

asb11 Is a Regulator of Embryonic and Adult Regenerative Myogenesis

Jin-Ming Tee,^{1,2} Maria A. Sartori da Silva,² Agnieszka M. Rygiel,² Vanesa Muncan,³ Robert Bink,² Gijis R. van den Brink,³ Paula van Tijn,² Danica Zivkovic,² Liudmila L. Kodach,² Daniele Guardavaccaro,¹ Sander H. Diks,² and Maikel P. Peppelenbosch²

The specific molecular determinants that govern progenitor expansion and final compartment size in the myogenic lineage, either during gestation or during regenerative myogenesis, remain largely obscure. Recently, we retrieved *d-asb11* from a zebrafish screen designed to identify gene products that are downregulated during embryogenesis upon terminal differentiation and identified it as a potential regulator of compartment size in the ectodermal lineage. A role in mesodermal derivatives remained, however, unexplored. Here we report pan-vertebrate expression of *Asb11* in muscle compartments, where it highly specifically localizes to the Pax7⁺ muscle satellite cell compartment. Forced expression of *d-asb11* impaired terminal differentiation and caused enhanced proliferation in the myogenic progenitor compartment both in in vivo and in vitro model systems. Conversely, introduction of a germline hypomorphic mutation in the zebrafish *d-asb11* gene produced premature differentiation of the muscle progenitors and delayed regenerative responses in adult injured muscle. Thus, the expression of *d-asb11* is necessary for muscle progenitor expansion, whereas its downregulation marks the onset of terminal differentiation. Hence, we provide evidence that *d-asb11* is a principal regulator of embryonic as well as adult regenerative myogenesis.

Introduction

THE ESTABLISHMENT OF THE RELATIVE sizes of the various compartments in the vertebrate body is one of the most important and defining processes of developmental biology. During embryonic development, tissue-specific progenitor compartments must undergo massive cell expansion to generate enough volume to produce functionally differentiated tissues. The increase and diversification of vertebrate compartments played a crucial factor in the terms of evolution and enabled organisms to cope with different environmental conditions [1–3]. Further, aberrant compartment regulation is implicated in many serious pathologies (eg, cancer) [4]. The factors that determine and regulate cell proliferation and differentiation, thus, defining compartment size and function, remain poorly understood and elucidation of the underlying molecular mechanisms driving expansion of progenitor compartments represents an important scientific question.

Likewise, little is known with respect to biological events triggering muscle compartment expansion [5–7]. In vertebrates the skeletal muscle arises from an embryonic compartment called myotome, originating from a transient

epithelial structure, the dermomyotome, which in turn is derived from somites. Somites are formed sequentially, as paired segments of the paraxial mesoderm on either side of the neural tube, from anterior to posterior, at regular time intervals. The somites are transient structures patterned by signals from the surrounding tissue into compartments 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 the muscle), and dermatome (precursor of the dermis) [8–10].

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. A similar molecular process of myogenesis occurs postnatally, to recruit adult muscle precursors into new myofibers during skeletal muscle damage [11]. Enhanced knowledge of the molecular determinants in the formation of muscular tissue, both in embryos and adult

¹Hubrecht Institute-KNAW and University Medical Center Utrecht, Utrecht, The Netherlands.

²Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, Rotterdam, The Netherlands.

³Tytgat Institute for Liver and Intestinal Research, Amsterdam, The Netherlands.

organisms, will help to elucidate important processes involved in developmental biology and give a better understanding of degenerative diseases, such as muscular dystrophy, as well as the process of repair, reproduction, or replacement of lost or injured cells, possibly leading to new ways of treatment.

Earlier we reported on efforts to discover the elements involved in progenitor compartment size regulation by isolating genes that are specifically repressed during terminal differentiation and thus may be responsible for the proliferation and expansion of the stem cell compartment [12]. To this end, mRNA from zebrafish embryos treated with 0.5 μ M all-trans retinoic acid (which terminates progenitor expansion and induces full terminal differentiation) was isolated and compared with untreated embryos. Differentially expressed mRNA fragments were tested using whole mount in situ hybridization (WISH) at different developmental stages. Finally, 1 fragment was singled out for detailed characterization based on its restricted spatiotemporal expression pattern during late gastrulation and early somitogenesis. The full-length sequence of the downregulated fragment revealed that the gene is homologous to the mammalian ankyrin repeat and suppressor of cytokine signaling (SOCS) box-containing protein 11 (*Asb11*). The amino acid sequence of zebrafish *Asb11* has 293 residues, composed of a series of 6 ankyrin repeats at the N-terminus, and a C-terminal SOCS box domain [12].

The *Asb* family constitutes a (hemi)chordate-unique gene family whose members are characterized by variable numbers of N-terminal ankyrin repeats and a C-terminal SOCS box [13]. ASB proteins act as substrate receptor subunits of E3 ligases, enzymes that mediate ubiquitylation and degradation of target proteins [14–16] and ubiquitin ligases regulate a plethora of physiologically important processes [17,18]. Although the zebrafish *Asb11* has the most functional homology with human ASB11, sequence analysis also showed high homology of zebrafish *Asb11* with other mammalian members of the ASB family, most markedly with ASB9. Considering the fact that mouse and human *Asb9* and *Asb11* are also located close to each other on the genome (which seems to have arisen as the result of a recent gene duplication event), we proposed that these genes are both mammalian homologues of zebrafish *asb11* [12]. Further, ASB proteins show very high pan-chordate conservation, *Homo sapiens* ASB11 and its orthologue in the urochordate *Ciona intestinalis* sharing 50% overall similarity on an amino acid basis, which points to very fundamental functions in chordate physiology.

Consistently, forced expression of *Asb11* in the presumptive nervous system of zebrafish embryos maintained cell progenitor proliferation and increased the neuronal compartment size, whereas in the absence of *Asb11* premature terminal differentiation was induced resulting in a reduced compartment size. The molecular mechanisms by which *Asb11* sustains progenitor expansion likely involve Notch activation as we established that *Asb11* is a positive regulator of canonical Notch signaling [12,19,20]. However, d-*Asb11* function in embryogenesis and adult organisms has not been fully explored, and thus, it is possible that d-*Asb11* is relevant for compartment definition outside the neuronal system, prompting more comprehensive analysis of its in vivo expression. Indeed, *Asb11* was well capable of acti-

vating Notch signal transduction outside the neuronal system as heterologous expression of this gene activates Notch reporters in a variety of cell types [19].

In this context, muscle development may constitute an interesting target for *Asb11* action as other ASB family members have been especially implicated in the regulation of this compartment [6,19]. ASB2 β was first identified in muscle cells during embryogenesis and in adult tissue, and was shown to regulate muscle differentiation by targeting actin filamin B (FLN β) for proteasomal degradation. Inhibition of ASB2 β blocked myoblast fusion and myotube formation, crucial processes in the later phase of muscle development [21]. Additionally, ASB15 has emerged as a regulator of protein synthesis and muscle growth [22], possibly mediated by the PI3K/Akt signal transduction pathway [23]. Besides, analysis of *Asb11* transcripts showed that the expression of this gene in muscle tissue has a pan-vertebrate characteristic, presenting a particularly high expression in mammalian muscle (mouse and human). Hence, we decided to characterize the function of d-*Asb11* during myogenesis. Further analysis showed that d-*Asb11* expression is specifically restricted to Pax7⁺ compartment and may be important for progenitor maintenance. Downregulation of d-*Asb11* activity, using a mutated form of d-*Asb11*, interfered with myotome formation during embryogenesis and adult muscle regeneration, whereas forced expression led to expansion of the muscle compartment both in vitro and in vivo. We conclude that d-*Asb11* constitutes an important regulator of primary and regenerative myogenesis.

