target gene lef1 in axin1/apc1 mutants shown with WISH for lef1 riboprobe. Scale bar, 100 µm.

feedback loop . We propose a model describing regulation of fast muscle fiber growth and maintenance during secondary myofibrillogenesis with respect to Myosatin and Wnt signaling (Fig. S4).

Discussion

Most zebrafish mutants such as after eight (aei) and deadly seven (des) that harbor mutations in genes of the Delta-Notch pathway [35] display a reduction in somite numbers secondary to patterning. Together with Delta-Notch signaling, Wnt/β-catenin signaling contributes to somite clock establishment, mediating early somite patterning [36,37]. The unique phenotype of the axin1/apc1 mutant is characterized by normal somite patterning followed by a gradual loss of approximately 10 most caudal somites. Our data show that this somite loss, resulting from sustained, ectopic and hyperactive Wnt/β-catenin, is secondary to cell fate alterations, ultimately leading to hypertrophic and degenerative fast muscle fibers. The hyperactive Wnt signal is transduced mainly through Lef1 and leads to an imbalance between proliferation and differentiation in the myotome. The late activation of Wnt/β-catenin in wild-type embryos by treatment with LiCl phenocopies the fast muscle fiber hypertrophy and degeneration observed in axin1/apc1 embryos. This corroborates the notion that the mutant muscle fiber phenotype arises independently and is subsequent to normal somite establishment, providing an opportunity to decouple roles of Wnt/β-catenin in myofibrillogenesis versus somite patterning.

High Wnt/β-catenin activity is required for somite patterning, as well as for proliferation i.e. expansion of the Pax3/7+ pre-myogenic progenitor compartment. Pax3 and Pax7 transcription factors, that mark the pre-myogenic progenitors in the developing dermomyotome [38] and satellite cells in the adult muscle, positively regulate cell proliferation [39,40]. Wnt/β-catenin has been implicated in induction of Pax3/7+ precursors in cell culture systems [41,42]. A high Wnt/β-catenin activity is probably also required for proliferation of differentiating myoblasts. Our data suggest that the sustained upregulation of myoD and myogenin in axin1/apc1 reflects propensity towards differentiation of hyperproliferating premyogenic Pax3/7+ precursors being consistent with the known role of Wnt/β-catenin signaling in myogenic differentiation in several systems [43,44].

We show that hyperproliferative fast muscle fibers underlie the fast muscle fiber degeneration in the axin1/apc1 mutants, as partial inhibition of proliferation restored to near normal impaired cell survival and fast

muscle fiber hypertrophy. The hypertrophic muscle fibers in axin1/apc1 embryos indicate that a myotomal cell population(s) hyperproliferates and differentiates, thus resulting in an increase in the mass of the muscle fiber. Simultaneously, conflicting instructions to myoblasts to undergo premature differentiation likely leads to apoptosis. To our knowledge, Wnt/β-catenin has as yet not been implicated in muscle hypertrophy in vivo. Ex vivo studies of the adult muscle reveal the synergistic effect of insulin and Wnt/β-catenin in causing myotube hypertrophy [45]. In addition, it has been shown that Wnt/β-catenin is upregulated in overload-induced hypertrophy of the adult muscle [46]. As a conserved transcriptional hierarchy is thought to regulate the myogenic differentiation in embryos and adults [47], these reported data may be extrapolated to the developing myotome.

Several in vitro and in vivo studies showed that Mstn overexpression prevents proliferation and differentiation of muscle precursors by inducing expression of the cell cycle inhibitor p21CIP/WAF, while endowing muscle progenitors with competence to respond to signals favoring muscle differentiation [32]. We showed that simultaneous knockdown of Lef1 and Myostatin, resulted in a hypertrophied muscle fiber, similar to knockdown of Myostatin. This suggests that Wnt/β-catenin signalling could lie upstream of the Mstn regulatory pathway, as knockdown of Lef1 is unable to rescue the myofiber phenotype (Fig. 6), while misexpression of mstn rescues the fast muscle hypertrophy in axin1/apc1 embryos (Fig. 5A). Thus, Wnt/β-catenin might mediate sustained proliferation of muscle progenitors by repressing mstn. However, there is also the possibility that the rescue of myofiber growth is non-specific and Myostatin might work independently of and/or in parallel with Wnt/β-catenin signalling in regulating myoblasts proliferation and differentiation, We favor the possibility of a Wnt/β-catenin-Mstn negative feedback loop, as our experimental evidence points towards a specific interaction, direct or indirect, between Wnt/β-catenin signalling and Myostatin as follows: (1) We observe an upregulation of mstn RNA transcripts and protein upon Lef1 knock-down which may reflect release from repression of mstn by Wnt/β-catenin (2) The downregulation of lef1 mRNAexpression upon mstn misexpression in axin1/apc1 and wildtype embryos suggests a negative feedback loop between Wnt/β-catenin and mstn, likely reflecting the mechanism that underlies phenotype-rescuing capacity of Lef1; (3) The identification of 3 putative TBE within a 2.8kb region upstream of the Myostatin ATG start site opens up a possibility of a molecular interaction between Mstn and Wnt/β-catenin signaling. However, Wnt/β-catenin could mediate repression of Mstn indirectly, through induction of its direct target follistatin [48] that is a known

negative regulator of Mstn [49]. Whether and how this genetic hierarchy regulating myofibrillogenesis translates into direct molecular interactions is an important avenue for further research.

Unlike mice expressing dominant negative Mstn, which equally affects both fast and slow muscle fibers, the axin1/apc1 embryo exhibits different phenotypes with both slow and fast muscle fibers. Although hypertrophy and hyperplasia is observed in the slow muscle fibers at 36 hpf, there is a reduction in the total amount of slow muscle myosin RNA. We speculate that the lack of quantitative differences observed in the slow muscle fibers could be due to the fact that slow muscle fibers only make up a small portion of the myotome. Therefore, a small increase of slow muscle myosin is not quantifiable by qPCR. On the contrary, the fast muscle fibers exhibit muscle fiber degeneration and disorganization at 36hpf, and at 54hpf, they appear hypertrophic (Fig. 2A). This is confirmed by a significant increase of the total amount of fast muscle myosin at 54hpf (Fig. 2B). The fast muscle hypertrophy is likely to reflect a compensatory response to decreased muscle stability. Significantly, it is only the fast muscle fibers that degenerate in response to hyperactive Wnt/β-catenin signaling even prior to overt hypertrophy. In agreement to the upregulation of fast muscle myosin in axin1/apc1 embryos, it has recently been shown in cattle that knock-out mutations in myostatin result in preferential downregulation of fast 2X myosin heavy chain [50]. Consistently, mstn, which we showed is affected by Wnt/β-catenin pathway is predominantly found in fast twitch muscle [51]. This study opens up a prospect to unravel the poorly understood difference in regulation of maintenance and growth of secondary slow versus fast muscle fibers.

Although our work showed that Mstn negatively regulates Wnt/β-catenin, it is very likely that there is an involvement of other signals that mediate timely and dosage-regulated restriction of the Wnt/β-catenin pathway, thereby safeguarding myofibrillogenesis and regulated muscle growth. The pathogenetic mechanism of the muscle hypertrophy in muscle degenerative diseases is still unclear. Our data, implicating a possible role of Wnt/β-catenin signaling in interaction with Mstn and p21CIP/WAF, which have been shown to be important in muscle diseases, might pave a way to approaching muscle diseases from a novel angle.

Materials and methods

Zebrafish embryos

Zebrafish embryos were raised and staged as previously described[52]. apcCA50a/CA50a is a lethal recessive zygotic mutation identified in a three generation forward mutagenesis screen [25] according to standard mutagenesis protocol. axin/mbl(tm13) is a recessive lethal zygotic mutant obtained in the large scale Tubingen screen[24]. axin1/apc1 compound mutants were generated from crossing apcCA50a/CA50a with axin/mbl(tm13).

Fish/embryo genotyping

To verify phenotype/genotype correspondence, nested PCR was performed to amplify the template. First amplification was done using outer primer pair apc1forward(1)-apc1reverse(4) for identification of apc1 mutants, and outer primer pair axin1forwards(1)-axin1reverse(4) for identification of axin1 mutants. Second amplification for apc1 and axin1 was done using primer pair apc1forward(2)-apc1reverse(3) and primer pair axin1forward(2)-axin1reverse(3) respectively. Primer sequences are as follows: apc1forward(1) 5'-GTGCCTTAGAGGTGCAGAAG-3', apc1forward(2) 5'-GCAGTGTCCTTGTGGTTATG-3', apc1reverse(3) 5'-TGCCTTTACACATTGGTGAG-3', apc1reverse(4) 5'-CACAATCCTAACAAGCCATTC-3', axin1forward(1) 5'-ATGTGTCCTCCATTTGTCTG-3', axin1forward(2) 5'-TTTGTCTGTCCACATACCTG-3', axin1reverse(3) 5'-ACACCAGGAAATTCATCCAG-3', axin1reverse(4) 5'-GATGCTCCTTCATTCCAAAC-3'. DNA sequencing was performed using apc1forward(2) and axin1forward(2) to identify the specific genetic mutations as described previously [24,25].

In situ hybridization and immunohistochemistry

Whole-mount in situ mRNA hybridization (WISH) was carried out as previously described[53]. Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4˚C and digoxigenin-tagged probes were made with Roche labeling mix to TOPdGFP, myoD, myogenin, lef1, and titin. For mstn, exonic fragments were generated with the primers: T3mstn(f) 5'-ATTAACCCTCACTAAAGGGAGAATGAACATGCCACCACAGAA-3' and T7mstn(r) 5'-TAATACGACTCACTATAGGGAGATAATCCAGTCCCAGCCAAAG-3', and digoxigenin-tagged probes were made. Embryos were fixed for antibody staining with 4% PFA or Carnoy's and whole-mount immunohistochemistry was performed according to Du et al.

[54], using primary antibodies A4.1025 (Developmental Studies Hybridoma Bank) 1:20, Eb165 (Developmental Studies Hybridoma Bank) 1:250, Pax3/7 1:20 (gift from Prof. N. Patel), PH3 (Upstate Biotechnology #06570) 1:1000, MyoD 1:250 (Santa Cruz, C-20, sc-302). Appropriate secondary antibodies were used at 1:200. Immunohistochemistry was analyzed at the level of yolk extension where there is minimal muscle degeneration, unless otherwise stated, as caudal to the yolk extension there is massive apoptosis.

Phalloidin staining

Phalloidin-TRITC (Sigma) staining (1:50) was performed at room temperature overnight. Muscle fibers were analyzed at the level of the yolk extension where there is minimal muscle degeneration, as caudal to the yolk extension there is massive apoptosis.

Microinjection of mRNAs and morpholinos (MO)

Morpholino antisense oligonucleotides were obtained from Gene Tool (Philomath, OR): zflef1 (ATG) 5'-CTCCTCCACCTGACAACTGCGGCAT-3'[22] and zMstn (ATG) 5'-TGCATGTTCCAAGGCGTGCTAAAGG-3 . Capped synthetic mRNA was prepared from pCS2+ constructs encoding zebrafish mstn (gift from L.D. Valle) or human p21CIP/WAF (gift from C.J. Weijer) using the mMessage mMachine kit (Ambion), and injected into one-cell stage embryos using a microinjector (World Precision Instruments). A concentration range of 2.5-100pg of mRNA was injected into one-cell stage embryos to test for viability and effect, and the concentration which had only a subtle effect on wild-type embryos was selected. For axin1/apc1 rescue experiments, 5pg of mstn or p21CIP/WAF mRNA was used.

Cell quantification and imaging

Fluorescent labelings were imaged using a Leica TCS SPE confocal microscope. For each set of experiments, all laser and software settings were standardized. Images from each embryo were cropped in Volocity (Improvision) to exclude the neural tube. Cell counts in the somites were done manually from a z-stack of the whole somite.

For each set of experiments, cells were counted and imaged at the first four somites of the yolk extension, unless stated otherwise. For quantification of

Pax3/7+ pre-myogenic progenitor cells, only weakly labeled Pax3/7+ nuclei were counted as previously described [38]. Digital pictures of WISH embryos were obtained using the Zeiss Axioplan Stereomicroscope (comparable available microscope is Zeiss Axio Imager) equipped with a Leica digital camera and were adjusted for brightness and contrast using Adobe Photoshop 7.0.

Western blot and quantification

Embryos (54 hpf) were dechorionated, deyolked in deyolking buffer (5mM KCl, 10mM D-glucose in PBS), and lysed by sonification for 15 seconds in 50 mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium deoxyocholate and protease inhibitor cocktail (Complete mini, Roche). An equivalent of 12 embryos per lane was fractionated by 17.5% SDS-PAGE gel and blotted semi-dry to PVDF membrane (Millipore).

Membranes were stained with Coomassie blue stain to verify loading. Membranes were blocked in blockbuffer (50mM Tris-HCL, 150 mM NaCl, 0.25% gelatin, 0.5% Triton X-100, pH7.4) and incubated overnight at 4°C with rabbit anti-Myostatin antibody (AB3239, Millipore, 1:2500), washed 3x10min with 100mM Tris HCl pH 7.5, 0.1% Tween-20 and incubated for 1h at RT with secondary horseradish peroxidase conjugated anti-rabbit IgG antibody (#554021, BD Transduction laboratories, 1:10000), followed by enhanced chemiluminescence (Sigma Aldrich).

For actin-loading control, membrane was stripped in 62.5mM Tris HCl pH6.8, 2% SDS, 0,14 % b-mercaptoethanol, blocked in TBS-0.05% Tween + 5% milk and incubated with rabbit anti-actin antibody (A5060, Sigma Aldrich, 1/5000) in TBS-0.05% Tween + 2% milk overnight at 4°C, followed by HRP-conjugated anti-rabbit IgG antibody in TBS-0.05% Tween for 1h at RT, and developed by enhanced chemiluminescence. The film was scanned with GS-800 Calibrated Densitometer (BioRad) and quantitated with Quantity One 4.6.7 program.

Lithium chloride treatment

LiCl treatment (0.3M) was repeated twice on the same clutch of embryos for each of the 3 developmental intervals: (1) Early: LiCl treatment pulse for 40 minutes at tailbud and again at 16 hpf, (2) Mid: LiCl treatment pulse for 40 minutes at 16 hpf and again at 24 hpf, and (3) Late: LiCl treatment pulse at 24 hpf and again at 30 hpf. Embryos were washed 3 times in between tre-

atments. Upon treatments, embryos were fixed at 36 hpf, and stained with Phalloidin to visualize all muscle fibers.

HUA treatment

Embryos were cultured in both 75 µM aphidicolin with 0.25% DMSO (Sigma-Aldrich) and 20 mM hydroxyurea (Sigma-Aldrich) from 24 hpf to 54 hpf. Embryos were then fixed for further experiments.

BrdU labeling

For BrdU labeling experiments, embryos (16 hpf, 28 hpf, 36 hpf) were dechorionated and placed in 10 mM BrdU with 15% DMSO on ice for 1 hour. After pulsing, embryos were washed in embryo medium several times and incubated at 28˚C for 12 hours. Embryos were then fixed with 4% PFA and immunohistochemistry was performed as above, with incubation in 2N HCl for 1 hour prior to blocking.

RNA isolation and qRT-PCR

Embryos were injected with 2 ng Lef1-MO. At 36 hpf, 40 of each wild-type, axin1/apc1 homozygous and lef1-MO injected embryos were collected. Total RNA extraction and purification was performed using standard Trizol and isopropanol precipitation. cDNA synthesis was performed using hexamers and M-MLV Reverse Transcriptase. Concentration of purified cDNA was measured with Nanodrop. 50 ng cDNA was used for each set of primers. Transcript levels of myhz2, myhz5, actin and mstn were quantified by real-time PCR using iQTM SYBR® Green Supermix (Bio-Rad) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad). Results were expressed as a relative ratio to the housekeeping gene actin according to a mathematical method as described[55]. Primer sequences are as follows: mstn(F) 5'-GATTAACGCATATGACGCGAAG-3', mstn(R) 5'-ACAGTGAGAGGGTACCTGCAG-3', myhz2(F) 5'-ACAGTTTTTCAACCACCACATGTT-3', myhz2(R) 5'- AATGCAAGCGGCCAAGTC-3', myhz5(F) 5'- GCTGGAGAATGAGGTGGAGTTG-3', myhz5(R) 5'- AGTCTGGTAGGTGAGCTCCTTGA-3', ActinControl(F) 5'-CAACAGGGAAAAGATGACACAGAT-3', ActinControl(R) 5'-CAGCCTGGATGGCAACGT-3'. Accession numbers for mstn is NM_131019, myhz2 is NM_152982, myhz5 is AY333451 and actin is AF025305. Triplicates were carried out for each amplification.

Statistical analysis

Shapiro-Wilk normality test was performed with SPSS 16.0. All data followed normal distribution, with the exception of wild-type DMSO controls in Fig. 3d. Unpaired two-tailed student's t-test was performed using SPSS 16.0. For wild-type DMSO controls in Fig. 3d, where no normal distribution was observed, non-parametric Mann-Whitney test was used. All significant differences ($p<0.05$) are marked with an asterisk (*) and highly significant differences ($p<0.005$) are marked with two asterisks (**). All bars in graphs depict mean values with error bars depicting standard deviations.

