Hemicentin 2 and Fibulin 1 are required for epidermal–dermal junction formation and fin mesenchymal cell migration during zebrafish development

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Abstract

Hemicentin 1 (Hmcn1) and Hemicentin 2 (Hmcn2) belong to the fibulin family of extracellular matrix (ECM) proteins that play pivotal roles during development and homeostasis of a variety of vertebrate tissues. Recently, we have shown that mutations in zebrafish Hmcn1, also called Fibulin 6, lead to massive fin blistering, similar to the defects caused by the Fraser syndrome gene Fras1. In contrast, the role of Hmcn2 during vertebrate development has thus far been uncharacterized. In zebrafish, hmcn2, like fibulin 1 (fbln1), another member of the fibulin family, is predominantly expressed in fin mesenchymal cells and developing somites, contrasting the strict epithelial expression of hmcn1. While antisense morpholino oligonucleotide (MO)-based knockdown of hmcn2 did not yield any discernable defects, hmcn2/fbln1 double knockdown fish displayed blistering in the trunk, pointing to an essential contribution of these proteins from mesodermal sources for proper epidermal–dermal junction formation. In contrast, and unlike hmcn1 mutants, epidermal–dermal junctions in the fin folds of hmcn2/fbln1 double knockdown fish were only moderately affected. Instead, they displayed impaired migration of fin mesenchymal cells into the fin folds, pointing to a crucial role of Hmcn2 and Fbln1 to remodel the ECM of the fin fold interepidermal space, which is a prerequisite for fibroblast ingrowth. TEM analyses suggest that this ECM remodeling occurs at the level of actinotrichia, the collagenous migration substrate of mesenchymal cells, and at the level of cross fibers, which resemble mammalian microfibrils. This work provides first insights into the role of Hmcn2 during vertebrate development, identifying it as an evolutionary conserved protein that acts in functional redundancy with Fbln1C and/or Fbln1D isoforms to regulate tissue adhesion and cell migration, while extending the current knowledge of the functions of vertebrate Fbln1.

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Introduction

Fibulins are secreted glycoproteins that can associate with various components of the extracellular matrix (ECM), including the basement membrane (BM) and elastic microfibrils. Being involved in the elaboration and stabilization of the ECM, they have been implicated in tissue organogenesis, vasculogenesis, fibrogenesis and tumorigenesis (de Vega et al., 2009). The different members of the fibulin family share a common multimodular organization divided into three domains, with an N-terminal domain I that varies among the family members, a central domain II consisting of a variable number of epidermal growth factor (EGF)-like modules, most of which contain a consensus sequence for calcium binding (cbEFG-like modules), and the C-terminal domain III, also called Fibulin C-terminal globular (FC) domain, which is specific to the seven fibulin family members (Fibulin 1–7) (de Vega et al., 2009).

The first fibulin discovered was Fibulin 1 (Fbln1) (Arguelles et al., 1989, 1990). Many ECM or BM proteins have been identified to bind Fibulin 1, including fibronectin, laminins, nidogens and the endostatin domain of Collagen XVIII (Balbona et al., 1992; Sasaki et al., 1995, 1998; Tran et al., 1997); but also elastic fiber components and growth factors (Perbal et al., 1999). In human, alternative splicing of FIBULIN 1 gives rise to four different isoforms, A, B, C and D, which differ in domain III, while in other vertebrate (mouse, chicken, zebrafish) as well as invertebrate species (the nematode C. elegans), only Fbln1C and -1D isoforms...
have been described (Argraves et al., 1990; Barth et al., 1998; Pan et al., 1993; Zhang et al., 1997). Fbln1 mutant mice lacking both Fbln1 isoforms display multiple developmental defects within blood vessels, renal glomeruli, lung alveoli and neural-crest derivatives, leading to perinatal lethality (Cooley et al., 2008; Kostka et al., 2001). To investigate the roles of the different isoforms, possibly justifying their conservation throughout metazoan evolution, splice variant-specific loss-of-function studies have been performed in C. elegans, re-introducing either fbln1c or fbln1d into fbln1-deficient mutants. These studies revealed both isoform-specific and shared functions, with a predominant requirement of Fbln1C for cell shape and adhesion regulation during tissue morphogenesis, and a specific requirement of Fbln1D to connect different tissues via flexible polymers (Muriel et al., 2005). Interestingly, however, the assembly of both Fbln1 isoforms in multiple locations is dependent upon the presence of Hemicentin (Hmcn), another member of the fibulin family also called Fibulin 6 (Muriel et al., 2005).

Like Fbln1, Hemicentins are ancient ECM proteins, with highly conserved orthologues in nearly all metazoans. While invertebrates like C. elegans have a single hmcn gene, usually two paralogues exist in vertebrates, called Hmcn1 and Hmcn2 (Vogel et al., 2006). With a mass of >600 kDa, Hmcns are by far the largest members of the fibulin family, with multiple, evolutionary conserved modules. The most highly conserved is an amino-terminal von Willebrand A (VWA) domain, followed by a long (>40) stretch of tandem immunoglobulin (Ig) domains. The vertebrate Hemicentins have an additional G2F motif between the Ig and EGF domains. Apart from Hmcn1 and Hmcn2, this G2F domain is only found in Nidogens, where it for instance mediates binding to the BM proteoglycan Perlecan (Hopf et al., 2001). Furthermore, mammalian Hmcn1, but not Hmcn2, has a series of six thrombospondin repeats inserted between the Ig and Nidogen G2F domains (Vogel et al., 2006). In C. elegans, the N-terminal VWA domain is involved in pericellular Hmcn localization, while the C-terminal Fibulin (FC) domain mediates Hmcn–Hmcn interactions as it assembles into higher order polymers (Dong et al., 2006; Vogel et al., 2006).