Materials and Methods

Fish and embryos

Embryos were obtained by natural matings, cultured in embryo medium, and staged according to methods previously described [24]. Zebrafish were kept at 27.5°C.

Cell culture

C2C12 mouse myoblasts were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (Gibco) with 4.5 g/L glucose and L-glutamine and supplemented with penicillin (50 U/mL), streptomycin (50 μ g/mL), and 10% fetal calf serum (FCS). Cells were grown in monolayers in a humidified atmosphere containing 5% CO₂. The differentiation medium contained 2% horse serum instead of 10% FCS.

Plasmid construction

Plasmids, containing *asb11* and related sequences, were constructed as described previously [12]. A partial cDNA fragment of *asb11* in pBluescript was used as a template to generate a riboprobe for in situ hybridizations.

mRNA synthesis, mRNA, and cDNA microinjections

Capped mRNAs were synthesized using the mMACHINE kit (Ambion). mRNAs were injected into 1-cell stage embryos or in 1 cell of the 2-cell stage blastomere. cDNA and mRNA were injected by using a microinjector (World Precision Instruments). About 300 pg of *Asb11*

mRNA or 10 pg of cDNA was used unless otherwise indicated. Total volume of the injection was set at 1 nL.

RNA isolation and quantitative reverse transcriptase–polymerase chain reaction

Total RNA extraction and purification was performed by using standard Trizol and isopropanol precipitation. cDNA synthesis was performed using hexamer primers and MLV reverse transcriptase. Transcript levels were quantified by real-time polymerase chain reaction (PCR) using ABsolute QPCR SYBR Green Fluorescein Mix (Westburg) on an iCycler iQ Real-Time PCR detection system (Bio-Rad). Results are expressed as a relative ratio to the housekeeping gene *actin*.

Primers

Zebrafish: *asb11*-F: CTGCAAAGAGAGGTCACACG and *asb11*-R: TCCTTTTGTCCAGTGAGC. Mouse: *Asb11*-F: GTCAGAAGGCCTGGACCAT and *Asb11*-R: CTCATGGAGTGGGGATCG. *Asb9*-F: TCCTCTTCATGATGCTGCAA and *Asb9*-R: CACGTGATCTGCTGTGATGA.

In situ hybridization

mRNA WISH was carried out as previously described [25]. Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and digoxigenin-tagged probes to myoD, muscle creatine kinase (mck), and myogenin were made with Roche labeling mix [26]. Images were obtained using a Zeiss Axioplan Stereomicroscope (Oberkochen) equipped with a Leica (Wetslar) digital camera.

Immunolabeling of zebrafish embryos

Embryos were fixed for antibody staining with 4% PFA and whole mount immunohistochemistry was performed according to Du et al. [27], using primary antibodies Pax7 1:20 (Developmental Studies Hybridoma Bank) and PH3 (Upstate Biotechnology; #06570) 1:1,000. Appropriate secondary antibodies were used at 1:200. Immunohistochemistry was analyzed at the level of yolk extension.

Immunolabeling of adult muscle

All stainings were initially performed as monostainings to assess the problem of bleed-through of the fluorors. Subsequently, concentrations were titrated to minimize the problem (ie, if staining in 1 channel was markedly stronger as in another channel, causing bleed-through antibody, then concentrations were adjusted to reduce staining intensity). In addition, only double stainings were accepted for further analysis when in the same field clear monostained structures could also be recognized.

Short-term (7 days) and long-term (2 months) bromodeoxyuridine (BrdU) incorporation and labeling was performed by immersing and allowing the zebrafish to swim in BrdU (150 mg/L for 4 h per day) for 7 days. Fish were fixed at 2 months after the BrdU pulse. Adult muscle tissue was isolated and frozen in liquid nitrogen, postfixed in 4% PFA, cryosectioned in 10 µm, and dried overnight. Sections were postfixed in acetone, washed, and kept in 100% methanol

overnight. Sections were permeabilized with 0.2% Triton X-100 and quenched with 100 mM glycine-NaOH (pH 10). Sections were washed in PBS-Tween-20 (PBS-T) and incubated in 2 N 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% bovine serum albumin (BSA), and 1% dimethyl sulfoxide (DMSO) in PBS-T] at room temperature for 1 h. 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 h and anti-rat conjugated secondary antibody (Cy2/Jackson Immunoresearch; 1:200) was incubated for 2 h at room temperature (RT). After washing, sections were blocked (20% goat serum and 0.1% BSA in PBS) for 1 h. *Asb11* antibody [12] was pre-incubated in fish powder and subsequently incubated in block (10% goat serum and 0.1% BSA) overnight at 4°C. After washing in PBS-T, sections were blocked for 1 h and incubated in anti-rabbit secondary antibody (Cy3/Jackson Immunoresearch) for 2 h at RT. After washing, sections were blocked (10% goat serum, 0.1% BSA, and 1% DMSO in PBST) for 1 h 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 (Rhodamine Red-X/Jakson Immunoresearch) for 2 h at RT. After immunolabeling, sections were counterstained in DAPI (Sigma) and analyzed by confocal microscopy as described earlier [28].

Tissue histology

For cryosections, adult muscle tissues were embedded in Jung Tissue Freezing Medium, and sectioned at 10 µm. For plastic sections, adult muscle tissues were 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 hematoxylin and eosin.

Zebrafish cell lysate for immunoblotting

Embryos were dechorionated at the appropriate stage and subsequently transferred to cell lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton-X 100] at 4°C (2 µL/embryo), and subsequently lysed by pipetting up and down and stored at -20°C. Before loading, 2× loading buffer was added and the sample was boiled for 5 min. A total of 7 embryos was loaded.

Immunoblotting

Cell lysates were sonicated and then centrifuged at 1,200 g for 10 min at 4°C. The pellet was discarded and the protein concentration was measured with the BCA protein assay kit (Pierce chemical Co.). Lysates were diluted 1:2 in protein sample buffer [125 mM Tris/HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, 20% glycerol, and 1 mg bromophenol blue] and incubated at 95°C for 5 min. Twenty-five micrograms of protein per lane was loaded onto SDS–polyacrylamide gel electrophoresis and subsequently transferred onto PVDF membrane (Millipore) as described earlier [29]. The blots were blocked with 2%

low-fat milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 1 h at room temperature and washed in TBST before overnight incubation at 4°C with primary antibody in 2% low-fat milk in TBST. Blots were then washed with TBST and incubated for 1 h at room temperature in 1:1,000 horse radish peroxidase conjugated secondary antibody in 2% low-fat milk in TBST. After a final wash with TBST, blots were incubated for 5 min in Lumilite plus (Boehringer-Mannheim) and then chemiluminescence detected using a Fuji LAS3000 illuminator (Fuji Film Medical Systems).

Luciferase reporter assay

C2C12 cells were transiently cotransfected with an MCK promoter-driven luciferase construct and one of the following constructs: myc-tagged *Asb11* (MT-*Asb11*), HA-tagged SOCS box deficient *Asb11* (HA-*Asb11*ΔSOCS), myc-tagged *Asb9* (MT-*Asb9*), or myc-tag empty vector (MT). To correct for transfection efficiency or dilution effects, cells were transfected as explained previously but reporter vector was replaced with a cytomegalovirus (CMV) promoter-driven Renilla luciferase (Promega). Lipofectamine Plus (Invitrogen) method was used according to manufacturer's instruction. After 48, 72, or 96 h of incubation in differentiation medium, cells were lysed with passive lysis buffer as provided by Promega and luciferase activity was assayed according to the Dual-Glo-Luciferase Assay System (Promega) protocol on a Lumat Berthold LB 9501 Luminometer (Berthold Technologies).