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CHAPTER 4

d-Asb11 Cullin box domain is essential for proper Notch activation in neural cell fate decisions *in vivo*

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Submitted

Introduction

Ubiquitination and subsequent proteasomal degradation of regulatory proteins is implicated in the control of numerous cellular processes, including cell cycle progression, gene transcription, signal transduction, proliferation and differentiation (Sedgwick & Smerdon, 1999). The molecular determinants involved in protein ubiquitination in vivo, are still largely unknown, but involve the conserved SOCS-box domain containing proteins. The SOCS box plays a role as a substrate recognition module in an ECS type E3 ubiquitin ligase complex (Fig. 1A) (Kile et al, 2002). Its function is divided over two sub-domains: the Elongin-BC box, an adaptor that links SOCS box proteins to the Cullin-Rbx module; and a motif termed cullin box, located immediately downstream of the BC-box. The cullin box is proposed to be important for determination whether a given SOCS box protein assembles into either a Cul2-Rbx1 or a Cul5-Rbx2 module, and subsequently recruits and activates E2 ubiquitin-conjugating enzymes for ubiquitination of substrates (Kamura et al, 2004; Kohroki et al, 2005; Krebs & Hilton, 2000; Mahrour et al, 2008). However, at present there are no in vivo data as to the importance of cullin boxes.

Recently, we showed that the zebrafish Ankyrin repeat and SOCS box containing protein 11 (d-Asb11) regulates neural compartment size (Diks et al, 2006) via the ubiquitination of Notch ligand DeltaA, enabling lateral inhibition and canonical Notch signal transduction to occur (Diks et al, 2008). These effects of d-Asb11 absolutely require the d-Asb11 SOCS box, as d-Asb11 variants with a deleted SOCS box do not show any activity with respect to Notch activation in vitro and in vivo (Diks et al, 2008). Thus, d-Asb11 is an attractive model protein to assess the elusive functionality of the cullin box motif within the SOCS box domain.

Results and Discussion

Generation and characterization of d-asb11 mutant

We performed a TILLING screen on an F1 N-ethyl-N-nitrosurea (ENU)-mutagenized zebrafish library for d-asb11 mutations mapping to the putative consensus sequence (Wienholds et al, 2003). A premature stop codon corresponding to amino acid 281 in the conserved LPϕP sequence of the d-Asb11 was identified (Fig. 1B-C), and the homozygous allele was designated asb11^{cul}. To our knowledge, this is the first report of a metazoan mutant pre-

senting a mutation in the consensus sequence of any SOCS box containing protein, allowing for the first assessment of the in vivo functionality of the cullin box.

Morphological analysis of asb11^{cul} embryos reveals a slightly shortened trunk, as well as a minor hyperpericardium at 72 hours post-fertilization (hpf) (Fig. 2A). The somites are also less well-defined in the asb-11^{cul} compared with wild-type embryos suggesting that d-Asb11 in general and the cullin box in particular also participates in morphological signaling outside the ectodermal lineage. Whole-mount in situ hybridization (WISH) with d-asb11 RNA probe on 10 hpf embryos exhibit enhanced levels of d-asb11 transcripts in asb11cul compared to wild type, showing expanded expression in the polster and along the margins of the neural plate (Fig. 2B). Quantitative RT-PCR (qPCR) confirmed the increase of mRNA transcripts in asb11^{cul} (Fig. 2B). Accordingly, higher protein expression levels were detected by Western blotting on 12 hpf lysates from mutant embryos (Fig. 2C). No significant quantitative differences between wild type and heterozygous embryos confirmed the recessive nature of the mutation. The higher mRNA transcripts and protein levels suggest a compensatory effect of a hypomorphic mutation in the asb11cul embryo, thus suggesting that the cullin box mutation has consequences for d-Asb11 functionality.

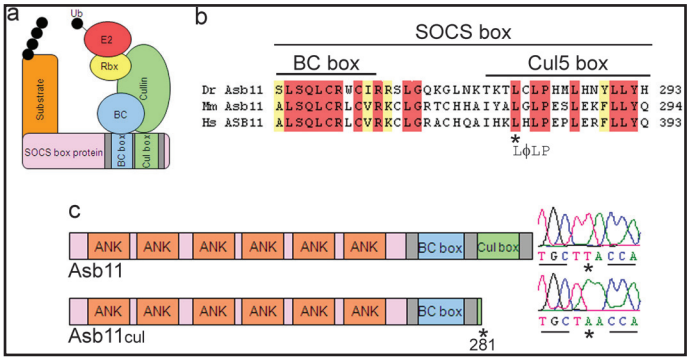

Fig. 1. Schematic representation of Asb11 proteins. (A), Asb11 functions as a substrate recognition module in a putative elonginC-cullin-SOCS-box (ECS) type E3 ubiquitin ligase complex. (B), Sequence alignment of conserved Asb11 SOCS box domain in different species. Consensus cul5-box sequence is indicated below the alignment. Identical amino acids are highlighted in red and very similar ones in yellow. Dr: Danio rerio; Mm: Mus musculus; Hs: Homo sapiens. (C), (left) Illustration of the wild type and mutant d-asb11 gene product. Mutated protein is represented as Asb11^{cul} showing the predicted residual fragment and the position of the identified mutation. The different domains are indicated ; (right) The T→Amutation changes a leucine to a stop codon.

Cullin box is required for correct expression of Notch genes

Morpholino-mediated knockdown of d-asb11 causes repression of

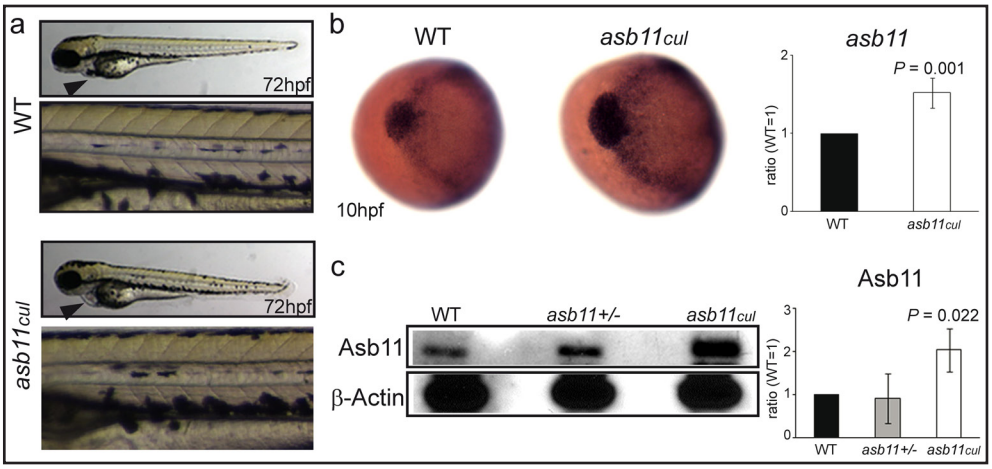

Fig. 2. Phenotypic assays on wild-type and asb11^{cul} mutant embryos. (A), Morphological analysis of wild type and mutant embryos at 72hpf. Somites phenotypes are shown in higher magnification. (B), (left) Anterior view of wild type and mutant embryos at 10hpf after whole mount in situ hybridisation, WISH, using probe against d-asb11. (right) Graph shows the quantification of the respective expressions using qPCR. (C), (left) Endogenous d-Asb11 protein was detected by immunoblotting of wild type (WT), heterozygous (asb11+/-) and mutant (asb11^{cul}) embryos at 12 hpf using anti-d-Asb11 antibody. (right) Graph quantifies 3 individual experiments, with 30 embryos/genotype/experiment.

specific Delta-Notch elements and their transcriptional targets, whereas mixexpression of d-asb11 induces Delta-Notch activity (Diks et al, 2008). To test whether the cullin box mutation has consequences for d-Asb11 functionality in regulating Delta-Notch signaling pathway, we first explored the capacity of the cullin box-deleted protein to activate, upon its overexpression, Notch-dependent transcription in vitro. We observed that overexpression of wild type d-Asb11 in human neuronal precursor cell line, NTera2 (Pleasure & Lee, 1993) leads to a strong activation of a Notch-mediated hes1 as expected based on previous results (Diks et al, 2008), however, overexpression of the mutant protein was not capable of doing so (fig. 4B). These results show that the d-Asb11 Cul5 box is essential for d-Asb11 activity in vitro. Subsequently, we investigated expression of Notch target genes in vivo by performing WISH on 12 hpf embryos for the Hairy/E(spl)-related transcription factors, her1, her4 and her5. At this timepoint, expression of her1 and her4 is considerably reduced in asb11^{cul} embryos (Fig. 3A-B). As her1 and her4 are known to be activated by constitutively active Notch signaling, this suggests that the Notch signaling pathway is disrupted in embryos lacking cullin box domain. In contrast, asb11^{cul} show a significant increase in her5 expression (Fig. 3C),

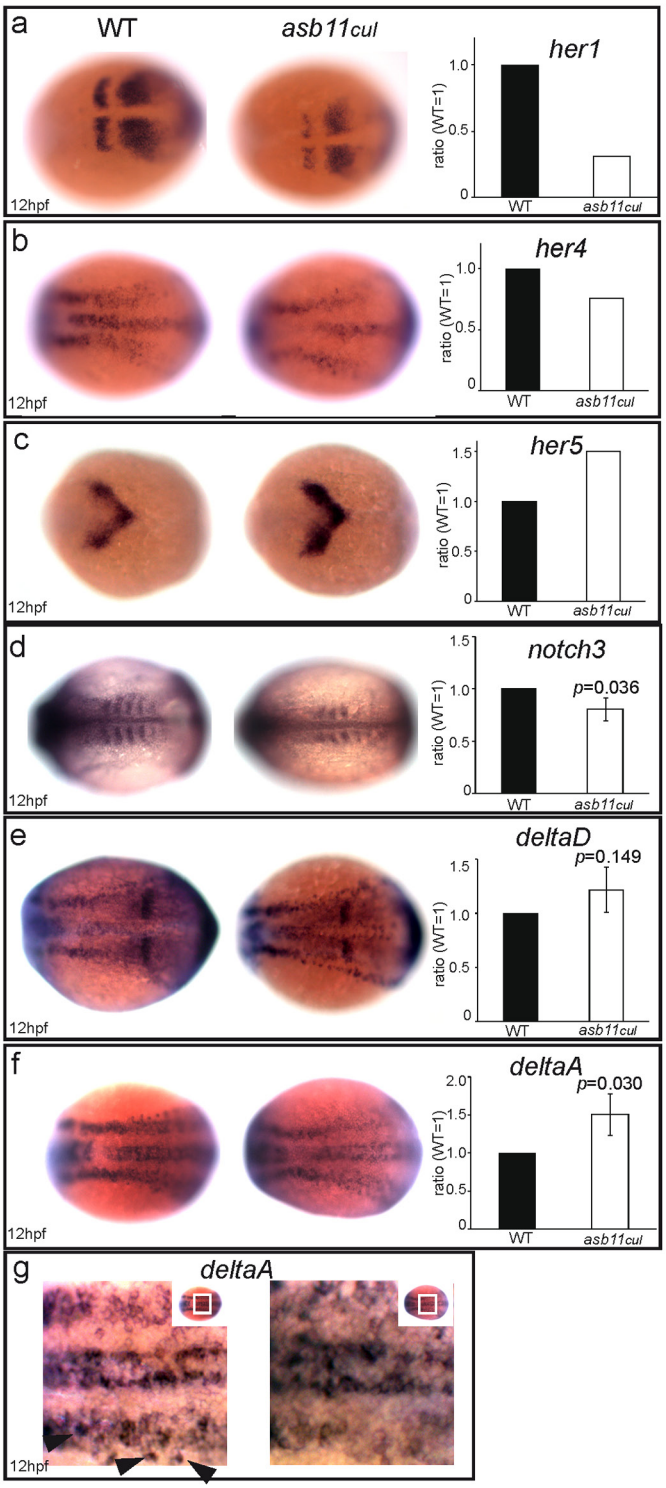

Fig. 3. asb11^{cul} presented altered expression of Delta-Notch pathway components. Wild type (left panel) and mutant (middle panel) embryos at 12 hpf were analyzed for WISH using probes against her1, A; her4, B; her5, C; notch3, D; deltaD, E; and deltaA, F. (G), Higher magnification shows detailed analysis of deltaA expression (arrows). (left) Graphs quantifies expression.

which is known to be downregulated by the Notch1A-intracellular domain (Hans et al, 2004). Consistently, we observe downregulation of notch3 (Fig. 3D), which has been shown to repress hes5, a mammalian homologue of zebrafish her5 (Beatus et al, 1999). Next, we analyzed expression of the Notch ligands DeltaA and DeltaD in asb11cul embryos. deltaA transcripts showed increased expression in mutants (Fig. 3F) whereas deltaD remained unaffected (Fig. 3E). Detailed examination of the WISH expression patterns of deltaA revealed a change in distribution of mRNA in the neural plate (Fig. 3G). Wild-type embryos exhibit a distinct "salt and pepper" aspect of deltaA mRNA distribution whereby some cells have stronger expression than their neighbors (Fig. 3G, arrows), consistent with the notion of Delta-Notch lateral signaling (Artavanis-Tsakonas et al, 1999; Skeath & Thor, 2003). In contrast, the asb11cul showed a smear of deltaA mRNA transcript across the neural plate, demonstrating loss of lateral inhibition in Notch signaling in mutant embryos.

Cullin box domain promotes Notch-mediated her4 induction

It was reported that Hairy/E(Spl) expression and activity can be independent of Notch signaling in vivo (Geling et al, 2003). Hence, to determine whether the altered regulation of Hairy/E(spl)-related transcription factors in mutants was dependent on d-Asb11-mediated Notch activity, we co-injected her4::gfp reporter DNA with d-asb11 full length or asb11^{cul} mRNA in zebrafish embryos. Injected embryos were treated with or without DAPT, a γ-secretase inhibitor which blocks Notch signaling (Geling et al, 2002). her4 transactivation was analyzed based on the GFP intensity, and classified as weak, medium or strong (Fig. 4A). When her4::gfp was injected with myc tag (MT) mRNA as a control, embryos presented 81%, 12.5% and 6% of weak, medium and strong GFP signals, respectively. Under DAPT treatment, the number of medium signal expressing embryos decreased to 9% and no embryos expressed strong GFP signal, showing that Notch signaling is disrupted with the DAPT treatment. Misexpression of MT-d-asb11 mRNA resulted in an increase in embryos expressing medium GFP signals (50%, c.f. 12.5% in MT-injected embryos), and strong signals (25% c.f. 6% in MT-injected embryos). Consistent with previous data MT-dAsb11 was unable to induce her4:gfp upon exposure of DAPT (Diks et al, 2008), showing the hierarchical upstream function of d-Asb11 in Notch activation.