Imaging and quantifications

Fluorescent labeling was imaged using a Leica TCS SPE confocal microscope. 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, heteroscedastic 2-tailed Student's *t*-test was performed using Microsoft Excel.

Results

Specific expression of *Asb11* in the Pax7⁺ muscle satellite cell

In an effort to determine possible extra-neuronal functions for *Asb11*, we explored the specific distribution of *d-asb11* transcripts in adult zebrafish tissues by reverse transcription-PCR. *d-asb11* cDNA from total mRNA extracted from muscle, heart, eye, intestine, gills, testis, pancreas, and brain was amplified. β-Actin was used as a control. *d-asb11* was expressed in all the tissues we explored (Fig. 1A), indicating functionality outside the nervous system, and possibly in muscle. This notion is reinforced by experiments attempting a pan-vertebrate comparison of *Asb11* expression.

In mammals and birds 2 orthologues of *d-asb11* are present, *Asb9* and *Asb11*, which are highly homologous, both sharing ~70% sequence similarity to *d-asb11*. *Asb9* and *Asb11* lie adjacent to each other on the X-chromosome, suggesting a *sarcopterygiic*-specific gene duplication event, not present in teleosts. We investigated specific-tissue expression of murine *Asb11* and murine *Asb9* and observed particularly prominent

Asb11 expression in skeletal and cardiac muscles and less, but significant, expression in brain (Fig. 1B), whereas *Asb9* was strongly expressed in testis and kidney (Fig. 1C). This is in agreement with the earlier established function of *asb11* in regulating compartment size in the central nervous system of zebrafish embryos, but the expression of *Asb9* in testis and kidney shows that high expression of *asb11* orthologues in distinct mammalian tissues is a phenomenon observed in evolutionary highly divergent vertebrates and calls for an investigation to the role for d-*Asb11* in zebrafish compartment expansion in these tissues.

To analyze the endogenous expression of d-*Asb11* during embryogenesis, we isolated total cell lysates of zebrafish embryos at 12, 14, 16, and 24 hours postfertilization (hpf) and compared protein levels of d-*Asb11* and MCK, a marker for terminal muscle differentiation whose onset of expression coincides with the end of progenitor expansion [30,31]. Indeed, as expected, expression of MCK protein increases throughout somitogenesis (Fig. 1D). Consistently with a role of *d-asb11* during progenitor expansion in muscle development, the expression of *Asb11* is high during early myogenesis and diminishes when the final stages in muscle differentiation ensue, being approximately complementary to the expression of MCK.

Next, we analyzed the histological localization of *d-asb11* and its relationship with progenitor cells. Cross sections of skeletal muscle tissue of zebrafish were costained with an antibody specific for d-*Asb11* and the well-established muscle satellite marker Pax7 [32,33]. *Asb11* staining displayed a distinct expression pattern on the sarcolemma where it colocalizes with a portion of the Pax7⁺ compartment (Fig. 1E). To confirm that these Pax7⁺/*Asb11*⁺ cells represent muscle progenitor cells, we tested their long-term BrdU label retention. The results, presented in Fig. 1E, confirmed that this double compartment truly represented slowly-cycling satellite cells. Thus, the muscle stem cell compartment in adult muscle is characterized by highly specific *Asb11* expression in this compartment.

Forced expression of *asb11* inhibits muscle cell differentiation and supports progenitor expansion in the C2C12 model system

Heterologous expression of *asb11* has been shown to support progenitor expansion while simultaneously inhibiting terminal differentiation in the PC12 and N-Tera2 in vitro model systems for neurogenesis [12]. Expression of *Asb11* in the satellite cell compartment may suggest that *Asb11* could also act in a similar manner in muscle formation. Mouse C2C12 myoblasts, upon application of differentiation medium, synchronously withdraw from the cell cycle, elongate, adhere, and finally fuse together to form myotubes exhibiting most mechanobiochemical adaptations associated with fully differentiated muscle [34] (Fig. 2A). Thus, this system represents a valuable model to study the effect of *Asb11* on muscle cell differentiation.

Confirmation of the inhibitory effects of *Asb11* on muscle cell differentiation was obtained from experiments in which C2C12 cells, upon differentiation medium, were transfected with different constructs in combination with MCK promoter-driven luciferase or a construct containing a CMV promoter-driven luciferase. Vectors containing either

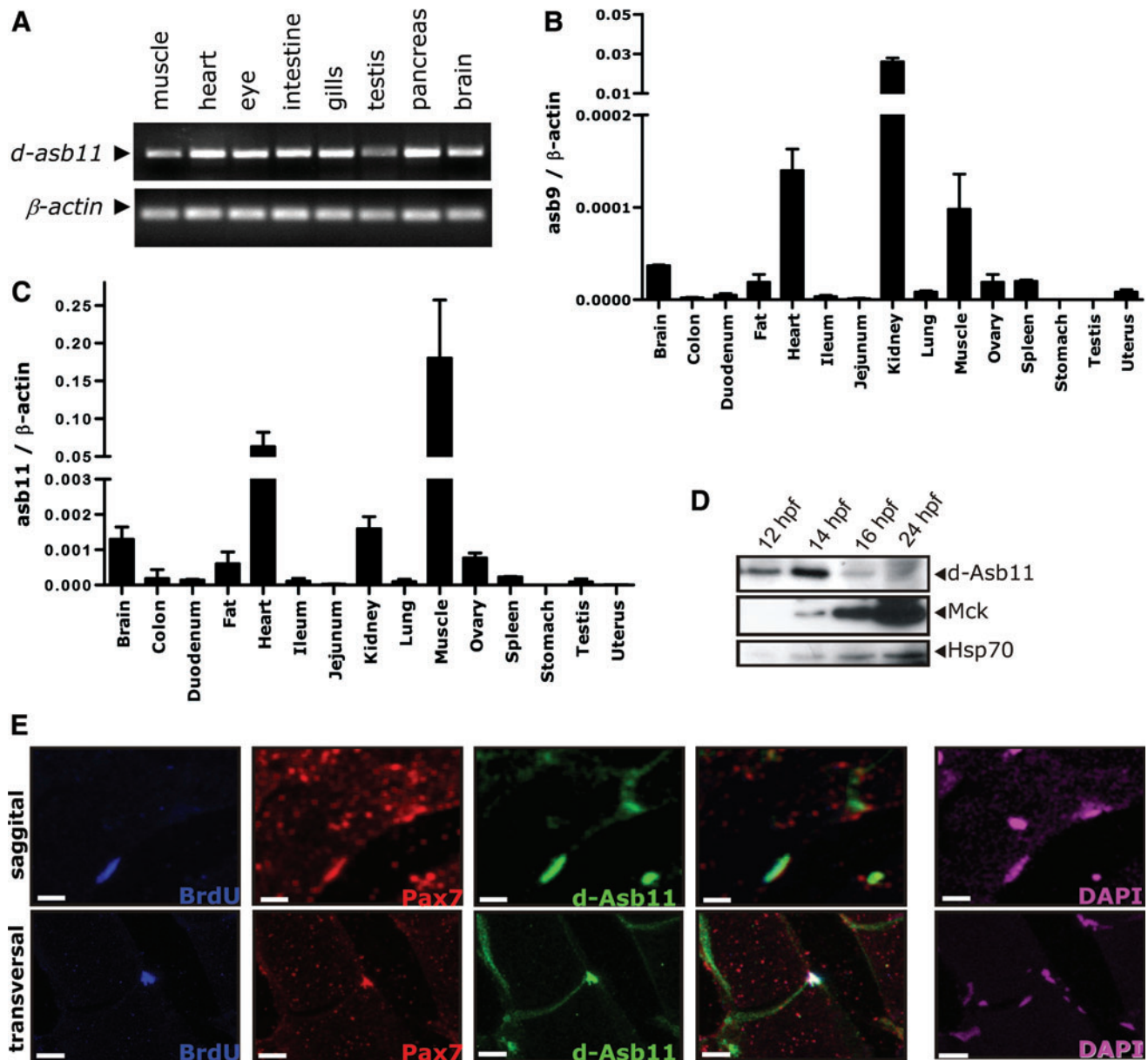


FIG. 1. Distribution of Asb11 expression in different tissues and colocalization with Pax7⁺ satellite cells. **(A)** RT-polymerase chain reaction analysis of zebrafish *d-Asb11* (*asb11*) in mRNA samples isolated from muscle, heart, eye, intestine, gill, testis, pancreas, and brain of adult fish. Zebrafish β -actin was used as template control. **(B, C)** Various types of tissue from adult mice were analyzed for mRNA expression of murine *Asb9* (*Asb9*) **(B)** and *Asb11* (*Asb11*) **(C)**. Results were normalized against β -actin expression. *Asb9* is showing the highest expression in kidney, heart, and muscle. *Asb11* shows the highest expression in muscle, heart, kidney, and brain. The bars indicate the standard error of the mean and were calculated from the results of 3 individual experiments. **(D)** Analysis of protein levels of Asb11 and muscle creatine kinase (MCK), a marker of muscle terminal differentiation, in different stages during embryogenesis. Hsp70 expression is used as input control. Expression of MCK and Asb11 showed inverse correlation during zebrafish embryogenesis. The images shown are representative for 3 independent experiments. **(E)** Adult zebrafish muscle was immunostained with bromodeoxyuridine (BrdU), Pax7, and d-Asb11 antibodies. *Top panels* show a representative staining of sagittal sections and *bottom panels* show a representative of transverse sections. d-Asb11 revealed a distinct expression pattern on the sarcolemma where it colocalizes with Pax7⁺ cells. The capacity of Pax7⁺/Asb11⁺ cells for long-term BrdU incorporation was tested and confirmed that this double-positive compartment truly represents slow-cycling satellite cells. 4',6-diamidino-2-phenylindole staining was included to determine cell locations (*white bars* are 10 μ m). Color images available online at www.liebertpub.com/scd

the HA-tag (HA) or a SOCS-box deficient version of *asb11* (*HA-Asb11 Δ SOCS*) were used as controls.

Differentiation induction of luciferase-mediated MCK promoter was significantly inhibited in the presence of *MT-Asb11* or *MT-Asb9* (orthologue of zebrafish *asb11* in the hu-

man genome, together with *Asb11*), while no significant effect was shown by coexpression with the *Asb11* lacking the SOCS box domain (Fig. 2B). Subsequently, we tested the effect of *MT-Asb11* as well as *MT-h-ASB9* on the expression of the early myogenic marker myogenin (MyoG) [35] and the

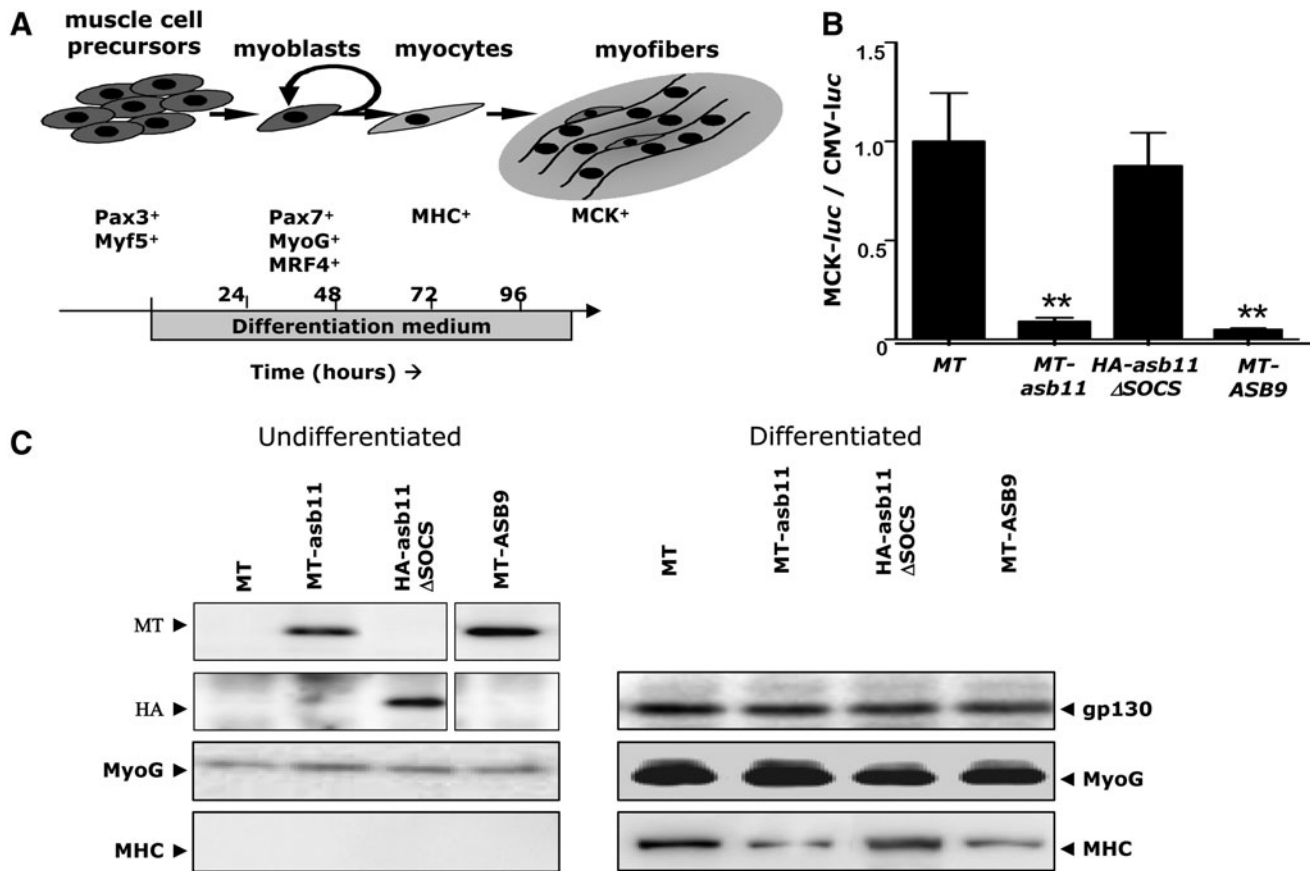


FIG. 2. Heterologous expression of *d-Asb11* or *h-Asb9* prevents terminal differentiation in the C2C12 model system of skeletal muscle differentiation. **(A)** Schematic illustration of the various stages of skeletal muscle differentiation as observed in the C2C12 model system and the associated expression of markers of muscle differentiation. **(B)** Following 96 h of exposure to differentiation medium, terminal differentiation of skeletal muscle, as assayed by luciferase activity driven by the MCK promoter, was inhibited by heterologous expression of either *asb11* or *h-Asb9*. Transfection of a vector expressing only myc-tag (MT) or a HA-tag suppressor of cytokine signaling (SOCS) box deficient *d-Asb11* (HA-*d-Asb11*ΔSOCS) showed no inhibitory effects. As a control for transfection efficiency, cytomegalovirus (CMV) promoter activity was used. Error bars show standard error of the mean (SEM) of 3 experiments. Statistical testing was done by comparing experimental groups to MT-only-transfected cells using a heteroscedastic paired Student's *t*-test. **(C)** C2C12 cells were transfected with a vector containing only the MT, MT-*asb11*, HA-*asb11*ΔSOCS, or MT-*hAsb9* and allowed to differentiate for 72 h in the appropriate medium. Positive expression of the constructs was assessed and is shown on the left side of the panel. Effects of these transgenes on the expression of myogenin (MyoG; a myoblast marker) and myosin heavy chain (MHC; a marker of terminal muscle cell differentiation) were tested either in undifferentiated cells (left) or after 72 h under differentiation medium treatment (right). A representative image from 3 independent experiments is shown. ***P* < 0.01.