Interestingly MT-Asb11^{cul} increased the number of embryos expressing medium signal whereas the number of embryos with strong her4::gfp

expression was slightly increased compared with control MT-injected embryos. However this effect was observed in both DAPT treated and untreated embryos (23% and 30%, respectively), suggesting that d-Asb11 lacking the cullin box domain (Asb11^{cul}) is much less efficient in inducing her4 reporter that wildtype Asb11 and its function is independent of Notch signaling. These data are consistent with studies showing that her4 may be expressed in a Notch-independent manner in specific domains of the nervous system (Yeo et al, 2007). All together our data suggest that cullin box domain is essential to regulate Notch targets genes through Notch signaling

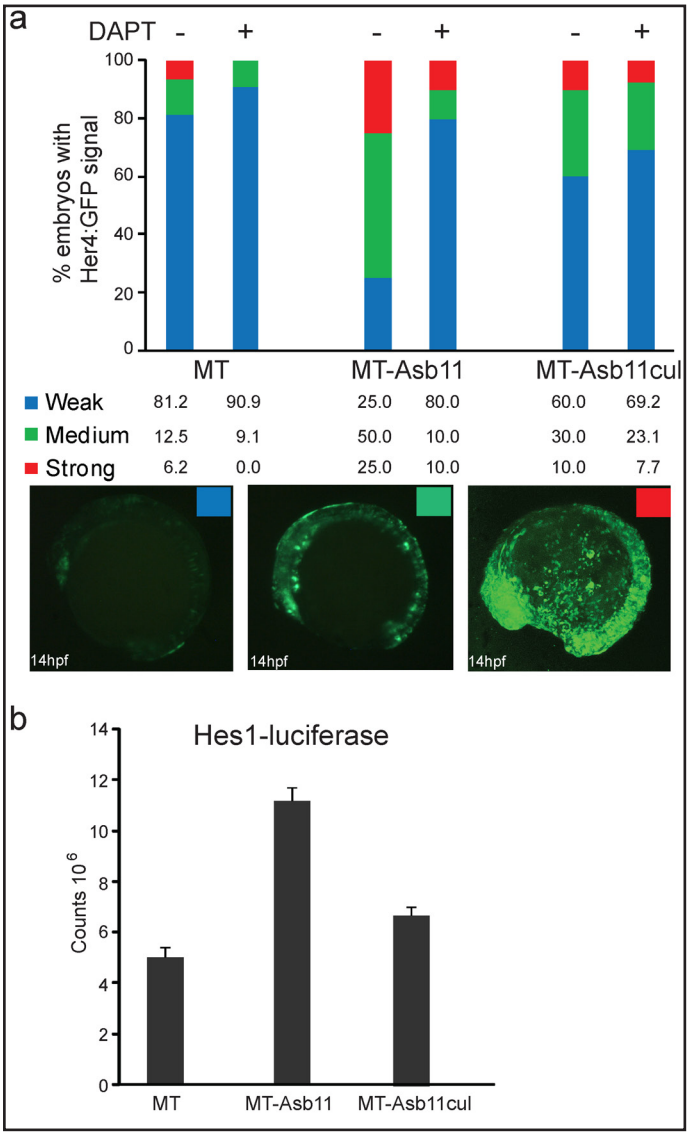

Fig. 4. Cullin box domain promotes induction of Notch-target genes. (A), her4::gfp reporter DNA was co-injected with myc-tag (MT) RNA as a control, myc-tagged d-asb11 full length (MT-Asb11) or myc-tagged asb11^{cul} (MT-Asb11cul) mRNA in zebrafish embryos. Injected embryos were treated with (+) (n=25) or without (-) (n=25) DAPT, from 1.5 hpf. At 14 hpf, embryos were analyzed for her4 transactivation based on the intensity of the GFP signal. Positive embryos were counted and percentages of embryos presenting weak (blue), medium (green) or strong (red) signal were given. (B), nTera-d1 cells were co-transfected with hes1-luciferase reporter and myc-tag (MT) as a control, or myc-tagged d-asb11 full length (MT-Asb11) or myc-tagged asb11^{cul} (MT-Asb11^{cul}) cDNA. Hes1-dependent Notch activity was analyzed by luciferase measurement.

activation although d-Asb11 lacking cullin box may yet affect proteins expression independently of Notch.

Cullin box is essential for DeltaA degradation in vivo

Previously, we have shown that d-Asb11 affects Delta-Notch signaling by targeting DeltaA for ubiquitination and subsequent degradation strictly dependent on the presence of the SOCS box (Diks et al, 2008). Likewise, others studies reported the importance of ASB SOCS box domain to recognize substrates and to act as a component of ECS complex (Chung et al, 2005; Kohroki et al, 2005). To study the role of the cullin box domain in d-Asb11 mediated DeltaA degradation, we injected wild type and mutant embryos with Myc-tagged deltaA (MT-dlA) and d-asb11 or asb11^{cul} mRNA at one-cell stage. Embryos were analyzed for the presence of DeltaA protein at 12 hpf. Wild type embryos injected with d-asb11 full length displayed substantial DeltaA degradation. In contrast, injected asb11^{cul} was not capable of degrading DeltaA when compared to control (Fig. 5). Thus, we show that the cullin box domain of d-Asb11 is essential for degradation of Notch ligand DeltaA in zebrafish embryos.

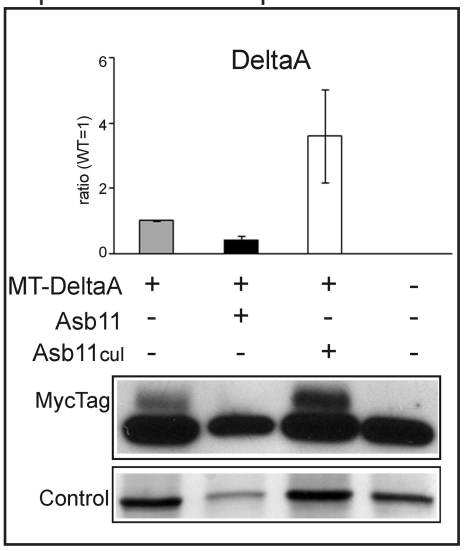

Fig. 5. Cullin box is essential for DeltaA degradation in vivo. Wild type and mutant embryos were injected with Myc-tagged deltaA (MT-DeltaA) and d-asb11 (Asb11) or asb11^{cul} (Asb11^{cul}) mRNA at one-cell stage. (lower panel) Lysates of 12 hpf embryos were obtained and analyzed for the presence of DeltaA on western blot. (higher panel) Graph quantifies 2 individual experiments, each with 30 injected embryos/group.

Absence of cullin box alters proliferation and differentiation

As Notch signaling drives precursor cell proliferation within the neurogenic regions of the embryo, a prediction from our findings would be that the loss of Asb11 cullin box would impair such proliferation. Fluorescent whole-mount antibody labeling with the mitotic marker anti-phosphohistone-3 (PH3) antibody showed a significant decrease in proliferation at 24 hpf in

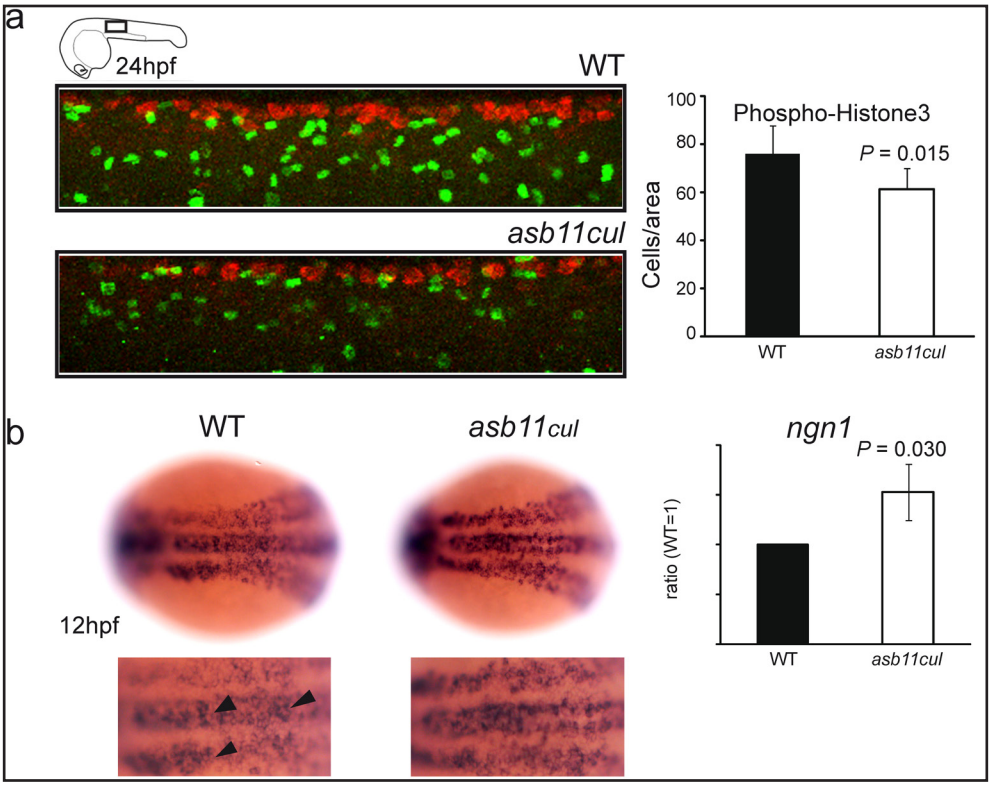

Fig. 6. Cullin box alters proliferation and differentiation patterns. (A), Fluorescent whole-mount antibody labeling of wild type (WT) and mutant embryos (Asb11cul) at 24 hpf for the mitotic marker anti-phosphohistone-3 (PH3) antibody. Graph shows the number of positive cells per area (5 somites from beginning of yolk extension) of 5 embryos of each genotype. (B), Wild type (left panel) and mutant (middle panel) embryos at 12 hpf were analyzed for WISH using probes against ngn1. Higher magnification shows detailed analysis of ng1 expression (lower panel, arrows). (right) Graph quantifies expression using qPCR.

asb11^{cul} embryos (fig. 6A), indicating that the d-Asb11 cullin box is necessary for proper cell proliferation. Subsequently, we performed WISH to investigate the expression of neurogenin1 (ngn1), a proneural bHLH transcription factor, which is expressed in neuronal precursors and differentiated neural cells (Ma et al, 1998) and is negatively regulated by Notch signaling (Blader et al, 1997). As expected, wild-type embryos at 12 hpf display the typical clustered expression of ngn1 (Fig. 6B, arrows). However, mutant embryos express ngn1 at a uniform high level without evidence of clustering (Fig. 6B). The increase in ngn1 mRNA expression was confirmed by qPCR. d-Asb11 morphants show a similar phenotype (Diks et al, 2006), confirming that the higher expression of ngn1 is caused by loss of d-Asb11 function in the mutant. Expression of ngn1 in the three longitudinal domains of zebrafish

neural plate corresponds to regions that express elevated levels of deltaA and in which the earliest neurons are born (Appel & Eisen, 1998). Based on these findings, we propose that the premature neuronal commitment in mutant embryos is a consequence of DeltaA accumulation in the neural plate, as asb11^{cul} is incapable of DeltaA degradation.

In conclusion, we show in this paper that the Cul5 domain of d-Asb11 is necessary for proper Notch activation in vitro and in vivo. Truncation of the cullin box, lacking the LϕLP motif, leads to alterations in expression of Notch pathway components. These changes may in part be regulated by a Notch-independent pathway. However, a complete cullin box is required for proper cell fate specification by Notch modulating function of d-Asb11 within the neurogenic regions of zebrafish embryo.

Methods

Fish and embryos. Zebrafish were kept at 27.5ºC. Embryos were obtained by natural matings, cultured in embryo medium and staged according to methods described previously (Kimmel et al, 1995).
Plasmid construction. Plasmids were constructed and/or provided as described previously(Diks et al, 2006; Diks et al, 2008). For asb11cul, mutant zebrafish cDNA was isolated and cloned into the EcoRI and XhoI sites of pCS2+MT and pCS2+.
mRNA synthesis, mRNA and DNA microinjections. Capped mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion). Fig.5, embryos were injected with 600pg MT-deltaA and 350pg d-asb11 or 350pg asb11cul mRNAs. Fig.4a, embryos were injected with 5pg her4::gfp DNA or 5pg her4::gfp + 300pg d-asb11 or asb11cul mRNA. Total volume of the injection was set at 1 nl.
DAPT treatment. Half of each injected group (n=50) (Fig.4a) was incubated in 100mM DAPT diluted in 1% DMSO in embryo-medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgSO4, 0.00005% Meth Blue. The other half was incubated in 1% DMSO in embryo-medium. The embryos were incubated from 1.5hpf till 14hpf, fixed with 4% PFA overnight at 4°C and analyzed for GFP expression.
In situ Hybridization. Whole mount in situ hybridizations were performed according to methods described previously (Oxtoby & Jowett, 1993). Probes were provided as described previously (Diks et al, 2006; Diks et al, 2008).
Immunoblotting. At 12hfp, chorion and yolk were removed. Embryos were

lysed in cell lyses buffer (50mM Tris-Cl pH7.5, 150mM NaCl, 1mM EDTA, 0.1% Na-deoxycholate, 1% NP-40, 10u, 1% protease inhibitor (ROCHE), 2uL/ embryo. Primary antibodies were diluted in PBS containing 1% milk (fig.2: rabbit anti-asb11 1:100, fig.5: rabbit anti-MT 1:1000, Bioke).

RNA isolation and qRT-PCR. Total RNA was extracted from wild type and mutants embryos at 10 or 12 hpf. Total RNA extraction, cDNA synthesis and qPCR quantification were performed according to methods described previously7.

Whole mount immunolabelling. Fig.6a, whole-mount immunohistochemistry was performed as described earlier (Diks et al, 2006).

Microscopy and image quantification. Pictures were obtained as described previously7. For analysis of fluorescent stainings, Leica Confocal TCS SPE was used. To quantify the intensity of signal, a z-stack (z-slices of 7µM) were made, scanning the whole embryo. Leica software (Application Suite 1.8.0) was used to create a maximum projection of the z-stack.

Cell cultures. nTera2/d1 cells were maintained in DMEM containing 10% FCS. The culture media were supplemented with 5mM glutamine and antibiotics/ antimycotics. Cells were incubated at 5% CO2 in a humidified incubator at 37ºC.

Luciferase reporter assay. NTera2/d1 cells were seeded in a 96-well plate and transfected using IBAfect and MA-enhancer (IBA Biosciences, GmbH) using the suppliers protocol. Luciferase was measured on a Packard TOPCOUNT Microplate Scintillation Counter (Packard). The experiments were performed two times in triplicate. Values were normalised with TAL-luc (nTera2/d1).

Statiscal testing. Statistical tests were performed using two-tailed t-test. All bars in graphs depict mean values with error bars depicting standard deviations.

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CHAPTER 5

d-Asb11 is a regulator of muscle precursor compartment size during embryonic and adult regenerative myogenesis

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Submitted

Introduction

One of the most important and defining processes during development is the establishment of the relative sizes of various compartments in the vertebrate body. During embryonic development, a progenitor compartment for any given tissue is formed, that subsequently expands before further differentiation ensues. The factors that govern the extent of progenitor compartment expansion remain in most cases obscure at best. Amongst the compartments with highly dynamic regulation of their relative size, the skeletal muscle is especially subjected to highly positional, temporal, and functional demand-dependent control. Although muscle atrophy is a serious clinical problem, and thus knowledge on the molecular factors that determine proliferation from muscle progenitors is hardly a pursuit of only academic interest, knowledge as to the factors driving expansion of the muscle progenitor compartment remains sketchy. Thus, enhanced knowledge as to the molecular determinants of skeletal muscle compartment size, both during embryogenesis, as well as in adult organisms, is an important scientific question.

The fundamental events in myogenesis that are common to all vertebrates are the specification of muscle stem cells to the myogenic lineage, and the subsequent proliferation, differentiation, migration and fusion of these myoblasts to become specific muscle fibers. Different fiber types within vertebrate muscles have been characterized and can be broadly classified as slow or fast fibers on the basis of their mechanical and metabolic properties (Rubinstein and Kelly, 1981). However, the molecular determinants that fundamentally govern the decision of a Pax7+ satellite cell to commit itself to terminal differentiation or to maintain proliferation and enlarge the pre-myogenic compartment remain poorly understood.

A possible candidate protein to function in the definition of muscle compartment size is Asb11. Asb11 is one of the 18 members of the ankyrin repeat-containing suppressor of cytokine signaling (SOCS) box protein family that are characterized by variable numbers of N-terminal ankyrin repeats. From a screen in zebrafish designed to identify genes downregulated by retinoic acid, we isolated the d-Asb11 gene. (Diks et al., 2006) Asb11 is one of the most conserved genes in the chordate phylum, with Ciona intestinalis Asb11 and human Asb11 being 70 % similar with respect to the constituting amino acids. Subsequent functional studies demonstrated that d-Asb11 regulates the size of the neural precursor department via the ubiquitination of DeltaA, providing the initial bias for lateral inhibition in Notch signaling

and thus allowing canonical Notch signaling to occur (Diks et al., 2008). Hence, Asb11 represents a first member of a novel class of regulators of compartment size in the embryo, regulating progenitor expansion by enabling Notch signaling and whose down regulation (e.g. by retinoic acid) defines final compartment size in the neural ectoderm. Whether Asb11 has functionality in the other germ layers apart from the ectoderm remains to be established, thus prompting us to explore its function in non-neuronal tissue types. Here, we show that d-Asb11 is a principal regulator of myogenesis, both during embryogenesis as well as during regenerative responses in adult animals, establishing Asb11 as a principal regulator of compartment size in vertebrate morphogenesis in multiple germ layers.

Results

High expression of d-Asb11 in the label-retaining Pax7+ muscle satellite stem cell compartment.

In an effort to determine possible extra-neuronal functions for Asb-11, we explored the tissue-specific distribution of d-Asb11 transcripts in adult zebrafish by reverse transcription-PCR amplification from total RNA extracted from various tissues. d-Asb11 specific RT-PCR products were amplified from the cDNA of total RNA extracted from the muscle, heart, eye, intestine, gills, testis, pancreas and brain. As a positive control to determine the integrity of the RNA samples used in the assay, transcripts for the constitutively expressed β-actin gene were amplified by RT-PCR. d-Asb11 is expressed in all the tissues we explored (Figure 1A), indicating functionality outside the nervous system, and possibly in muscle. The latter notion was supported by experiments in which we directly investigated protein expression of endogenous d-Asb11 in adult muscle. To this end, protein extracts were made from the adult zebrafish muscle fibers and subjected to Western blot employing a rabbit anti-d-Asb11 polyclonal antibody (Diks et al., 2006); Figure 1B), revealing low constitutive expression of d-Asb11 in this tissue. For subsequent experimentation as to the spatial expression of d-Asb11 in muscle fibers cross sections of skeletal muscle tissue of zebrafish, body musculature were co-stained with an antibody specific for d-Asb11 and a well known muscle satellite marker Pax7. It appeared that d-Asb11 showed a distinct expression pattern on the sarcolemma where it co-localized with Pax7. To confirm that these Pax7+/d-Asb11+ represent true muscle stem cells, the capacity of these cells for long term BrdU label retention was

investigated and confirmed that this double compartment truly represented slow cycling satellite cells (Figure 1C). Thus, the satellite cell muscle stem cell compartment in adult muscle is characterized by high specific d-Asb11 expression.

d-Asb11 is important for myogenenic proliferation during embryogenesis

As it is becoming clear that Asb family members in general may have functions in muscle development (Bello et al., 2009; McDaneld et al., 2006)

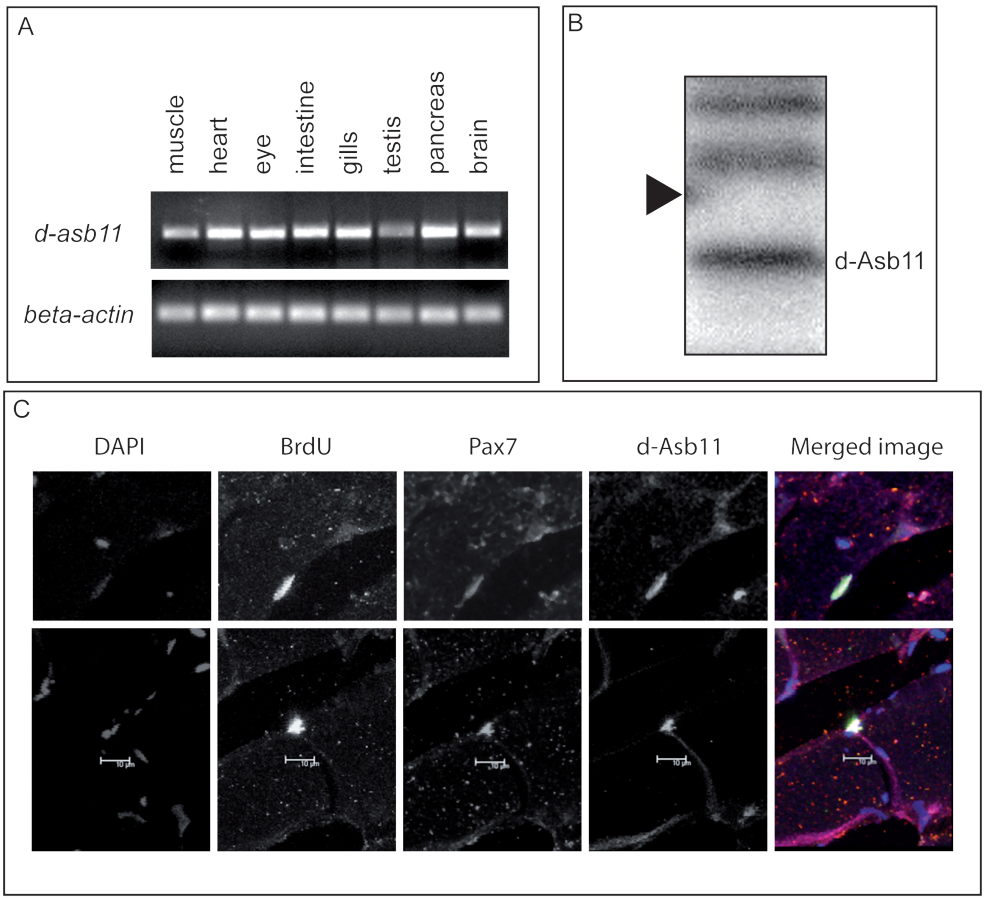

Figure 1
(A) RT-PCR of Asb11 from adult zebrafish RNA samples isolated from: muscle, heart, eye, intestine, gills, testis, pancreas, and brain tissues, showing expression of Asb11 in all tissues analyzed. β-actin was used as template control. (B) Western blot from zebrafish adult muscle probed with anti-Asb11. (C) Adult muscles were fixed, cryosectioned (10µm) and triple immunostained with anti-BrdU, anti-Pax7 and anti-Asb11. Top panels show a representative of sagittal sections, and bottom panels show a representative of transverse sections.

and because we observed clear expression of d-Asb11 in zebrafish muscle stem cell compartment, we decided to investigate the possible functionality of d-Asb11 in muscle development. To this end, we employed a germline zebrafish d-Asb11 mutant which we recently generated and lack the C-terminus of the Cullin box resulting in a hypomorph allele (d-Asb11Cul-/-) (da Silva, Tee et al., unpublished data). The d-Asb11Cul-/- mutants survive until adulthood but have, amongst other aberrancies, truncated trunks and

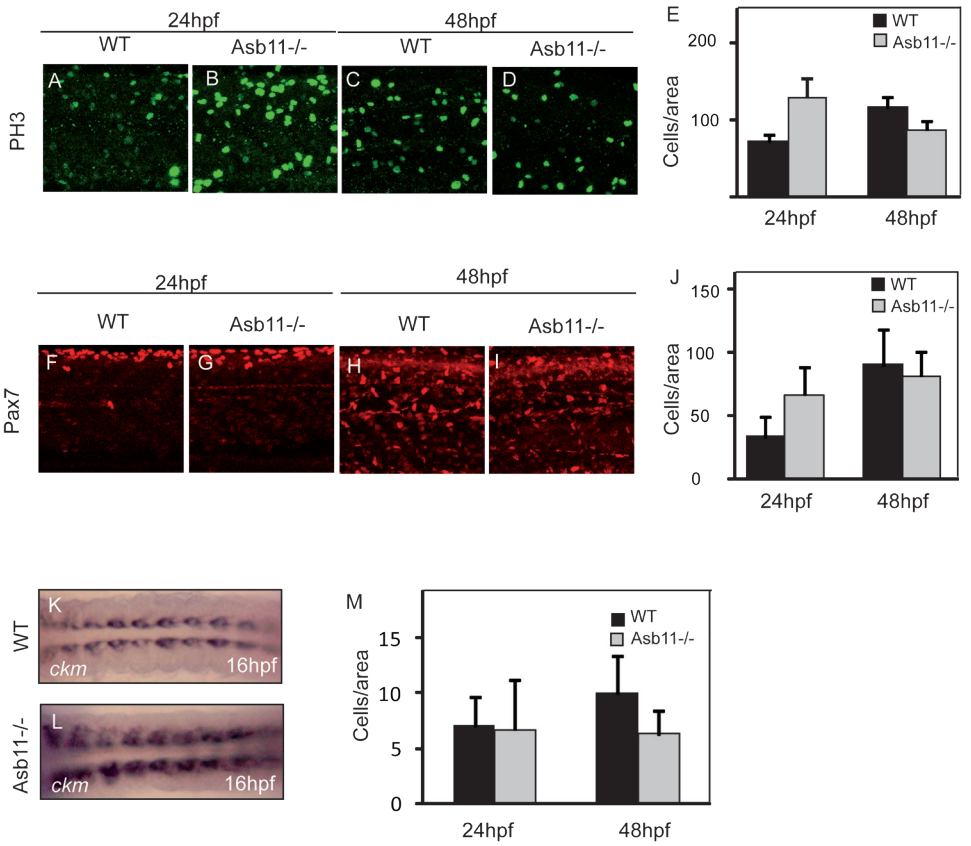

Figure 2

Embryos from wild-type and Asb11cul-/- embryos were fixed at 24 and 48hpf, and subjected to immunostaining with (A) anti-phosphohistone3 (PH3) (24hpf, n=7; 48hpf, n=7) and (B) anti-Pax7 (24hpf, n=3; 48hpf, n=7). Embryos were imaged in comparable positions at the level of the yolk extension. Quantification of the number of PH3+ and Pax7+ cells, are as shown in (E) and (J) respectively. Whole mount in situ hybridization of 16hpf embryos using creatine kinase muscle (ckm) riboprobe was compared between (K) wild-type and (L) Asbllcul-/- embryos. Asbllcul-/- embryos show a higher expression of ckm. (M) Quantification of proliferating Pax7 cells (PH3+ and Pax7+ cells) comparing between wild-type and Asbllcul-/- embryos at 24hpf and 48hpf. Scale bar, 50µm.

poorly defined somites (da Silva, Tee et al., unpublished data). Interestingly, we see an increase in proliferation in the myotome of Asb11Cul-/- at 24hpf (Figures 2A-B, E), and this is consistent with the increase in Pax7+ muscle precursors at the same time point (Figures 2F-G, J). These Pax7+ cells, however, show strong commitment to further differentiation along the myogenic lineage as evident from their expression of creatine kinase - muscle (CKM), a late terminal muscle differentiation marker (Figure 2K-L), and suggest that the absence of functional d-Asb11 accelerates differentiation in muscle. In apparent agreement, at 48hpf, there is a significant decrease in proliferation in d-Asb11Cul-/- mutants (Figures 2C-E), with no significant difference in the number of Pax7+ cells between wild-type and d-Asb11Cul-/- mutants at 48hpf (Figures 2H-J). When we quantified the number of co-localized Pax7+ and BrdU+ cells at 24 and 48hpf, we observe a dramatic decrease in proliferating Pax7+ cells in d-Asb11Cul-/- at 48hpf (Figure 2M). There is no significant difference at 24hpf (Figure 2M). Thus, functionally, d-Asb11 is essential for maintaining myogenic proliferation in the stem cell compartment during embryogenesis.

Forced expression of d-Asb11 is sufficient to inhibits embryonic muscle differentiation

A prediction from the proposed role of d-Asb11 as a factor inhibiting differentiation and maintaining proliferation in the Pax7+ muscle stem cell compartment would be that forced expression of d-Asb11 is associated with impaired differentiation in zebrafish muscle. Thus, we injected d-Asb11-MT mRNA into embryos, and observe the whole mount in situ hybridization expression of myoD, a bHLH transcription factor that is expressed in adaxial cells and in the posterior region of each developing somite (Weinberg et al., 1996). Consistently, overexpression of d-Asb11-MT mRNA resulted in the loss of somitic myoD expression accompanied by variably reduced or absent expression in adaxial cells at 13-14 hpf (Figure 3B-D; Supplementary Table 1) or in a bended and/or displaced field of myoD expression (Figure 3B and D). Furthermore, when d-Asb11-MT RNA was injected in only one of the blastomeres of two-cell stage embryos, the expression pattern of d-Asb11-MT in the embryo coincided with exclusion of myoD expression (Figure 3E-F). When injected in zygotes, overexpression of d-Asb11-MT affected myoD expression in the entire myotome at 16hpf (Figure 3G-I). Moreover, employing myogenin and creatine kinase-muscle (CKM) as markers for advanced muscle cell differentiation, this process was substantially impaired

upon overexpression of d-Asb11-MT (Figure 3J-O). Thus, d-Asb11 acts to prevent muscle differentiation in vivo.

Forced expression of d-Asb11 inhibits muscle cell differentiation in the C2C12 model system

Independent confirmation for the inhibitory effects of d-Asb11 on muscle cell differentiation was obtained from experiments in which the influence of forced d-Asb11 expression was tested in the controlled environment of C2C12 mouse myoblast differentiation. Upon serum withdrawal, proliferating C2C12 myoblasts synchronously withdraw from the cell cycle, elongate, adhere, and finally fuse together to form myotubes exhibiting most, if not all mechano-biochemical adaptations associated with fully differentiated muscle. Thus, this system offers a possibility to study the effect of d-Asb11 on muscle

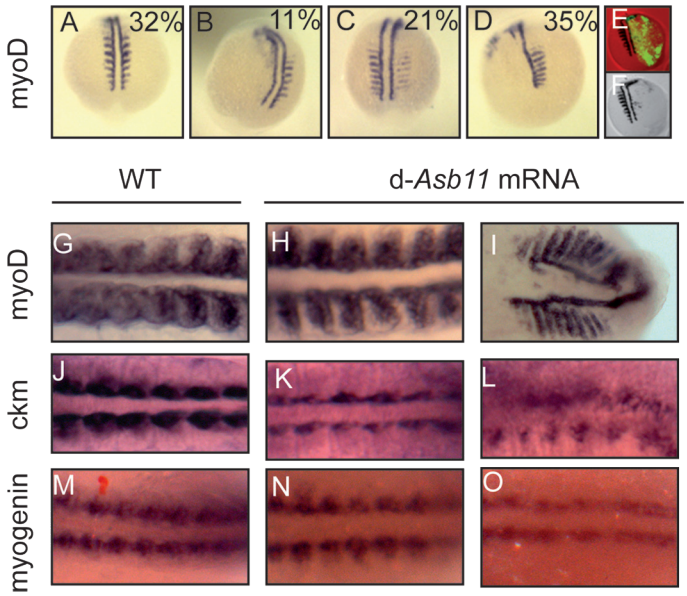

Figure 3
(A-F) Zebrafish embryos were injected at the two-cell stage in one of the two blastomeres with mRNA encoding full-length d-Asb11-MT (300pg mRNA) resulting in patterning defects in the injected half of the embryo. At 13-14hpf, embryos were fixed and categorized with respect to expression patterns for myoD. Percentages indicate the relative number of defects in expression that were obtained following d-Asb11-MT overexpression. (A) wild-type myoD expression; (B) bended adaxial expression of myoD and somites absent; (C) myoD expression and the somites absent/reduced, adaxial expression of wild-type; (D) adaxial and somite expression absent; (E-F) immunolabelling of d-Asb11-MT overlay with (F) myoD in situ hybridization. (G-O) Embryos were injected at one-cell stage with d-Asb11 mRNA, fixed at 16hpf. Whole mount in situ hybridization was performed using (G-I) myoD, (J-L) ckm and (M-O) myogenin riboprobes, and expression was compared between (G, J, M) uninjected wild-type controls and (H, I, K, L, N, O) d-Asb11 mRNA injected embryos.

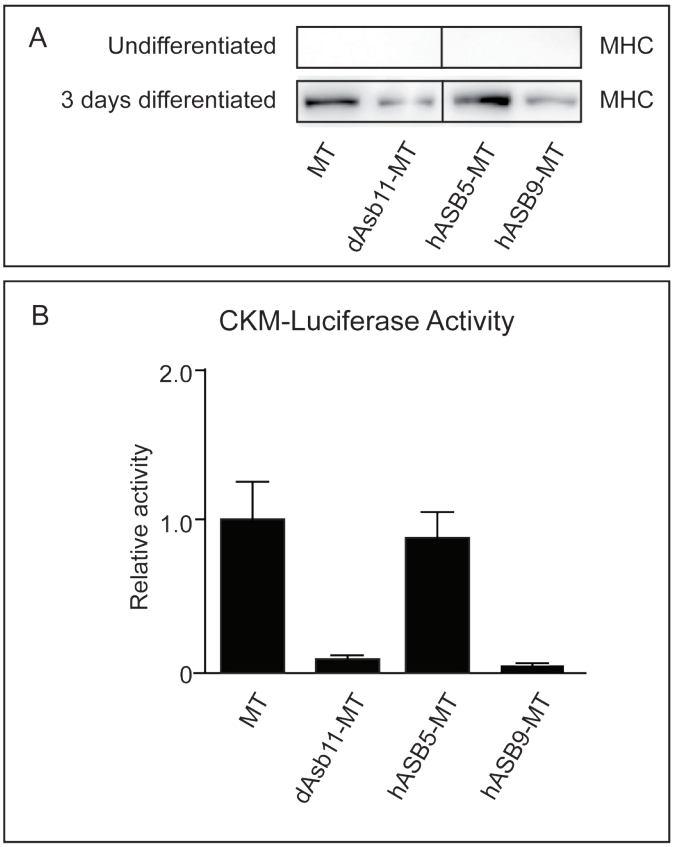

Figure 4
C2C12 cells were transfected with MT, d-Asb11-MT, h-ASB5-MT or h-ASB9-MT and allowed to differentiate for 3 days in differentiation media. The image is a merged photo of 2 images from the same Western blot, removing a middle non-relevant band in between. (B) C2C12 cells were co-transfected with CKM or CMV promoter-driven luciferase and MT, d-Asb11-MT, h-ASB5-MT or h-ASB9-MT, and allowed to differentiate for 3 days in differentiation media. The CKM promoter driven luciferase activity was normalized against the CMV promoter-driven luciferase activity.

cell differentiation in isolation. Indeed, transfection of C2C12 cells with a CKM promoter-driven luciferase construct or a construct containing a CMV promoter-driven luciferase construct shows substantial activation of the CKM promoter following serum withdrawal (data not shown), showing that also in our hands can recapitulate important aspects of muscle cell differentiation. In apparent agreement, under these conditions myosin heavy chain was substantially induced (Figure 4A). Importantly, however, when cells were transfected with expressing CMV promoter-driven d-Asb11-MT or CMV promoter-driven h-Asb9-MT (together with h-Asb11 the orthologue of d-Asb11 in the human genome), withdrawal of mitogenic factors no longer induced either the CKM promoter or cellular levels of myosin heavy chain (Figure 4A-B). Together, these observations show that forced expression of d-Asb11 prevents muscle differentiation. Interestingly, the inhibition of cell differentiation is specific to d-Asb11 or h-Asb9, as forced expression of h-Asb5 did not show significant differences (Fig 4A-B).

d- Asb-11 is essential for regenerative myogenesis?