terminal myocyte marker myosin heavy chain (MHC) [36] (Fig. 2C). As expected, both genes were substantially induced under differentiation conditions in control cells. After verification that the different *Asb* constructs were sufficiently expressed (Fig. 2C) we investigated the influence of such heterologous expression on in vitro muscle differentiation. Importantly, when cells were transfected with expressing MT-*asb11* or MT-*h-ASB9* constructs, differentiation no longer induced MHC expression (Fig. 2C). However, induction of MyoG was unaffected by expression of either MT-*Asb11* or MT-*ASB9*. Together, these observations showed that forced expression of *Asb11* prevents terminal myocyte differentiation but not myoblast differentiation of muscle precursors, at least in the C2C12 model system, consistent with a possible role of *d-asb11* in maintaining progenitor proliferation in muscle.

Forced expression of Asb11 in vivo deregulates differentiation in the presumptive myotome and causes hyperplastic myotome formation

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 *asb11* is associated with impaired differentiation in zebrafish muscle. Thus, we injected MT-*asb11* mRNA into 2-cell stage blastomeres, and investigated the expression pattern of *myoD*, a bHLH transcription factor that is expressed in adaxial cells and in the posterior region of each developing somite [31,37], by using WISH. An anti-Myc tag antibody was used to confirm expression of injected mRNA and to identify the half of the embryo derived from the injected blastomere. Compared with wild-type expression

(Fig. 3A), forced expression of *MT-asb11* resulted in loss of somitic *myoD* expression accompanied by variably reduced or absent expression in adaxial cells at 13–14 hpf in >80% of the injected embryos ($n=82$) (Fig. 3B–D). Further, the expression pattern of *MT-d-Asb11* in the embryo coincided with

exclusion of *myoD* expression (Fig. 3E–F). Additionally, when expressed in the whole embryo, *Asb11* affected *myoD* in the entire myotome, showing a banded and/or displaced expression pattern at later stages (16 hpf) (Fig. 3G–I). Hence, forced expression of *d-Asb11* delays acquisition of *myoD*

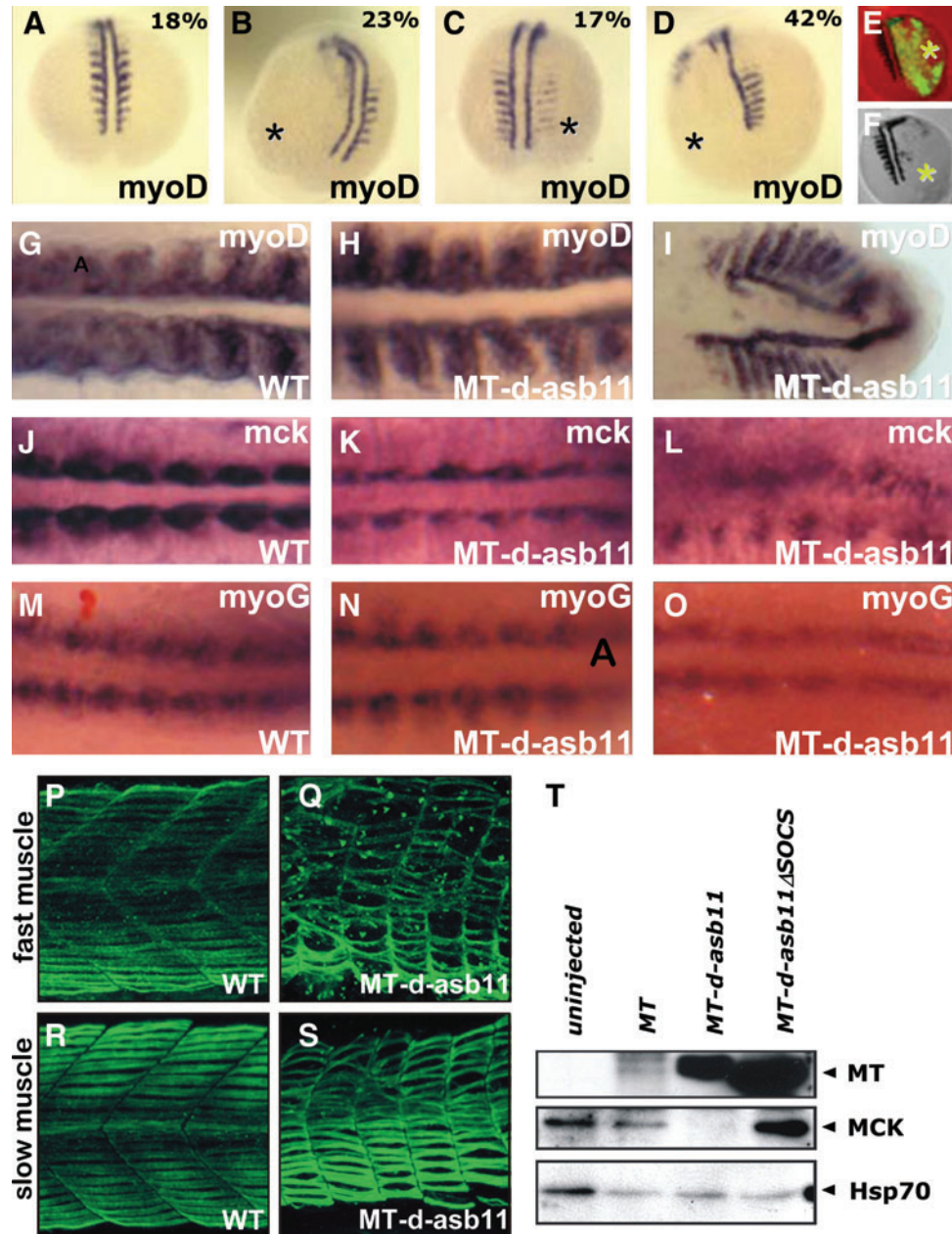


FIG. 3. Forced expression of *asb11* mRNA interferes with muscle differentiation in vivo. (A–D) Zebrafish embryos were injected at the 2-cell stage in 1 of the 2 blastomeres (marked with asterisk) with encoding full-length *MT-d-asb11* mRNA (300 pg). The numbers indicate the percentages of injected embryos displaying the particular phenotype (total $n=82$). Panel A shows an uninjected wild-type embryo. (E, F) Immunolabeling of *MT-Asb11* overlay with *myoD* in situ hybridization. (G–O) Embryos were injected at 1-cell stage with *Asb11* mRNA and fixed at 16 hours postfertilization (hpf). Whole mount in situ hybridization was performed using (G–I) *myoD*, (J–L) *mck*, and (M–O) *myoG* riboprobe. Expression was compared between uninjected (G, J, M) and injected embryos (H, I, K, L, N, O). (P–S) Abnormal axial muscle formation at 36 hpf in response to *d-asb11* mRNA injections. Fast and slow muscle fibers were stained by immunolabeling as described earlier [33]. (T) Confirmation of *d-asb11*-forced expression effects on *mck* at the protein levels. Zygotes were injected *MT-asb11* mRNA, *MT* only, or *MT-asb11*ΔSOCS-injected embryos, serving as controls. Total cell lysates were isolated at 16 hpf. The protein was analyzed using an anti-Mck antibody. Anti-*MT* antibody was used to verify induced expression of *asb11* variants, and as a loading control an anti-Hsp70 antibody was used. A representative image from 3 independent experiments is shown. Color images available online at www.liebertpub.com/scd

positivity in the presumptive axial myotome and deregulates important aspects of differentiation during zebrafish embryogenesis. As judged by *mck* expression—a marker for advanced muscle cell differentiation—*d-asb11* misexpression inhibited terminal muscle differentiation (Fig. 3J–L), whereas *myoG* expression—a more early marker of muscle differentiation—was only marginally affected by such *asb11* misexpression (Fig. 3O).