Regenerative myogenesis is still a poorly understood process. To determine whether regenerative myogenesis like primary embryogenesis is possibly regulated by d-Asb11, we induced mechanical injury with a 30G needle on the adult zebrafish body musculature, dorsal to the anus and we investigated the number of proliferating BrdU+ cells, Pax7+ cells and Asb11+ cells in lesion-induced versus uninjured wild-type zebrafish at 7 days post injury. Although this procedure does not produce significant changes in the number of BrdU+ cell or Pax7+ cells, the d-Asb11+/Pax7+ double positive compartment substantially increase in size following muscle injury, indicating that d-Asb11 may be involved in response to trauma in this tissue (Figure 5A). Subsequently, we compared the proliferation between d-Asb11Cul-/- and wild-type zebrafish. Axin1+/-;Apc1+/- double heterozygous zebrafish were used a positive controls as Wnt signaling is shown to positively regulate satellite cell proliferation on adult muscle fibers during wound healing response in CD34+ cells (Polesskaya et al., 2003) as well as cultured myofibers (Otto et al., 2008). d-Asb11Cul-/- mutants showed a significant diminished numbers of BrdU+ cells (Figure 5B), suggesting that regenerative proliferation is dependent on functional d-Asb11. At Day 0 and Day 1 post-injury, muscle fibers were comparable in wild-type, Axin1+/-;Apc1+/- and dAsb11Cul-/- zebrafish (Figure 5C, G, K). At Day 1 post-injury, there was a marked cellular invasion, and evident degeneration and necrosis of mature fibers. At Day 5 post-injury, Axin1+/-Apc1+/- zebrafish showed remarkable recovery and numerous small diameter regenerating muscle fibers were observed (Figure 5I). However, in wild-type and d-Asb11Cul-/- zebrafish, the muscle fibers were still necrotic (Figure 5E and M). At Day 10 post-injury, Axin1+/-;Apc1+/- zebrafish showed a clear recovery and regeneration of the muscle fibers (Figure 5J), whereas the aspect of wild type fish only moderately worse. In dAsb11Cul-/- zebrafish, however, at day 10 post-injury, only a slight improvement could be noted with small regenerating fibers emerging (Figure 5N). Thus, d-Asb11 is permissive not only for embryonic myogenesis but for regenerative myogenesis as well.

Discussion

The determinants of the size of the muscle cell compartment remain poorly understood. During embryogenesis a group of Pax7+ stem cells forms and proliferates until final compartment size is reached and in most precursor

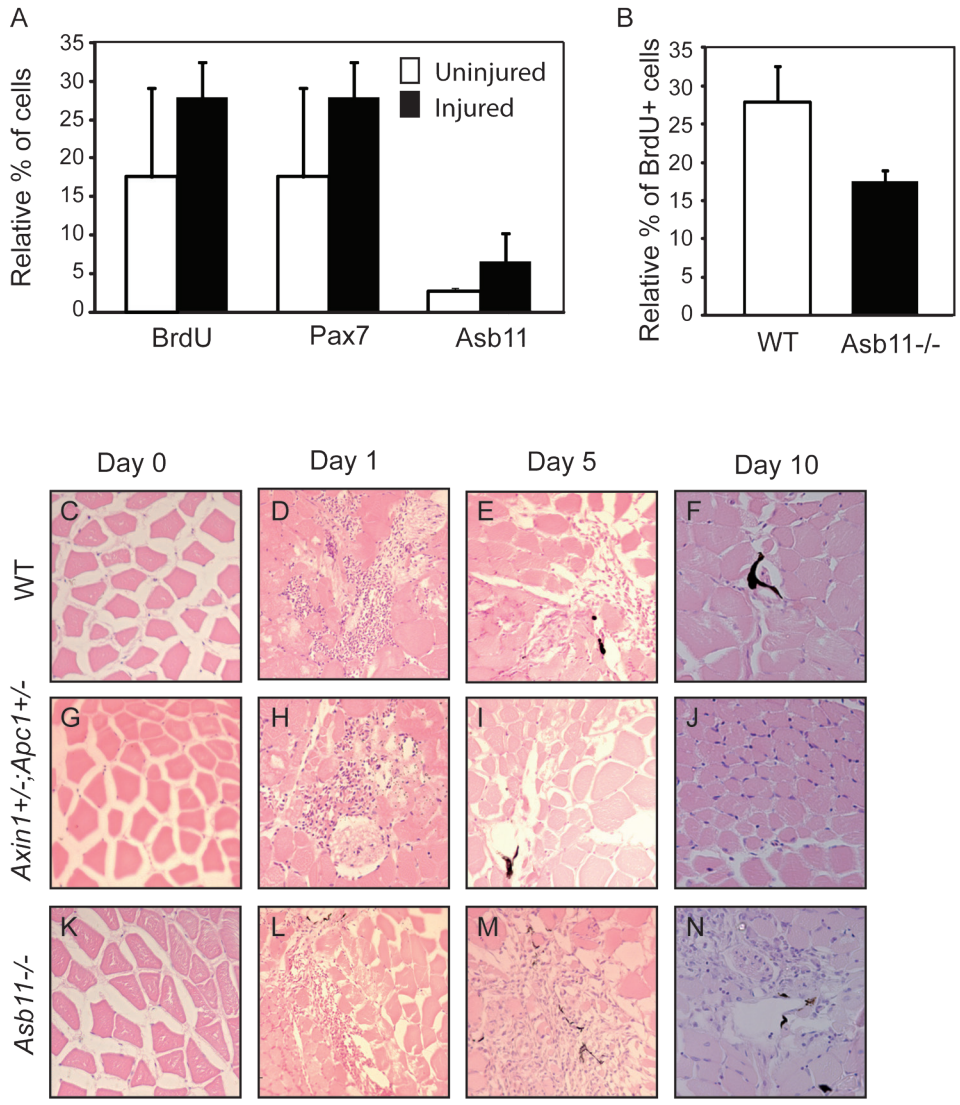

Figure 5

(A-N) Induced mechanical injury was performed with a needle on adult zebrafish on the dorsal flank of the muscle, at the level of the anus. Zebrafish were allowed to swim until fixation. (A) Zebrafish were fixed and frozen 7 days post-injury. Adult muscle tissues were cryosectioned and triple immunostaining with anti-BrdU, anti-Pax7 and anti-Asb11 was performed. (B) Wild-type and Asb11cul-/- zebrafish were fixed and frozen 7 days post-injury. Adult muscle tissues were cryosectioned and immunostaining with anti-BrdU was performed. (C-N) Zebrafish were fixed at (C, G, K) 0, (D, H, L) 1, (E, I, M) 5 and (F, J, N) 10 days post-injury. The adult musculature was sectioned and counterstained with HE. 3 adult zebrafish per group were analyzed. Evans Blue Dye was used as a marker to identify the area of injury.

cells a genomic programme responsible for terminal differentiation is started finally resulting in functional muscle fiber whereas a small subpopulation remains Pax7+ and form satellite cell population from which regenerative myogenesis can be restarted following injury. Our data presented here show that d-Asb-11 is both essential and sufficient for maintaining stem cell proliferation during zebrafish embryogenesis and is required for regenerative responses during injury as well. Importantly, we show that d-Asb11 is expressed beneath the basal lamina of an adult zebrafish muscle fiber, and co-localizes with a well-accepted muscle satellite cell specific marker Pax7 (Zammit et al., 2006). This, together with the co-localization of d-Asb11 with slow-cycling label retaining term BrdU cells, suggests that the d-Asb11 positive cells are the muscle satellite cells themselves. Interestingly, there is significantly less d-Asb11 cells compared with Pax7 cells in the adult muscle fibers. It is tempting to speculate that the d-Asb11 cells are the primary stem cells, and thus, is activated and proliferates in response to muscle damage/ injury (Zammit, 2008).

d-Asb11 shows high homology to both mammalian Asb-9 and Asb-11 whereas no obvious Asb 9 homologue is present in fishes. As h-ASB9 and h-Asb11 lie adjacent on the same chromosome (X), h-Asb11 and h-Asb9 seem to represent the result of an evolutionary relatively recent genetic duplication event. In silico analysis have revealed that 46.6% of the ancestral chordate genes appear in duplicate in one or more of the vertebrate lineages, with 34.5% having at least one duplication before the divergence of fish from tetrapods and 23.5% having at least one duplication afterward (Dehal and Boore, 2005). This suggests that possibly, the zebrafish d-Asb11 functions similarly to both h-Asb9 and h-Asb11, the muscle functions of d-Asb11 apparently being represented by h-Asb9 (as judged from its expression pattern in Human Protein Atlas) and the neuronal functions of d-Asb11 being represented in h-Asb11 (again, as judged from its expression pattern).

d-Asb11 maintains the neuronal progenitor compartment, implying an important role in the ectodermal compartment size. In zebrafish, this function of d-Asb11 does not seem restricted to this germ layer, as we now show that it is important for mesodermal lineage as well and hence d-Asb11 appears a regulator of vertebrate compartment size of more general importance. In embryos, proliferation is increased during the first wave of myogenesis, and decreased during the secondary wave of myogenesis. This, together with the decreased proliferation in adult zebrafish indicates that the muscle precursor pool is diminished. This is remarkably similar to the effects of d-Asb11 on embryonic neural precursors (Diks et al., 2006),

suggesting that d-Asb11 functions possibly in a similar way in regulating both the neuroectodermal and mesodermal cell fate. Whether d-Asb11 is important for compartment size in the endodermal lineage, however, is questionable. Neither d-Asb11 nor its human homologues h-Asb9 and h-Asb11 are strongly expressed in this compartment. Thus, proliferation in the endodermal progenitor compartment is maintained either via other mechanisms or other Asb proteins (e.g. h-Asb6 shows strong expression in the proliferating endodermal compartment). Further investigations as to how endodermal progenitor expansion is regulated are called for.
Recently we showed that the functions d-Asb-11 in neurogenesis are mediated by its potential to enable canonical Notch signalling. Interestingly, canonical Delta-Notch signaling (Diks et al., 2008) is an important signaling pathway in satellite cell activation and muscle regeneration (Brack et al., 2008; Luo et al., 2005). There is a temporal switch between Notch and Wnt signaling, whereby, Notch signaling has to be downregulated for myogenesis to proceed (Brack et al., 2008). This is consistent with our data, showing expression of d-Asb11 in muscle satellite cells, and the requirement of d-Asb11 to maintain the muscle precursor pool. What is more fascinating is the requirement of d-Asb11 in efficient muscle regeneration. It is important to note, that albeit slower, muscle regeneration is still evident in d-Asb11Cul-/- zebrafish. Furthermore, based on the evolutionary conservation of d-Asb11 with human h-ASB9 and h-ASB11, it is tempting to hypothesize that the phenotypes we observe in the d-Asb11 mutants could be linked to human muscular diseases, prompting an investigation into the role of h-Asb9 in muscle pathology.

Experimental procedures

Zebrafish embryos

Zebrafish embryos were raised and staged as previously described (Westerfield, 1995). Asb11cul1/cul1 is homozygous viable mutation identified in a TILLING screen from a library of ENU-mutagenized zebrafish according to standard mutagenesis protocol. axin1+/-;apc1+/- compound heterozygous mutants were generated from crossing apcCA50a/CA50a (Hurlstone et al., 2003) with axin/mbl(tm13) (van de Water et al., 2001) as described in (Tee et al., 2009). For adult muscles (Figures 1 and 5), zebrafish between ages 6-9 months were used.

RT-PCR

RT-PCR primers (10µM) used was as follows: Asb11-For-RTPCR-483: 5'- CTGCAAAGAGAGGTCACACG -3' and Asb11-Rev-RTPCR-877: 5'- TCCTTTTTGTCCCAGTGAGC -3'. The PCR program used is 94˚C 2', 30 cycles of (94˚C 20", 65˚C 30" -0.5˚C per cycle, 72˚C 60"), 10 cycles of (94˚C 20", 58˚C 30", 72˚60") and 72˚C 3'.

Adult muscle immunolabeling

Short term (7 days) and long term (2 months) BrdU incorporation and labeling was performed by immersing and allowing the zebrafish to swim in BrdU (150mg/L for 4 hours per day) for 7 days. Fish were fixed at 2 months after the BrdU pulse. Adult muscle tissue was isolated and frozen on liquid nitrogen, post-fixed in 4% PFA, cryosectioned in 10 µm and dried overnight. Sections were post-fixed in acetone, washed and kept in 100% methanol overnight. Sections were permeabilized with 0.2% Triton X-100 and quenched with 100mM Glycine-NaOH pH10. Sections were washed in PBS-Tween20 (PBS-T) and incubated in 2N Hydrochloric acid. To restore the pH, sections were washed in 0.1 M Sodium borate. After washing in PBS-T, sections were blocked (2% goat serum, 1% BSA, 1% DMSO in PBS-T) at room temperature for one hour. BrdU antibody (Abcam Ab6326) was incubated 1:200 in block overnight at 4°C.
After washing in PBS-T, sections were blocked again for 1 hour and anti-rat secondary antibody (Jackson Immunoresearch; 1:200) was incubated for 2 hours at RT. After washing, sections were blocked (20% Goat serum, 0.1% BSA in PBS) for 1 hour. Asb11 antibody (Diks et al., 2006) was pre-incubated in fish powder and subsequently incubated in block (10% goat serum, 0.1% BSA) overnight at 4°C.
After washing in PBS-T, sections were blocked for 1 hour and incubated in anti-rabbit secondary antibody (Jackson Immunoresearch) for 2 hours at RT. After washing, sections were blocked (10% goat serum, 0.1% BSA, 1% DMSO in PBST) for 1 hour at RT and incubated in Pax7 antibody (Developmental Studies Hybridoma Bank) 1:20 in block overnight at 4°C.
After washing in PBS-T sections were blocked and incubated in anti-mouse secondary antibody (Jackson Immunoresearch) for 2 hours at RT. After immunolabelings, sections were counterstained in DAPI (Sigma).

Immunolabelling of zebrafish embryos

Embryos were fixed for antibody staining with 4% PFA and wholemount immunohistochemistry was performed according to Du et al. [54], using primary antibodies Pax7 1:20 (Developmental Studies Hybridoma Bank) and PH3 (Upstate Biotechnology #06570) 1:1000. Appropriate secondary antibodies were used at 1:200. Immunohistochemistry was analyzed at the level of yolk extension.

In situ hybridization

Whole-mount in situ mRNA hybridization (WISH) was carried out as previously described (Jowett, 2001). Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4˚C and digoxigenin-tagged probes were made with Roche labelling mix to myoD, creatine kinase-muscle (ckm) (Xu et al., 2000) and myogenin. Images were obtained using a Zeiss Axioplan Stereomicroscope (Oberkochen, Germany) equipped with a Leica (Wetslar, Germany) digital camera, and were adjusted for brightness and contrast using Adobe Photoshop 7.0.

Microinjection of mRNAs

Capped synthetic mRNA was prepared from pCS2+ constructs encoding zebrafish d-Asb11 using the mMessage mMachine Kit (Ambion, Austin, Texas), and injected into one cell-stage embryos using a microinjector (World Precision Instruments). A concentration of 300pg d-Asb11 mRNA was used.

Plasmid construction

Full-length h-Asb5 and hAsb-9 was amplified from commercially available EST clones using primers containing an EcoRI or XbaI site and subsequently cloned into the EcoRI and XbaI sites of pCS2+MT.

Western blot

C2C12 cells were differentiated by adding 5% Horse serum. For western blot the cells were washed and taken up in Laemli sample buffer. Cell lysates were heated to 95°C for 5 minutes and afterwards stored at -20°C if necessary. Next, the samples were loaded on SDS-polyacrylamide gels. The material was

transferred to Immobilon (Millipore) by wet blotting in transfer buffer (50 mM Tris pH 8.0, 40 mM Glycine, 0.0375% SDS, 20% methanol) and the blots were incubated for 1 hour in blocking buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 5% non-fat milk). Blots were incubated overnight at 4°C in TBS-T (50mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 10% blocking buffer and 1:2000 MyHC (kindly provided by L. Puri). The blots were washed in TBS-T. After incubation with the appropriate secondary antibody and extensive washing in TBS-T, the blots were developed by ECL.

Luciferase reporter assay

C2C12 cells were seeded in a 24-well plate at a density of 25%. The following day, cells were transfected with the expression construct (0.1mg) and the reporters (0.1mg CKMM-luc or CMV-luc) using Effectene (Qiagen) with the standard protocol. After 24 hours, luciferase was detected using Luclite (Perkin Elmer) and was measured on a Packard TOPCOUNT Microplate Scintillation Counter (Packard). Transfected constructs used were as described previously (Diks et al., 2006; Diks et al., 2008).