In an effort to confirm these effects at the protein level, zygotes were injected with *MT-asb11* and *MT-Asb11ΔC* mRNA. Uninjected embryos and embryos injected with *MT* only served as controls. Total cell lysates were isolated at 16 hpf and analyzed using an anti-MCK antibody, while an anti-MT antibody was used to verify induced *asb11* variants (Fig. 3T). Indeed, forced expression of *asb11* caused reduction of *Mck*, in agreement with its inhibitory function of cell differentiation.

Finally, to analyze the effects of forced *d-Asb11* expression in slow and fast muscle fiber formation, we injected *MT-Asb11* mRNA in zygotes that were then immunostained at 36 hpf. At this time point, aberrant expression of *d-Asb11* caused gross abnormalities in both slow and fast muscles (Fig. 3P–S), accompanied by disruption of normal muscle architecture. Together, these results indicate an important role for *d-asb11* in muscle progenitor expansion.

Earlier we have shown that in the presumptive neuronal system forced expression of *d-Asb11* increases proliferation [12] concomitant with the delay in the acquisition of terminal differentiation markers. The in vitro experiments suggest that *d-Asb11* can also maintain proliferation in the developing myotome. Thus, we assume that the misexpression of *d-Asb11* will coincide with increased proliferation of the presumptive musculature. To analyze this possibility further, we injected different concentrations of *d-Asb11* mRNA in zygotes, using GFP mRNA as a control. Misexpression of *d-Asb11* mRNA in 3 independent experiments, each consisting of at least 60 *asb-a* mRNA-injected embryos, resulted among others in cyclopia, notochord defects, and tail phenotypes (23%), including no tail (*ntl*)-like phenotype and in a loss of 1 of the 2 pectoral fins (4%), whereas control mRNA never induced these effects, even when the control RNA was injected at twice the concentration of *d-Asb11* mRNA. The notochord and tail phenotypes and the cyclopia are especially evident at higher *d-Asb11* mRNA concentrations (Fig. 4A), which finally may cause severe dysmorphogenesis but also the myotome appears significantly enlarged (Fig. 4A). Morphometric analysis confirms this notion (Fig. 4B). Thus, it appears that the block in muscle differentiation provoked by *d-Asb11* overexpression coincides with compartment expansion.

Fish homozygous for a hypomorphic Asb11 mutation are impaired in muscle progenitor expansion and myotome formation

The expression of *d-Asb11* in the muscle progenitor cell compartment and the inappropriate expansion of the myotome following its misexpression suggest that *d-Asb11* is important for progenitor expansion. Thus, we decided to investigate the effect of moderate *d-Asb11* deficiency during muscle development. To this end, we employed a mutant *d-asb11* zebrafish germline (*d-asb11^{cul}*) that lacks the cullin box, a SOCS box subdomain, resulting in a hypomorphic allele.

We recently described the isolation of *d-asb11^{cul}* mutants in detail and demonstrated that these are defective in Notch signaling and have impaired cell fate specification within the neurogenic regions of the embryos [20]. We did not analyze effects on muscle development, however. The *d-asb11^{cul}* mutants survive until adulthood; however, they present a subtle shorter trunk and less well-defined somites. Interestingly, when the developing myotome was analyzed for the number of proliferating muscle progenitor cells, as defined by phospho-histone 3 (pH3) and Pax7 double staining, this number was significantly reduced in the *d-asb11* mutants (Fig. 5A). Further, as judged by the expression of *mck*, muscle terminal differentiation in *d-asb11^{cul}* embryos is temporally enhanced (Fig. 5B), whereas the cell proliferation in the myotome of these mutants is substantially reduced (Fig. 5C–E). Thus, we conclude that *d-asb11* is required for maintaining myogenic proliferation in the stem cell compartment during embryogenesis.

Expression of d-Asb11 in proliferating satellite cells is important for adult regenerative myogenesis

To determine whether regenerative myogenesis like primary embryogenesis is regulated by *Asb11*, we induced mechanical injury with a 30G needle on the adult zebrafish body musculature, dorsal to the anus, and investigated the number of BrdU⁺, Pax7⁺, and *Asb11*⁺ cells in lesion-induced versus uninjured wild-type zebrafish at 7 days postinjury. Although this procedure does not produce significant changes in the number of BrdU⁺ or Pax7⁺ cells, the double-positive *d-Asb11*⁺/Pax7⁺ compartment substantially increases in size following muscle injury, indicating that *d-asb11* may be involved in response to trauma in this tissue (Fig. 6A). Subsequently, we compared the proliferation between injured *d-asb11^{cul}* and wild-type zebrafish. Injured *d-Asb11^{cul}* mutants showed a significant diminished numbers of BrdU⁺ cells (Fig. 6B), suggesting that regenerative proliferation is dependent on functional *Asb11*.

Next, we performed a muscle regeneration comparative analysis using injured tissues of wild-type, *d-Asb11^{cul}*, and *axin1/apc1* mutants. *axin1/apc1* double heterozygous mutants were used as positive controls as Wnt signaling is shown to positively regulate satellite cell proliferation on adult muscle fibers during wound-healing response in CD34⁺ cells [38] as well as cultured myofibers [39].

At day 0, muscle fiber morphology was comparable in wild-type, *axin1/apc1*, and *d-Asb11^{cul}* zebrafish lines (Fig. 6C, G, K). At day 1 postinjury, there was a marked cellular invasion, and evident degeneration and necrosis of mature fibers of all tissues (Fig. 6D, H, L). At day 5 postinjury, *axin1/apc1* mutants showed remarkable recovery and numerous small-diameter regenerating muscle fibers were observed (Fig. 6I), whereas wild type showed a slight recovery (Fig. 6E) and in *d-Asb11^{cul}* the muscle fibers were still necrotic (Fig. 6M). At day 10 postinjury, *axin1/apc1* showed a clear recovery and regeneration of the muscle fibers (Fig. 5J), while the aspect of wild-type fish appeared only moderately worse (Fig. 6F). In *d-Asb11^{cul}*, however, little improvement could be noted with small regenerating fibers emerging (Fig. 6N). Altogether these results establish a crucial role of *Asb11* in both embryonic and adult regenerative myogenesis.