Tissue histology

For cryosections (Figure 1), adult muscle tissues were embedded in Jung Tissue Freezing Medium, and sectioned at 10µm. For plastic sections (Figure 5), adult muscle tissues was dehydrated and rehydrated in an ethanol gradient. Adult muscle tissues were embedded in Technovit 8100 (Kulzer) and sectioned at 7 µm. Sections were then counterstained with Mayer's haemotoxalin and eosin.

Muscle injury and EBD incorporation

Muscle injury and Evans Blue dye (EBD) incorporation was performed as described in Chapter 8. The adult muscle were fixed and processed at 0, 1, 5 and 10 days post-injury. EBD was used to identify the site of injury.

Imaging and Quantifications

Fluorescent labelings were imaged using a Leica TCS SPE confocal microscope. The number of BrdU+, Pax7+ and Asb11+ cells in the adult muscle tissue were counted at comparable positions at the dorsal part of the myotome. For statistical analysis, two-tailed Student's t-test was performed using

Microsoft Excel.

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CHAPTER 6

Identification of a d-Asb11 induced Notch independent pathway expanding the muscle progenitor compartment

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In preparation

Introduction

The ankyrin repeat-containing suppressor of cytokine signaling (SOCS) box protein family (Asb) constitutes a chordate-unique gene family whose members are characterized by variable numbers of N-terminal ankyrin repeats and a C-terminal SOCS box. Within this family zebrafish d-Asb11 and its mammalian orthologues h-Asb11 and h-Asb9 (which seem to have arisen as the result of a recent gene duplication event) is special as it shows very high pan-chordate conservation, Homo sapiens Asb11 and its orthologue in the urochordate Ciona intestinalis sharing 70 % overall similarity on a amino acid basis. The amino acid sequence of zebrafish d-Asb11 has 293 residues, composed of a series of six ankyrin repeats at the N-terminal, and a C-terminal SOCS box. Its N-terminal domain together with the ankyrin repeats are important for specific protein-protein interactions, while the SOCS box domain is necessary for the ubiquitination and degradation of the attached proteins via the E3 ubiquitin proteosomal pathway. Our group has previously identified the zebrafish d-Asb11 gene in a differential display screen for genes downregulated upon neural differentiation (Diks et al., 2006) and established that its expression maintains proliferation and inhibits terminal differentiation in the neural progenitor compartment, whereas it down regulation when compartment size has reached its final extent allows terminal differentiation to occur. However, the functionality for differentiation in cell types originating from non-ectodermal germ layers remains unexplored.

The mechanisms by which d-Asb11 drives neural progenitor expansion have been partially elucidated. Asb11 specifically ubiquitinates and degrades DeltaA in neurogenic regions of the embryo, providing the initial bias essential for lateral inhibition in Notch signaling and thus allowing canonical Notch signaling to drive cell proliferation. From ectopic expression experiments in Hela cells, it is evident that d-Asb11 can also activate Notch signaling outside the neural progenitor compartment, although the levels of transactivation observed are ten-fold less as in neuronal committed cell lines (Diks et al., 2008). Thus the functional meaning for d-Asb-11-mediated activation of Notch signaling outside the ectoderm remains uncertain.

One of the tissue types in which d-Asb11 might function is muscle. Muscle cell differentiation proceeds to an ordered sequence of events, each characterized by expression of specific proteins. It has become clear that different stages of muscle development require expression of specific Asb proteins. For instance, Asb2β was identified in embryonic chick as well as

adult skeletal muscle, and regulates muscle differentiation by targeting actin filamin B (FLNb) for proteosomal degradation (Bello et al., 2009). Inhibition of Asb2β blocks myoblasts fusion and myotube formation, and thus this protein seems important for later phase of muscle development (Bello et al., 2009). On the other hand, Asb15 is expressed in fully differentiated muscles, and has emerged as a regulator of protein synthesis and muscle growth following physical challenge (McDaneld et al., 2006), partly mediated through the PI3K/Akt signal transduction pathway (McDaneld and Spurlock, 2008). Interestingly h-Asb9 (together with h-Asb11 the human orthologues of d-Asb11) is expressed in muscle, suggesting it might have a function there. Furthermore, morpholino-mediated knock down of d-Asb-11 seems to result in aberrant somite formation (Tee et al., unpublished data; Chapter 6). Hence, it is possible that d-Asb11 controls specific aspects of muscle cell development and thus has functionality outside the ectodermal lineage, prompting investigations into this direction.
Here, we provide evidence that d-Asb11/h-ASB9 expression delays expression of muscle creatine kinase (CKM) during myogenenic differentiation both in vitro and in vivo, in a seemingly Notch-independent fashion. Thus, although these results support the notion that d-Asb11 is involved in the regulation of compartment size in derivatives of multiple embryonal germ layers, the signaling mechanisms involved can apparently involve Notch-dependent and independent pathways.

Results

dAsb11/ hASB9 is important for late myogenesis in vitro

For investigating the possible role of dAsb11/ hASB9 in muscle cell differentiation in vitro, we decided to use C2C12 myoblasts, which can differentiate into striated muscles and have shown to be a successful tool to analyze the role of Asb15 in myogenesis . C2C12 cells were transfected with MT, dAsb11-MT, dAsb11^SOCS-HA, hASB5-MT or hASB9-MT constructs, as described previously (Diks et al., 2006) (Figure 1A). After transfection, cells were allowed to differentiate for 3 days. Total cell lysates were obtained from both undifferentiated cells and 3 days differentiated cells.
Transient overexpression of d-Asb11-MT, d-Asb11^SOCS-HA and hASB5 in C2C12 cells does not affect myoblast proliferation or myogenin expression as shown by Western Blot using anti-PCNA and anti-myogenin antibody respectively (Figure 1B, C). However, overexpression of hASB9 resulted in a

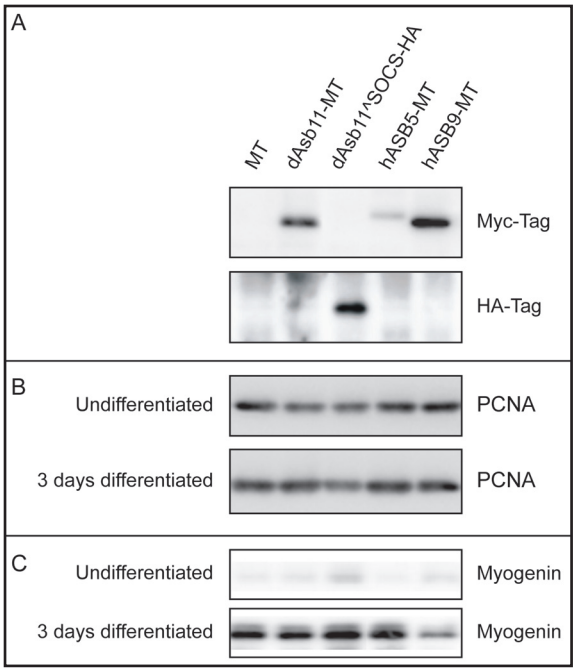

Figure 1. dAsb11 regulates late myogenic markers. C2C12 cells were transfected with MT, dAsb11-MT, dAsb11^SOCS-HA, hASB5-MT or hASB9-MT constructs. One set of cell lysates were isolated from undifferentiated cells, and another set from 3 days differentiated cells which are transfected with the different constructs. Western blot was performed on C2C12 cell cultures, and probed with (A) Myc-tag and HA-Tag, (B) PCNA, (C) Myogenin.

decrease in myogenin protein, while overexpression of both dAsb11 and hASB9 decreases myosin heavy chain expression (Tee et al., unpublished data; Chapter 6). Contrastingly, overexpression of hASB5 and dAsb11^SOCS does not affect proliferation or differentiation of the C2C12 myoblasts (Figure 1A-C). Furthermore, we have previously shown that d-Asb11 is important for modulating the levels of CKM protein, which is another late differentiation marker (Tee et al., unpublished data; Chapter 6). A reporter assay using CKM-Luciferase in C2C12 cells was utilized. Cells co-transfected with Asb11-MT or hASB9-MT and CKM-Luc showed a significant decrease in CKM-luciferase activity, while cells co-transfected with Asb11^C-MT showed a dramatic increase in CKM-luciferase activity. Together, this suggests that d-Asb11/h-ASB9 is important for late myogenic events, but not for early myogenesis.

dAsb11 regulates endogenous CKM protein in vivo

To examine if d-Asb11 functions in a similar way to regulate CKM protein in vivo, we isolated total cell lysates of zebrafish embryos at various timepoints during somitogenesis, and analyzed the endogenous proteins by Western blot with anti-CKM or anti-d-Asb11 antibody. As expected, endogenous CKM protein increased throughout somitogenesis, starting at 14 hpf (Figure 2A-B). Interestingly, endogenous dAsb11 protein decreased from 14 hpf onwards, coinciding with the upregulation of CKM protein (Figure 2A-B).
Next, we injected dAsb11-MT and d-Asb11^C-MT into a one-cell stage

embryos. Total cell lysates were isolated at 16hpf. Consistent with the in vitro data, we see downregulation of endogenous CKM protein with misexpression of Asb11-MT (Figure 2C-D). In contrast, the levels of endogenous CKM was slightly upregulated in embryos injected with Asb11^C construct (Figure 2C-D), indicating that the level of CKM is dependent on having an intact SOCS

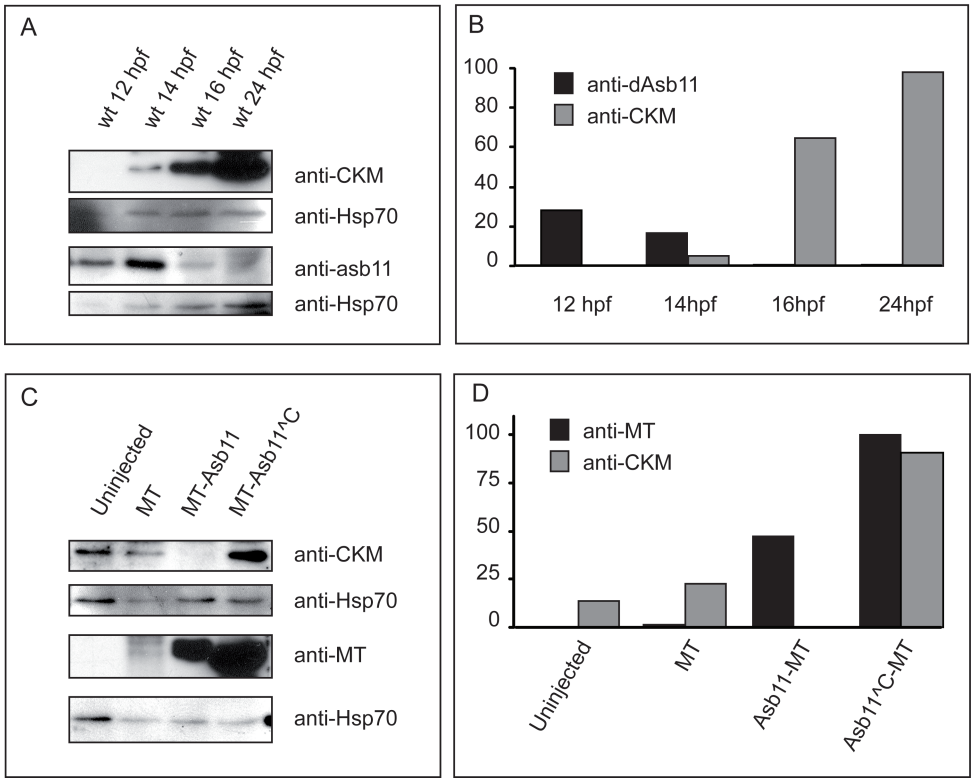

Figure 2. d-Asb11 downregulates CKM. (A) Embryos were collected at 12, 14, 16 and 24hpf, and total cell lysates were isolated. The protein was analyzed using anti-CKM and anti-dAsb11 antibody, and as a control anti-Hsp70 was used. (B) Quantification of the Western Blot in (A). The intensity of the band of interest was normalized against the intensity of the control band. (C) Embryos were injected with MT, dAsb11-MT or dAsb11^C-MT constructs, and total cell lysates were isolated at 16hpf. The protein was analyzed using anti-CKM and anti-MT antibody, and as a control anti-Hsp70 was used. (D) Quantification of the Western Blot in (A). The intensity of the band of interest was normalized against the intensity of the control band.

box.

Notch-dependent and -independent regulation of CKM

As d-Asb11 is known to modulate Notch signaling, it is possible that the effect we are seeing is indirectly, through Notch signaling pathway. To test this hypothesis, we injected NICD mRNA and/or Asb11 morpholino (MO) into a one cell stage embryo. Misexpression of 200pg NICD mRNA and NICD+Asb11MO mRNA resulted in a severely defected embryo, with disrupted somitogenesis. Therefore, there is only very little CKM protein observed (Figure 3A). Misexpression of 100pg NICD mRNA resulted in an increase in CKM protein level (Figure 3A). Similarly, knockdown of Asb11 with Asb11 MO resulted in CKM protein increase (Figure 3A). However, injection of both NICD mRNA and Asb11 MO showed an even bigger increase in CKM protein (Figure 3A). This suggests that Notch signaling regulates CKM, probably by regulating differentiation, and dAsb11, independent of Notch signaling also plays a role in regulating CKM.

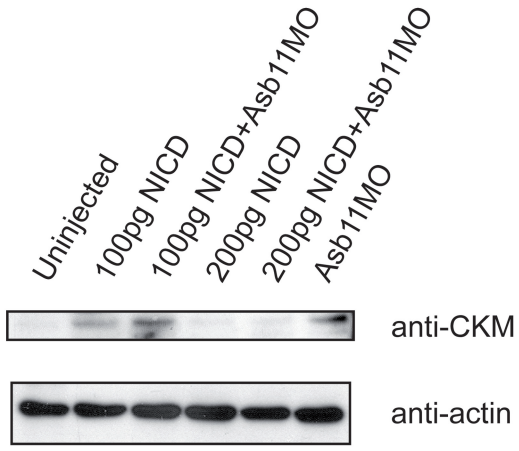

Figure 3. dAsb11 downregulates CKM independent of Notch signaling pathway. Embryos were injected with 100pg NICD, 100pg NICD+Asb11MO, 200pg NICD, 200pg NICD+Asb11 MO or Asb11MO, and total cell lysates were isolated at 16hpf. The protein was analyzed using anti-CKM antibody, and as a control anti-actin was used.

Discussion

The present data implicate d-Asb11/h-Asb9 in the regulation of muscle differentiation, at least as assayed by CKM expression. d-Asb11 is expressed in embryos during the early phases of somitogenesis, with the highest expression at 14 hpf and is downregulated at 16 hpf onwards. On the other hand, CKM protein is expressed in embryos from 14 hpf onwards and increases as muscle cells differentiate. As CKM is expressed relatively late during myogenesis, these data would fit well with a scheme in which expression of d-Asb11 maintains the proliferation of progenitor cells and its down-regulation allow further differentiation and CKM expression, analogous to it action in neurogenic regions of the embryo. Alternatively,

Asb9 was reported to target creatine kinase B (CKB) for proteosomal degradation (Debrincat et al., 2007). CKB is an isoform of CKM and is expressed predominantly in the kidney and testes (Debrincat et al., 2007). Thus as d-Asb-11 regulation of CKM is SOCS-box specific, it would appear that possibly dAsb11 interacts with CKM, leading to the SOCS box-dependent ubiquitination of CKM, and diminished CKM protein levels. Importantly, however, we observe d-Asb11/h-Asb9-dependent down regulation of the CKM promoter in reporter experiments, and hence regulation of CKM seems to occur at a more fundamental genomic level. Nevertheless, it is possible that direct d-Asb11/h-Asb9-mediated proteolytic breakdown of CKM serves as a secondary mechanism as well.

Support for a role for d-Asb11 as a driver of muscle cell progenitor expansion comes from experiments in which d-Asb11 inactivating morpholinos are injected into zebrafish embryos, leading to premature induction of CKM and thus suggesting that the switch between progenitor proliferation and differentiation is temporally brought forward. Conversely misexpression of d-Asb11 in embryos leads to an apparently delayed induction of CKM. Finally, in vitro experiments both d-Asb11 and h-Asb9 suppress differentiation-induced transactivation of both the CKM promoter as well as the differentiation-dependent of myosin heavy chain kinase, another protein expressed fairly late during muscle development. Hence we interpret our results as providing important support for the notion that d-Asb11 expression can suppress terminal differentiation in the progenitor compartment also outside the ectoderm. It could now also prove interesting to investigate its effects on differentiation in endodermal derivatives.