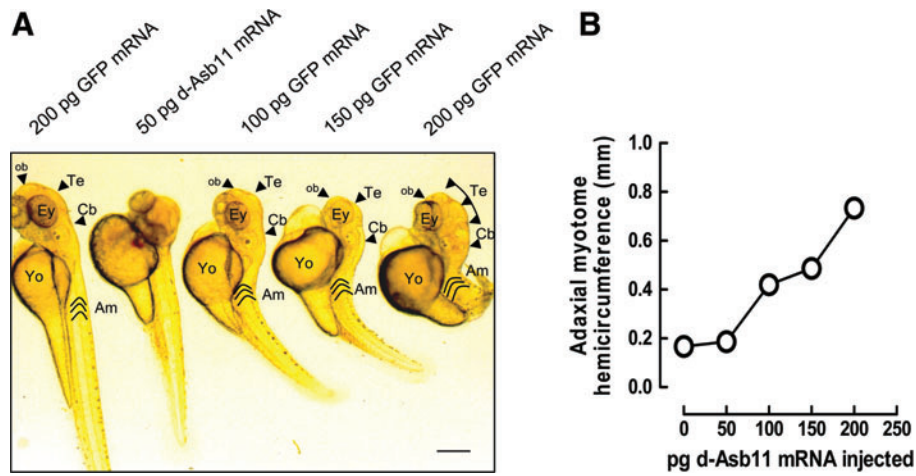


FIG. 4. Forced expression of *asb11* mRNA increases muscle compartment size. (A) Zebrafish zygotes were injected at the 2-cell stage with encoding full-length *MT-d-asb11* mRNA at different concentrations or 200 pg of GFP-encoding mRNA as a control. Injection of increasing amounts of *d-Asb11* mRNA (50, 100, 150, and 200 pg) in zygotes causes CNS and especially brain hypertrophy, which was described earlier at 48 h [12], but also the muscle compartment appears enlarged. Ob, olfactory bulb; Te, telencephalon; Cb, cerebellum; Am, adaxial myotomes; Ey, Eye; Yo, yolk sac; CNS, central nervous system. Bar indicates 100 μ m. (B) Morphometric analysis of the adaxial myotome hemicircumference at equivalent position in the embryo is consistent with an increased muscle compartment size following *d-Asb11* misexpression. Color images available online at www.liebertpub.com/scd

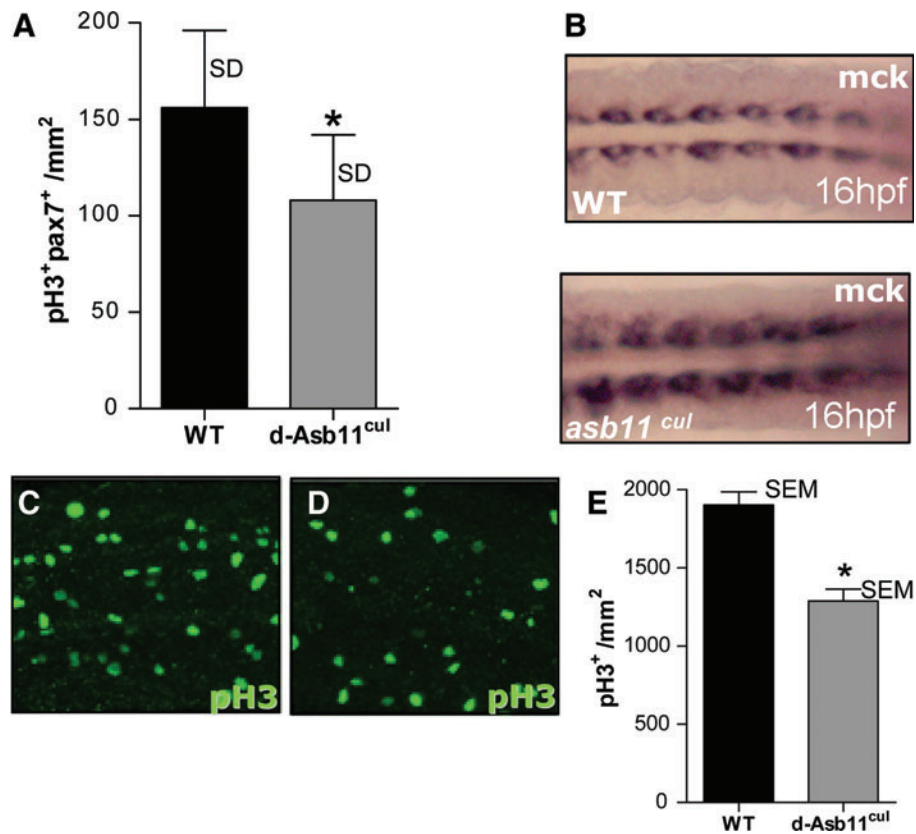


FIG. 5. Fish harboring a hypomorphic *asb11* allele show reduced size of the pax7^+ progenitor compartment and precocious terminal differentiation. Isolation of the hypomorphic *asb11* allele-containing *asb11^{cul}* fish has been described before [20]. (A) At 24 hpf, the size of the expanding muscle progenitor compartment was determined by double labeling with anti-PH3 antibody (a marker for proliferating cells) and an anti-Pax7 antibody. Cells were counted at equivalent positions in the developing dorsal myotome. The *asb11^{cul}* embryos have significantly lower numbers of proliferating muscle progenitor cells ($n=3$). The bars show standard deviation, the asterisk indicates $P < 0.05$ (using a heteroscedastic paired Student's *t*-test). (B) Wild-type and mutant embryos were fixed at 16 hpf and tested for advanced muscle differentiation using *mck* riboprobes. (C–E) Proliferation in the presumptive myotome of wild-type (C) and *d-asb11^{cul}* (D) embryos was visualized by anti-pH3 staining at 48 hpf, representative image ($n=3$). Quantification of total proliferation in zebrafish embryos using the number of PH3-positive cells as a surrogate measure (E). The results represent the average of 8 embryos. The bars depict SEM; $*P < 0.05$ (using a heteroscedastic Student's *t*-test). Color images available online at www.liebertpub.com/scd

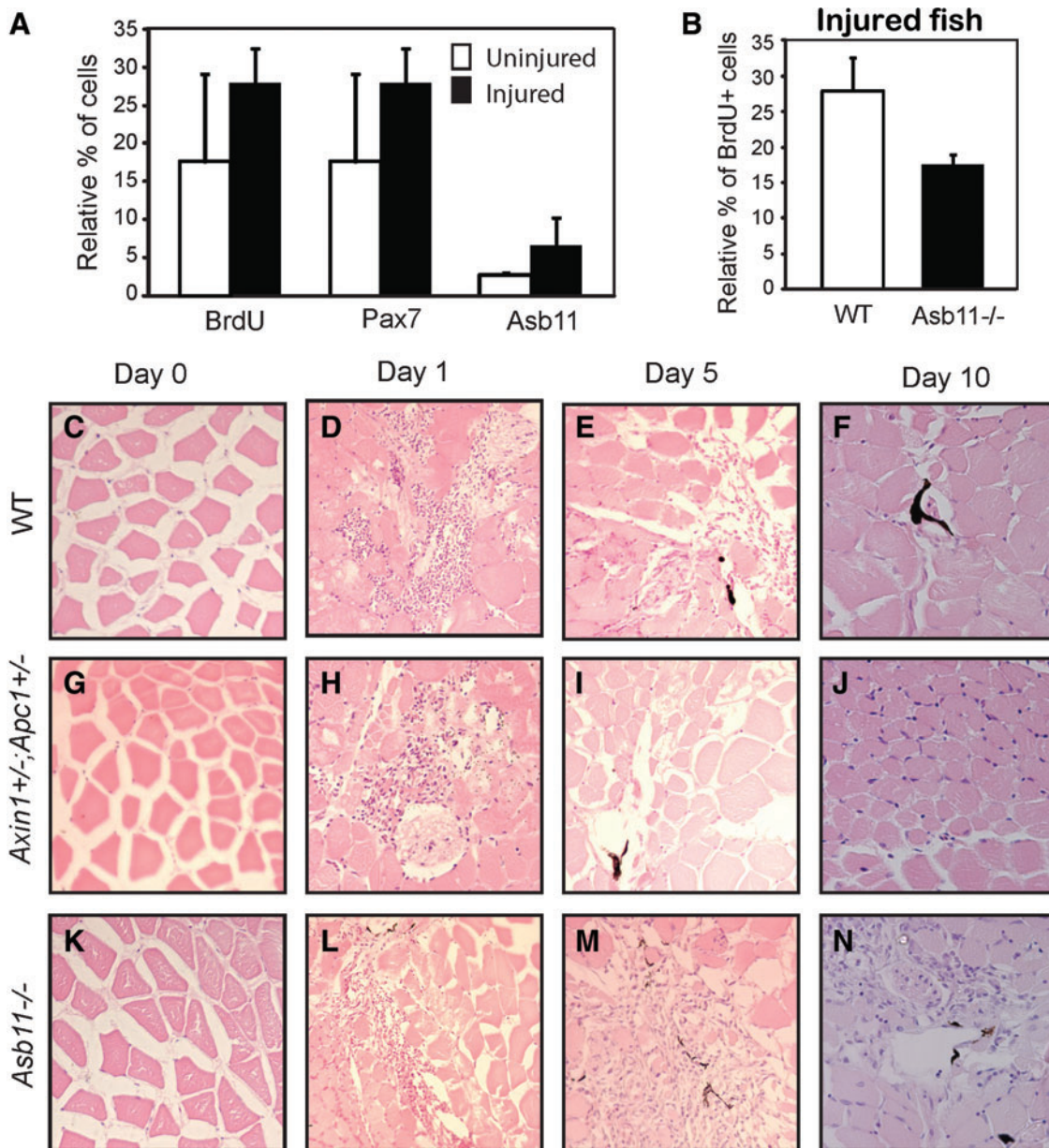


FIG. 6. The hypomorphic *asb11^{cul}* allele is associated with impaired regenerative responses following mechanical injury. Mechanical injury was induced using a needle to inflict trauma on the dorsal flank of the muscle in adult zebrafish. **(A)** Zebrafish were fixed and frozen 7 days postinjury. Adult muscle tissues were cryosectioned and immunostained with BrdU, Pax7, and d-Asb11. Quantification of the percentage of positively stained cells was performed. Error bars indicate the standard deviation calculated from data obtained from 8 different embryos. **(B)** Wild-type, *axin⁻/apc⁻*, and *d-Asb11^{cul}* zebrafish were fixed and frozen 7 days postinjury. Adult muscle tissues were cryosectioned and immunostaining with BrdU antibody 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 postinjury. Evans blue dye was used as a marker to identify the area of injury. The adult musculature was sectioned and counterstained with haematoxylin-eosin (HE). Color images available online at www.liebertpub.com/scd

Discussion

The determinants of the size of the muscle cell compartment remain poorly understood. During embryogenesis a group of Pax7⁺ stem cells arises and proliferates until the final compartment size is reached. In most precursor cells, a genomic program, responsible for terminal differentiation, is started resulting in functional muscle fiber, whereas a small subpopulation remains Pax7⁺ cells forming the satellite cell

population from which regenerative myogenesis can start in response to injury. Our data presented here show that d-Asb11 is essential for maintaining muscle stem cell proliferation during zebrafish embryogenesis and is required for regenerative responses during injury as well. Importantly, we demonstrated that d-Asb11 is expressed beneath the basal lamina of adult zebrafish muscle fiber, and colocalized with a well-accepted muscle satellite cell specific marker Pax7. This, together with the coexpression of d-Asb11 with

label-retaining BrdU slow-cycling cells suggested that the d-Asb11-positive cells are the muscle satellite cells themselves. Interestingly, there are significantly less 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, are activated and induced to proliferate in response to muscle damage/injury.

The *d-Asb11* gene has high homology to both mammalian *Asb9* and *Asb11*; however, no obvious *asb9* homologue is present in fish. As mammalian *Asb9* and *Asb11* lie adjacent on the same chromosome (X), it seems to represent the result of an evolutionary relatively recent genetic duplication event. In silico analysis 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 1 duplication before the divergence of fish from tetrapods and 23.5% having at least 1 duplication afterward [40]. This suggests that zebrafish *asb11* functions similarly to both mammalian *Asb9* and *Asb11*, as it seems that d-Asb11, in zebrafish, bears a more varied number of functions, while in mammals the function of ASB proteins appears to be more specific. This hypothesis is supported by our results where zebrafish *asb11* transcripts were present in all tissues analyzed, whereas mice showed more tissue-specific expression for both *Asb9* (testis and kidney) and *Asb11* (muscle and heart) transcripts, although there is no information of the function of these genes in male germ cell compartment, urogenital system, or muscle development. However, ASB1 [41] and ASB17 [42], which are also highly expressed in mice testis, have been implicated in mammalian spermatogenesis. Moreover, human and murine *Asb9* were reported to interact with the creatine kinase isoforms, brain creatine kinase [14] and ubiquitous mitochondrial creatine kinase [43], targeting them to proteosomal degradation. In agreement with the notion that zebrafish use *Asb* proteins in multiple compartments, whereas in mammals ASB proteins function in specific compartments, zebrafish present a smaller number of *Asb* proteins compared to humans and mice. Clearly, more in-depth analysis of expression and function of different *Asb* proteins in different tissues is required; however, it is tempting to speculate on a function of *d-Asb11* in zebrafish spermatogenesis and kidney development.

Earlier, we have demonstrated that *d-Asb11* maintains the neuronal progenitor compartment, implying a critical role in the ectodermal compartment size. In zebrafish, this function does not seem to be 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. The effects of *d-Asb11* on embryonic myogenesis are remarkably similar to its effects on embryonic neural precursors [12], suggesting that *d-Asb11* functions in a similar way in regulating both the neuroectodermal and mesodermal cell fates. Whether *d-Asb11* is important for compartment size in the endodermal lineage, however, is questionable. Interestingly, ASB9 expression has recently been linked to the maintenance of cell proliferation in colorectal cancer [44]. In this context it is interesting to note that we observe higher expression of the CysLT1 receptor in *Asb9*-expressing colon cancer cell lines (unpublished observations) and that expression of this receptor has been linked to compartment expansion in colon cancer [45]. Thus, further investigations on how endodermal

progenitor expansion is regulated and whether they involve ASB-like proteins should be warranted.

Recently we showed that the functions of *Asb11* in neurogenesis are mediated by its potential to enable Notch signaling activity [19]. Canonical Delta-Notch signaling plays a key role in satellite cell activation and muscle regeneration. There is a temporal switch between Notch and Wnt signaling, whereby Notch signaling has to be downregulated for myogenesis to proceed [46]. Consistently, our data showed that *asb11* expression in muscle satellite cells is required to maintain the muscle precursor pool and efficient muscle regeneration. It is important to note, that although slower, muscle regeneration is still evident in *d-asb11^{cul}* zebrafish.

In conclusion, based on the evolutionary conservation of *asb11* with murine *Asb9* and *Asb11*, it is tempting to hypothesize that the phenotypes we observed in the *asb11* mutants could be linked to human muscular diseases, prompting an investigation into the role of *Asb11* in muscle pathology.

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Author Disclosure Statement

All authors do not have any conflict of interests.

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Address correspondence to:

Prof. Maikel P. Peppelenbosch
Department of Gastroenterology and Hepatology
Erasmus MC-University Medical Center Rotterdam
's-Gravendijkwal 230
NL-3000 CA Rotterdam
The Netherlands

E-mail: m.peppelenbosch@erasmusmc.nl

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