If, however, d-Asb-11 regulates expansion of muscle progenitors in way analogous to its role neural development, regulation of this expansion seems fundamentally different from its mode of action in neurogenesis. In the latter process, d-Asb11 effects are mediated through induction of Notch signaling. Notch signaling is necessary for the proper development of numerous cell types and tissues. It is typically viewed as a transcriptional cascade in which the cleaved intracellular domain of the Notch receptor, NICD, enters the nucleus and activates transcription of downstream target genes. Notch signaling inhibits muscle cell differentiation via multiple pathways (Buas et al., 2009). The upregulation of CKM in zebrafish embryos in overexpression of NICD is thus rather surprising, as it suggest that rather as inhibiting differentiation and stimulating proliferation, in the context of myogenesis it promotes differentiation. Thus d-Asb-11 employs mechanisms alternative from Notch to inhibit the induction CKM during muscle development. This

would fit well with the notion that d-Asb11 acts specifically through DeltaA in Notch signaling, whereas most of the Delta-Notch signaling pathway involved in myogenesis involves DeltaD, which even negatively feeds back to regulate d-Asb11 (Diks et al., 2008). The Notch-independent pathways mediating its effects in muscle remain unclear but probably involve ubiquitination and proteolytic breakdown of direct binding partners. Recent studies suggest that Asb proteins, including d-Asb11 regulate a number of biological processes by this mechanism (Chung et al., 2005; Debrincat et al., 2007; Diks et al., 2008; Wilcox et al., 2004). Therefore, a possible explanation for our data is that dAsb11^C-MT or dAsb11^SOCS-HA, can interact with such targets through the intact ankyrin repeats, but as it lacks the SOCS box, it cannot target it for ubiquitination and degradation. And as dAsb11^C is expressed at higher levels than endogenous Asb11, it allows dAsb11^C to act as a dominant negative and blocks the normal function of Asb11. Therefore, CKM is increased in dAsb11^C-MT injected or dAsb11^SOCS-HA transfected embryo/cells respectively.

In short, we have shown that d-Asb11 is important for late myogenesis events and downregulates CKM in a Notch independent manner. It is clear that the physiological significance of dAsb11-CKM regulation needs to be further examined.

Material and Methods

Zebrafish and embryos

Zebrafish (Danio rerio) were kept at 28.5˚C on a light/dark cycle of 14/10 hour. Embryos were obtained by natural matings and cultured in embryo medium (Westerfield, 1994) until the desired age was reached.

RNA synthesis and microinjection

Capped mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's instructions. As a template, MT, dAsb11-MT and dAsb11^C-MT constructs were used. Approximately 300pg mRNA was injected into the blastomere of 1-cell stage embryos.

Zebrafish cell lysate for immunoblotting

Embryos were dechorionated at the appropriate stage and subsequently

transferred to cell lysis buffer (50mM HEPES mH7.5, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 10% glycerol, 1% Triton-X 100) at 4˚C (2µl/embryo), and subsequently lysed by pippeting them up and down and stored at -2-˚C. Before loading, 2X loading buffer was added to the cell lysis buffer and the sample was boiled for 3 min. Per lane, a total of 7 embryos was loaded.

Morpholinos

Antisense morpholinos were obtained from Gene Tools, LLC. The sequence was as follows: dAsb11 MO: 5'-AGAACCTCGCAGACAGCAACGGTC-3'. Approximately 5ng was injected per embryo, into one cell stage zebrafish embros.

Plasmid construction

Full-length hAsb5 and -9 was amplified from commercially available EST clones using primers containing an EcoRI or XbaI site and subsequently cloned into the EcoRI and XbaI sites of pCS2+MT.

Western blot

C2C12 cells were differentiated by adding 5% Horse serum. For western blot the cells were washed and taken up in laemli samplebuffer. Cell lysates were heated to 95˚C for 5 minutes and afterwards stored at -20˚C if necessary. Next, the samples were loaded on SDS-polyacrylamide gels. The material was transferred to Immobilon (Millipore) by wet blotting in transferbuffer (50 mM Tris pH 8.0, 40 mM Glycine, 0.0375% SDS, 20% methanol) and the blots were incubated for 1 hour in blocking buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 5% non-fat milk). Blots were incubated overnight at 4˚C in TBS-T (50mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 10% blocking buffer and 1:1000 PCNA (Sigma, P8825), 1:100 Myogenin (kindly provided by L. Puri), or β-actin (Santa Cruz Biotech, SC-1615). The blots were washed in TBS-T. After incubation with the appropriate secondary antibody and extensive washing in TBS-T, the blots were developed by ECL.

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CHAPTER 7

Evans Blue dye labelling in injured adult zebrafish muscle

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Submitted

Introduction

Zebrafish regenerates efficiently without any side effects like scarring. In injured zebrafish, the regenerating new muscle fibers can be seen clearly from 7 days post-injury and by 2 months, the muscle is completely healed (Poss et al., 2002; Rowlerson et al., 1997). The ease of injury to the muscles without much stress to the zebrafish makes this model organism ideal for muscle regeneration studies. Zebrafish has been shown to be a good model to study muscular dystrophy, although there has been limited knowledge regarding viable zebrafish showing massive muscle degeneration. We report here the usage of Evans Blue dye to label compromised muscle fibers in zebrafish. For physical injury to the muscle, it is necessary to identify the area of injury in retrospect, as only compromised fibers will incorporate EBD. EBD is widely used in various dystrophic models in both zebrafish and mice to locate degenerating fibers and in such experiments in injected into the peritoneum (Bassett and Currie, 2004; Hamer et al., 2002). We show that the EBD is clearly visible at whole muscle isolate and also in transverse plastic sections. Therefore, this procedure will ease identification of site of injury, as the outer epidermal layer of the zebrafish is healed by 5 days post-injury.

Protocol

PART 1. Preparation of material

The tools needed for this procedure include:
Part 2: MS222, 30G needle, Gentamycin, 10cm petri dish
Part 3: MS222, Scissors, damp piece of cloth or tissue, 10cm petri dish, Evans Blue dye (EBD), Gentamycin, 30G needle, 1mL syringe.
Part 4: Surgical blade, 10cm petri dish.

PART 2. Introduction of muscle injury in the muscles of the dorsal trunk

1. When the tools are ready, place zebrafish into system water containing the MS222 anesthetic.
2. Leave zebrafish in anesthetic until it is unresponsive to external stimuli. Then, remove the zebrafish and place it in a 10 cm petri dish.
3. Carefully take a sterile needle and prick the dorsal trunk of the zebrafish. The injury should always be performed at the same spot to reduce variation.

In our experiment, we have chosen the dorsal trunk right above the anus of the zebrafish. The zebrafish might bleed a little.
4. Quickly return the zebrafish to system water containing Gentamycin (5mg/ml) and allow it to regain consciousness, and swim for an hour in the tank.
5. Put the zebrafish in a clean tank, and put it back into the system until desired age post-injury.

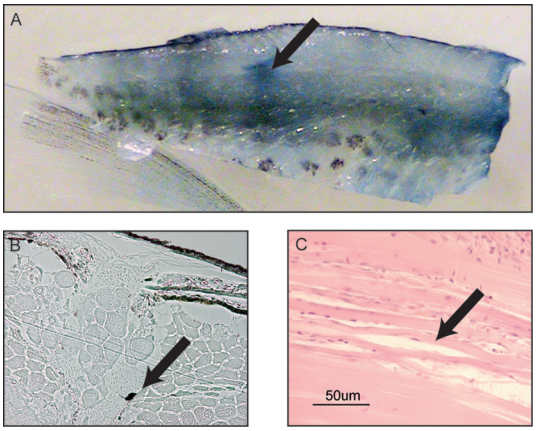
PART 3. EBD intraperitoneal injection (8-16 hrs prior to sacrificing zebrafish)

1. Set up the 10cm petri dish, scissors and damp tissue arrangement on the stereomicroscope.
2. When the tools are ready, place zebrafish into system water containing the MS222 anesthetic.
3. Using a 1mL syringe, aspirate appropriate amounts of EBD (1% volume relative to body mass). Ensure not too much air bubble is incorporated into the syringe.
4. Place anesthetized zebrafish on damp tissue/sponge.
5. Insert tip a little behind the heart at a 90˚ angle, and angle downwards.
6. Inject intraperitoneally into the body cavity of the zebrafish. You should see the blue dye entering the body cavity and into the belly of the zebrafish.
7. Quickly return the zebrafish to system water containing Gentamycin (5mg/ml) and allow it to regain consciousness, and swim for an hour in the tank.
8. Put the zebrafish in a clean tank, and put it back into the system until you are ready to sacrifice the zebrafish (8-16 hours). It is important to keep careful watch, as zebrafish may die. If zebrafish shows signs of distress, sacrifice them immediately, and isolate the muscle are described below.

PART 4. Isolation of zebrafish muscle

1. Sacrifice zebrafish by killing them in high doses of MS222 and ice-cold water. Ensure that the zebrafish is dead.
2. Remove the head of the zebrafish.
3. Carefully hold the zebrafish with a forcep in one hand, and use a surgical blade to scrape away the zebrafish scales.
4. Place surgical blade in the middle of the zebrafish (from dorsal view) and carefully cut the muscle away from the bone and central nervous system.

PART 5. Representative results



A. The inside flank of the zebrafish muscle, showing blue EBD staining (black arrow) at the injured part 1 day post-injury.
B. A transverse section of the injured muscle 1 day post-injury. The black arrow shows the blue EBD staining, while the red triangle shows the part where the needle was inserted.
C. A sagittal section of the injured muscle, counterstained with HE. Regenerating fiber was observed at 11 days post-injury.

Discussion

To obtain favorable outcome of the physical injury to the muscle, it is critical that injury is performed at the dorsal part of the trunk, to prevent excessive damage to the zebrafish main blood vessels. The zebrafish has to be anesthetized properly to avoid excessive stress to the zebrafish. For wild-type zebrafish, the EBD dye can be detected up to 11 days post-injury. We have not tested longer duration of EBD incorporation. However, muscle regeneration is visible at 11 days post-injury and it is expected that after muscle regeneration, and degradation of the injured muscle tissue, the EBD will not be visible. For intraperitoneal injections, the procedure has to be performed quickly, as the zebrafish may wake up after some time outside of the anesthetic. Ensure that the needle is inserted slightly behind the heart at a sharp angle of approximately 90˚, and angled downwards. This is to prevent accidental injection of the EBD into the heart. The EBD will be diffused throughout the whole zebrafish, and will appear blue. Therefore, it is advisable that the EBD intra-peritoneal injection is done only 8-16 hours before your desired age for sacrificing them.

Possible applications:

(1) Identification of site of induced muscle injury

(2) Muscle regeneration studies
(3) Characterizing degenerating muscle fibers in dystrophic mutants

Difficulties:

(1) Precision of muscle injury in the dorsal flank of the muscle is necessary to avoid damage to main blood vessels, which may cause death.
(2) After EBD incorporation, the zebrafish has to be monitored closely for distress, and should be sacrificed 4 hours minimum, and 16 hours maximum. We observe high percentage of death after 16hpf.

Materials

Name	Type	Company	Catalog Number	Comments
30G needle	Equipment	Becton Dickinson	304000	
Surgical blade	Equipment	Swann-Morton	BS2982	Any surgical blade can be used according to personal preference.
1mL syringe	Equipment	Becton Dickinson	300013	
MS222	Reagent	Sigma	18003253010	
Gentamycin solution	Reagent	Sigma-Aldrich	G1397	
Evans Blue Dye	Reagent	Sigma-Aldrich	E2129	

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CHAPTER 8

Discussions

Why study muscle development in zebrafish?

Muscle development studies in zebrafish have recently gained wide interest amongst researchers and clinicians. Especially are the characterization of a small class of 'dystrophic' mutants, which undergo rapid degeneration of the embryonic skeletal muscle (Bassett et al., 2003; Bassett and Currie, 2003; Granato et al., 1996; Hall et al., 2007; Parsons et al., 2002), thus placing zebrafish as an informative model of human muscular dystrophy. Large scale forward genetic strategies in zebrafish have the promise to unravel many more mutations and aid design possible novel strategies to alleviate muscle damage. For example, uncovering the mechanisms underlying the genetics and physiology of a recently identified *sof* zebrafish mutant capable of recovering from severe, early muscle degeneration and thrive as adults, may ultimately have important ramifications (Jacoby et al., 2009).

During embryonic development, muscle formation is critical for normal locomotion and viability of all vertebrate animals. Zebrafish embryos are particularly suited to the study of muscle development for several reasons, most importantly, they develop externally, are transparent, somitic muscle comprises a large proportion of the body and is accessible, and they begin to move very soon after gastrulation. Both embryological and genetic studies have taken advantage of these qualities to examine early stages of muscle development in zebrafish with great success. However, the later stages of development have been relatively little studied beyond the initial identification of mutations that affect muscle fiber differentiation, function and integrity.

In this thesis, we utilized the strengths of zebrafish in loss of function and gain of function studies to identify a novel role of d-Asb11 and the canonical Wnt/β-catenin signaling pathway in early and late myogenesis, respectively. Furthermore, we exploited the muscle regenerative capacity of zebrafish, by examining the adult muscle regeneration in loss of function of d-Asb11 and gain of function of Wnt/β-catenin signaling pathway.

Wnt/β-catenin signaling in myogenesis

In Chapter 3, we discussed the implications of upward deviation from the tightly controlled physiological level of Wnt/β-catenin activity by genetic and chemical intervention in zebrafish embryos. Wnt/β-catenin signaling has been shown to control various aspects of myogenesis through morphogenetic

gradient from the neural tube and dorsal ectoderm. Table 1 lists the known Wnt target genes involved in muscle formation, from segmentation to muscle growth, with the highlighted contribution from Chapter 3 of this thesis to the muscle fiber specification and growth.

Different members of the Wnt family, synergistically and independently of Shh, regulate activation of myogenesis by inducing expression of myogenic bHLH proteins in the presomitic mesoderm (Munsterberg et al., 1995; Stern et al., 1995), and has been thoroughly reviewed by (Cossu and Borello, 1999) and (Bryson-Richardson and Currie, 2008). However, there have been limited studies relating to the role of Wnt/β-catenin signaling in myofibrillogenesis and muscle growth. Recent work have shown that the non-canonical Wnt ligand, Wnt11 is important for elongation of myocytes and organization of early muscle fibers via the Wnt/PCP pathway (Gros et al., 2009). Our study has added value to this research field by documenting the direct/indirect role of Wnt/β-catenin signaling pathway in modulating the expression of Myostatin and p21CIP/WAF, and thus regulating muscle growth. Furthermore, we observe a differing effect between slow and fast muscle fibers.

Although we do not understand as yet the mechanism underlying this observation, there have been studies showing modulation of genes by Wnt/β-catenin during muscle fiber specification. Six1 can reprogram adult muscle from the slow twitch phenotype into the fast twitch phenotype (Grifone et al., 2004) and is required for the onset of fast muscle differentiation in zebrafish (Bessarab et al., 2008). A recent study employing computational methods predicted the TCF4 and Gli-binding sites in the promoters of Six1 and Six4, suggesting that Wnt signalling pathway together with Hedgehog signalling may regulate Six genes (Hallikas et al., 2006). It would be interesting to look if Wnt signalling (synergistically with Hedgehog signalling) directly regulates Six1 or indirectly through regulation of Pax3 (Ridgeway and Skerjanc, 2001; Taneyhill and Bronner-Fraser, 2005) in skeletal muscles fiber determination and differentiation.

Hagiwara et al. have reported that Sox6 could function as a transcriptional repressor of slow fiber specific genes in the developing mouse fetal skeletal muscle (Hagiwara et al., 2005). In Sox6-/- mutant mouse, the slow fiber type-specific gene expression is significantly increased, whereas fast fiber type specific gene expression is significantly decreased. In zebrafish, Prdm1 holds the key, switching between alternative muscle fiber type programs. Prdm1 accomplishes its function by repressing Sox6, and also acts directly as a global repressor of fast-specific differentiation genes (Baxendale et al., 2004; von Hofsten et al., 2008). Although Pdrm is expressed in the mouse

myotome (Chang et al., 2002; Vincent et al., 2005), at present it is still unclear whether the fiber type specification regulatory network is conserved among different vertebrate species. Sox6 mediates expression of Wnt1 signalling in embryonic carcinoma cells (Hamada-Kanazawa et al., 2004). As all these genes are also present in the muscles, it would be interesting to speculate that during embryogenesis, slow muscles are specified through regulation by Sox6, which in turn mediates expression of Wnts. Subsequently, upregulation of Wnts either through regulation by Sox6 or independently, induces expression of Six1 required for fast muscle differentiation.

Muscle growth and regeneration

Although a wealth of knowledge is known about the influence of Wnt proteins on embryonic muscle development, there is only sketchy knowledge regarding the role of Wnt signaling in adult muscle regeneration. Formation of new muscle fibers relies on the existence of satellite cells, which reside between the myofiber basal lamina and plasma membrane. Contradictory observations have been observed regarding the involvement of Wnt signalling in adult muscle regeneration. In Chapter 5, we briefly show that double heterozygous gain of function Wnt/β-catenin adult zebrafish exhibit a more efficient regeneration compared to wild-types. Although we did not explore the mechanism underlying this phenotype, it is frequently stated that muscle regeneration recapitulates embryogenesis. Polesskaya et al., demonstrated that injured muscle produces several Wnt isoforms and that Wnt signaling through the canonical β-catenin pathway induces Pax7 expression and myogenic specification in a subpopulation of muscle-derived side population of stem cells, which could then participate in muscle regeneration (Polesskaya et al., 2003). In agreement with this, a recent study implicated the role of Wnt proteins in regulating muscle satellite cell proliferation by maintaining the expression of Pax7 to MyoD ratio (Otto et al., 2008). On the contrary, Wnt signalling has been shown to modulate the myogenic versus fibrogenic (Brack et al., 2007) or adipogenic (Taylor-Jones et al., 2002) activity of regenerating adult muscles. However in the work by Otto et al., no detectable level of fibrogenic conversion was observed in satellite cells exposed to different Wnt proteins (Otto et al., 2008). Zhao and Hoffman presented an analysis of their Affymetrix expression profiling data, comprising of cardiotoxin (CTX)-induced muscle regeneration series, and proposed that in vitro myogenesis assays more like recapitulate embryonic development than they do regeneration (Zhao and Hoffman, 2004). No

evidence of differential expression of Wnt pathway members were observed, contrary to what is reported in (Polesskaya et al., 2003).
It would be interesting to test whether models of muscular dystrophies would express higher levels of Wnt proteins as a proliferative response to regenerate the degenerating fibers. In various gene expression profiling of human patients with muscle degenerating diseases, whereby Wnt signaling has shown to be differentially expressed (Pescatori et al., 2007; Saenz et al., 2008). Interestingly, induction of Wnt antagonists has been observed in gene expression profiling of young pre-symptomatic Duchene Muscular Dystrophy (DMD) patients (Pescatori et al., 2007). Coinciding with that, expression of genes involved in fibrosis was also upregulated. This is contrary to the role of Wnt signalling in driving fibrogenesis (Brack et al., 2007).

Perspectives on application of Wnt/β-catenin signalling in muscle diseases

Over the past few years, strategies employing inhibition of the myostatin signalling pathway for clinical applications to increase muscle size and strength have emerged as promising avenues. Nevertheless, several pharmacologic approaches, such as androgen steroids and glucocorticosteroids, have been tested with only limited and minor success. For genetic diseases, gene manipulation strategies were employed, but have also been unsuccessful. Follistatin, a direct downstream Wnt signalling target gene, has recently shown potential as a therapeutic agent for muscle diseases (reviewed in (Rodino-Klapac et al., 2009).
Both regeneration and muscle development are dependent upon the presence of muscle satellite cells, and these processes will therefore compete for satellite cell availability when activated simultaneously. In DMD patients, the pool of satellite cells seem to be exhausted before growth is finished. Together with the accumulation of fibrotic and adipose tissue, exhaustion of satellite cells is thought to be the reason of lethality in human patients. Therefore, a strategy to prolong the regenerative capacity would prove to be an attractive approach in the treatment of muscular dystrophy. As Wnt signalling is reported to be involved in both satellite cell proliferation, as well as preferentially undergoing fibrogenesis and adipogenesis during regeneration, a careful study of gene expression profiles in human patients, mdx dystrophic mice, as well as further in vivo studies on various animal models might provide an answer in carefully regulating the signalling pathways or genes involved in functional muscle regeneration.

Ankyrin and SOCS box containing protein (Asb11) mutant: A role for Cullin box?

The gene d-Asb11 was recovered from a differential display screen, showing perturbations in neural plate patterning (Diks et al., 2006). The d-Asb11 protein contains six ankyrin repeats and a C-terminal SOCS box domain. In Chapter 4, we describe a zebrafish mutant identified from a TILLING screen on an F1 N-ethyl-N-nitrosurea (ENU)-mutagenized zebrafish library. The mutation was mapped to the conserved LPϕP sequence, located in the Cullin box domain. The Cullin box domain is important for direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 Modules to Elongin BC-based ubiquitin ligases (Mahrour et al., 2008). In addition to the fundamental importance of the Cullin box domain as critical participant in the control of cellular ubiquitination (at least in vitro), they have recently received additional attention due to the discovery of mutations in cullin-encoding genes in several human diseases (Maksimova et al., 2007; Tarpey et al., 2007; Zou et al., 2007). We showed that in zebrafish, the Cullin box functions in both a Notch-dependent and –independent pathway. Cullin box has been shown to be involved in Notch signaling, for example in the SCF/Cullin family in Drosophila (Lai, 2002), and we show for the first time in vivo the specificity of Cullin box for degradation of the Notch ligand DeltaA. The binding probably occurs through the ankyrin repeats and SOCS box domain, which are known to be important for specificity of protein-protein binding; and the degradation machinery requires the SOCS box/Cullin box domain.

What is interesting is the decrease in proliferation in asb11cul-/- embryos at 24hpf, contrasting to the increase in proliferation of morpholino knockdown of d-Asb11 at the same time-point, suggesting a specific role of Cullin box in this process. Other protein complexes containing Cullin box (e.g. Skip1-Cullin-F-box) have been shown to control proliferation through degradation of critical regulators such as cyclins, CDK inhibitors and transcription factors (Willems et al., 2004). However, to date, there has been no literature supporting this ubiquitination process in Asb proteins.

The specific function of Cullin box relating to Notch-dependent or -independent pathway remains to be identified, as in Chapter 4, we observe activation of her4:gfp in embryos whereby Notch signaling is inhibited using the chemical DAPT. However, in Chapter 6, we described the Notch-independent role of d-Asb11 (with specific requirement for the Cullin box/ SOCS box) in regulating the expression of muscle-specific creatine kinase (ckm). We showed that overexpression of d-Asb11 lacking the C-terminus

Cullin box/SOCS box (d-Asb11^C-MT) both in vitro and in vivo resulted in a significant increase in CKM protein. We speculate that d-Asb11 interacts with CKM, leading to a SOCS box dependent ubiquitination of CKM, and a decline in CKM protein level. Thus, in the case of d-Asb11^C-MT, the ankyrin repeats domain is able to bind to CKM, but cannot target it for ubiquitination. Therefore, the d-Asb11^C-MT acts as a dominant negative and blocks the normal function of d-Asb11.

Ankyrin and SOCS box containing protein (Asb11) mutant: A role for muscle regeneration?

In Chapter 5, we utilized the characterized Asb11cul-/- zebrafish to look into the adult regeneration myogenesis process. What prompted us to explore this angle was the expression of Asb11 in adult muscle tissues, both at RNA and protein level. Interestingly, we show the co-localization of Asb11 with a satellite-specific marker, Pax7 (which has also been identified in juvenile and adult zebrafish muscle (Hollway et al., 2007)). These satellite cells have the ability to contribute to fiber repair and are bona fide fiber associated self-renewing stem cells (Collins et al., 2005; Montarras et al., 2005). It has also been shown that satellite cells undergo asymmetrical cell division in vivo and in vitro, and that they segregate the asymmetric fate determinants Delta and Numb, as well as Myf5 into one or other Pax7-expressing daughter cell (Kuang et al., 2007; Shinin et al., 2006). It is highly likely that Asb11 may play a role in the specification of these Delta/Numb cells, as we have previously described in the case of neurogenic precursor cell population (Diks et al., 2008).

In mammals, the skeletal muscle possesses a remarkable ability to regenerate. Muscle regeneration includes necrosis of the damaged tissue, inflammation, activation of myogenic stem cells, and as a result of this activation, formation of new myofibers and reconstitution of a functional contractile apparatus. The regenerative process is usually initiated by some form of damage to the muscle fibers, whether through direct mechanical trauma, ischemia, thermal insults or toxic chemicals. Characteristically, there are two phases of degeneration of damaged muscle fibers: the first in an early intrinsic phase, whereby calcium-activated proteases causes disruption of the sarcomeric units. The permeability of the sarcolemma, which is increasing as a result of the degradation of the sarcolemma is indicated by the uptake of low-molecular weight dyes like Evans blue (Hamer et al., 2002). In Chapter 7, we

described the feasibility of this labeling procedure in zebrafish.
The second phase of muscle degeneration is the immune response whereby there is an invasion of the damaged muscle fiber by neutrophils (Orimo et al., 1991) and macrophages, which secret specific cytokines that triggers the activation of satellite cells associated with that muscle fiber (Nathan, 1987). Several Asb proteins (e.g. Asb1 in mice) have been shown to be expressed in macrophages, and thus a truncated d-Asb11 may very well have effects on macrophages. However, it is important to note that there is a clearing-up and recovery of the muscle fibers, making it unlikely that the less-efficient regeneration observed could be mainly due to macrophage deficiency. Muscle degeneration is followed by activation of the muscle repair process. Satellite cells migrate (Bischoff and Heintz, 1994) and extensively proliferate before fusing with one another to form young multinucleated myotubes, or with the ends of the damaged muscle fibers (Robertson et al., 1990).

Ankyrin and SOCS box containing protein (Asb11): A universal regulator for precursor compartment?

We show in Chapter 5 that d-Asb11 is important for maintenance of the muscle precursor compartment. d-Asb11 maintains the neuronal progenitor compartment, implying an important role in the ectodermal compartment size. In zebrafish, this function of d-Asb11 does not seem restricted to this germ layer, as we now show in Chapter 5 that it is important for mesodermal lineage as well and hence d-Asb11 appears to be a regulator of vertebrate compartment size of more general importance. In embryos, proliferation is increased during the first wave of myogenesis, and decreased during the secondary wave of myogenesis. This, together with the decreased proliferation in adult zebrafish indicates that the muscle precursor pool is diminished. This is remarkably similar to the effects of d-Asb11 on embryonic neural precursors (Diks et al., 2006), suggesting that d-Asb11 functions possibly in a similar way in regulating both the neuroectodermal and mesodermal cell fate. Whether d-Asb11 is important for compartment size in the endodermal lineage, however, is questionable. Neither d-Asb11 nor its human homologues h-Asb9 and h-Asb11 are strongly expressed in this compartment. Thus, proliferation in the endodermal progenitor compartment is maintained either via other mechanisms or other Asb proteins (e.g. h-Asb6 shows strong expression in the proliferating endodermal compartment). Further investigations as to how endodermal progenitor expansion is regulated are called for.

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Table 1 Muscle genes regulated by the Wnt signalling pathway

Gene	Organism/System	Canonical/Non-canonical(Wnts?)	Direct/Indirect (Up-/Down-regulate)	Musculoskeletal expression/Function	References
Proliferation					
C-myc	Human colon cancer	Canonical	Direct (Up)	Transcriptional regulator	He et al., 1998
CyclinD	Human colon cancer	Canonical	Direct (Up)	Cell cycle kinase regulator	Tetsu et al., 1999; Shtutman et al., 1999; Disputed by Sansom et al., 2005
Segmentation and patterning					
Axin2	Mouse	Canonical (Wnt3A)	Direct (Up)	Tailbud, PSM	Aulehla et al., 2003
Cdx1	Mouse	Canonical (Wnt3A)	Direct (Up)	Tailbud	Lickert, 2000
Cdx4	Mouse	Canonical (Wnt3A)	Direct (Up)	Tailbud	Pilon, 2006
Myogenesis					
Engrailed2	Xenopus	Canonical	Direct (Up)	Zebrafish slow MPC	Degenhardt, 2001; McGrew, 1999
Twist	Wnt1 induced mammary cancer	Canonical (Wnt1)	? (Up)	Sclerotome, Dermatome	Howe, 2003, Morin-Kensiki, 1997
Selp	Mouse PSM explants	Non-canonical (Wnt3A)	Direct (Up)	Posterior PSM, Dermomyotome	Buttitta et al., 2003
Troy	Mouse PSM explants	Canonical (Wnt3A)	Direct (Up)	Dermomyotome	Buttitta et al., 2003
Arl4	Mouse PSM explants	Non-canonical	? (Up)	Dermomyotome	Buttitta et al., 2003
Paraxis	Mouse	Canonical (Wnt6)	? (Up)	Dermomyotome	Linker et al., 2005
Pax3					
Follistatin	Human embryonic carcinoma cell	Canonical	Direct (Up)		Willert et al., 2002
Myofibrillogenesis and muscle growth					
Follistatin	Human embryonic carcinoma cell	Canonical	Direct (Up)		Willert et al., 2002
Pax3					
Mrp/Plf	Murine mammary C57MG	Canonical	Direct (Up)	Actin-binding protein, muscle cells	Ziegler, 2005
Myostatin	Zebrafish	Canonical	? (Down)	Cell cycle regulator	Tee et al., 2009

Summary

Muscle tissue is generated when myoblasts, or muscle precursor cells, stop proliferating and undergo terminal differentiation to become myotubes or mature muscle cells. We are interested in how the genetic basis of muscle precursor cells determines the outcome of the muscle cell fate, and thus leading to disruption in muscle formation and maintenance.

We utilized the zebrafish carrying mutations in both Axin1 and Apc1, resulting in overactivation of the Wnt/β-catenin signaling pathway. We discovered that loss of function of these two genes results in aberrant muscle fiber formation in zebrafish embryos, differentially affecting fast and slow muscle fibers. The aberrant fast muscle development in gain-of-Wnt/β-catenin-function derives from hyperproliferating pre-myogenic progenitors. Chemical intervention that attenuates hyperproliferation rescues fast muscle fibers to normality. Epistatic analyses show that normal fast muscle myofibrillogenesis requires (a) restriction of Wnt/β-catenin signaling and the corresponding upregulation of Myostatin; (b) Myostatin functions in the same process in a negative-feedback loop to repress the Wnt/β-catenin pathway. The downstream target of Wnt/β-catenin in this process is p21CIP/WAF, which is known to be downstream of Myostatin.

Next, we investigated the role of Asb11, a positive regulator of Notch signaling pathway, in embryonic and adult muscle development. Our group has previously reported the role of Asb11 in maintenance of the neural progenitor compartment. We showed that an intact Asb11 protein is required for proper myogenesis to proceed. In adult muscles, Asb11 co-localized with a subpopulation of Pax7 cells, a marker for muscle progenitor and stem cells. Furthermore, long-term BrdU retaining cells, which are slow cycling, co-localizes with Asb11 cells, suggesting a stem cell-like role of Asb11 in adult myogenesis. Importantly, we observed a less efficient regeneration in Asb11 adult zebrafish mutants. These mutants lack a Cullin box domain which we reported to be functionally important in vivo for Notch signalling activation and cell fate specification.

Finally, we described a role of Asb11 in myogenic differentiation, both in vitro and in vivo. We provide evidence that d-Asb11/h-ASB9 expression delays expression of muscle creatine kinase (CKM) during myogenic differentiation in a seemingly Notch-independent fashion. Thus, although

these results support the notion that d-Asb11 is involved in the regulation of compartment size in derivatives of multiple embryonal germ layers, the signaling mechanisms involved can apparently involve Notch-dependent and independent pathways.

Samenvatting

Ik heb ontwikkeling, groei en regeneratie van het spierweefsel onderzocht. Hiertoe gebruik ik een zebravismodel gemuteerd voor zowel Axin1 als Apc1, leidend tot Wnt/â-catenin signaaltransductie overactivering. Mijn resultaten tonen dat functieverlies van deze genen leidt tot verstoorde spiervezel aanmaak in zebravis embryos, met afzonderlijke effecten op snelle en langzame spiervezels. Epistatische analyses tonen aan dat de aanmaak van spierfibrillen in snelle spiervezels de restrictie van Wnt/â-catenin signaaltransductie en de bijbehorende opregulatie van Myostatin vereist.

Vervolgens heb ik Asb11 onderzocht. Hier laten de resultaten zien dat Asb11 noodzakelijk is voor correcte myogenese. Ik laat zien dat een mutant zonder cullin box een inactief eiwit codeert en dat zowel myogenese als myoregeneratie in de mutant gemankeerd zijn. In volwassen spieren co-localiseert Asb11 met de Pax7-positieve subpopulatie en dat de Asb11-positieve populatie op lange termijn BrdU incorporatie behouden (langzaam delende cellen). Asb11 is dus een stamcelmarker. .

Tenslotte beschrijven wij een rol voor Asb11 in differentiatie van spiercellen in vitro en in vivo. Onze resultaten laten zien dat gedurende myogene differentiatie de expressie van creatine kinase (CKM) in de spier wordt vertraagd door expressie van Asb11, op ogenschijnlijk Notch-onafhankelijke wijze. De betrokken signalerings mechanismen kunnen echter gereguleerd worden via Notch-afhankelijke en -onafhankelijke signaaltransductie.

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Jin Ming

Curriculum vitae

Jin-Ming Tee was born on 10th October 1981 in Kuala Lumpur, Malaysia. She finished her pre-university education in year 2000 at St. John's Institution in Kuala Lumpur. She continued her tertiary education at Faculty of Food Science and Biotechnology (now known as Faculty of Biomolecular Sciences), Universiti Putra Malaysia, where she obtained a Bachelors in Biotechnology (1st class Honours) in 2004. After graduation, she was employed as a research assistant in the group of Dr. Chai-Ling Ho, before she embarked on her Masters studies in University of Bath, United Kingdom. In 2005, she obtained her Masters in Research in Regenerative Medicine. In January 2006, she started her PhD research under the supervision of Dr. Dana Zivkovic. After obtaining her PhD, she will start as a postdoctoral researcher in Prof. Dr. Thomas Perlmann's group at Ludwig Institute of Cancer Research/Karolinska Institute, Sweden

List of publications

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