In C. elegans, Hmcn has pleiotropic functions in transient cell contacts that are required for cell migration and BM invasion, as well as for stable cell–ECM contacts at hemidesmosome-mediated cell junctions and elastic fiber-like structures (Vogel and Hedgecock, 2001; Vogel et al., 2006). In vertebrates, loss-of-function experiments have thus far only been performed for Hmcn1, but not Hmcn2. In mouse, homologous recombination of Hmcn1 has been reported to cause early defects in cytokinesis and death of homozygous embryos at preimplantation stages (Xu and Vogel, 2011), corresponding to a similar role of Hmcn in the C. elegans germline (Xu and Vogel, 2011).

Recently, we have described zebrafish hmcn1 mutants, which in contrast to the mouse mutants are viable, but display specific blistering in the developing fins (Carney et al., 2010). These defects are similar to those found in zebrafish bearing mutations in Fras1, Frem1 or Frem2 (Carney et al., 2010), BM-associated proteins which upon mutation in human cause Fraser syndrome, a recessive multisystem disorder characterized by embryonic epidermal blistering, cryptophthalmos, syndactyly, renal defects and a range of other developmental abnormalities (Smyth and Scambler, 2005). The elevating fin fold of teleosts constitutes a relatively simple yet distinctive structure, consisting of two opposing epidermal sheets, each with an underlying BM, which are attached to each other via so-called cross fibers spanning the interepidermal/dermal space between the two sheets (Dane and Tucker, 1985). In addition, the dermal space contains well-ordered arrays of collagenous actinotrichia, which run along the proximodistal axis of the elevating fin fold (Dane and Tucker, 1985). Constituents of these fibers are most likely produced by epidermal cells (Duran et al., 2011), while the dermal space is initially cell-free (Dane and Tucker, 1985). Only later, it is invaded by fibroblast-like fin mesenchymal cells that use the actinotrichia as a migration substrate (Wood and Thorogood, 1984). Like fras1 and most other ECM component-encoding genes, hmcn1 is expressed in apical-most epidermal cells of the elevating fin fold, while fras1 and hmcn1 mutants display compromised epidermo–dermal junction formation, with blisters directly underneath the BM (Carney et al., 2010). In contrast, hmcn2 shows prominent expression in the invading fin mesenchymal cells (Carney et al., 2010), whose biology and role during fin morphogenesis is poorly understood.

Here, we report that all of zebrafish fibulin family members, fbln1 and hmcn2 are co-expressed in fin mesenchymal cells and in somitic muscle progenitors. Morpholino-based knockdown of fbln1 causes defects during the formation of intersomitic blood vessels, while hmcn2 knockdown neither leads to any discernible defects, nor enhances the fin blistering of hmcn1 mutants. However, concomitant loss of both fbln1 and hmcn2 leads to compromised epidermal–dermal attachment at somite levels, and to compromised migration of fin mesenchymal cells through the dermal space of the fin folds. The obtained results with specific splice-blocking morpholinos and via rescue experiments further indicate that Fbln1C and Fbln1D can compensate for each other during epidermal–dermal junction formation, but not during mesenchymal cell migration. This work represents the first functional analysis of Hmcn2 function in any vertebrate species, and unravels thus far unappreciated redundant roles between Fbln1 isoforms and Hemicentins, which are required both for ECM junction formation and for ECM remodeling to allow proper mesenchymal cell migration.

Materials and methods

Fish lines and handling

Fish lines used in this project were TLEK (wild type), the transgenic enhancer trap line ET37 (Choo et al., 2006), and the transgenic line Tgf(fli1a:EGFP), labeling blood vessels (Lawson and Weinstein, 2002). Embryos and larvae were kept at standard conditions (28 °C) and staged in hours post fertilization (hpf) as described (Kimmel et al., 1995).

Morpholinos, efficacy controls, and mRNA rescues

Morpholino oligonucleotides (MOs) were purchased at Gene Tools, LLC. About 1 mM stock solutions in distilled water were prepared and aliquots were stored at −20 °C. Before use, morpholinos were preheated at 70 °C for 10 min, diluted in Danieau’s buffer and phenol red (0.05%) (Sigma) to indicated concentrations (Table 1), and microinjected into 1–4 cell stage embryos (1.5 nl per embryo) as described (Nasevicius and Ekker, 2000). As a negative control, standard morpholino (std MO) was obtained from Gene Tools, LLC. The morpholinos used were fbln1-atg-1: ACAACACGATCATGTAGAGATCCAT fbln1-atg-2: ATTATCCAGACGCTGAGTTTTAT fbln1c-splice: CGGCTGCAGACGCACACAAGAAGAC fbln1d-splice: GTCTGCTAGAGGAATGACACACAAT hmcn2-atg-1: TACACGAAACCTTTTTTACTTCTAC hmcn2-atg-2: CTACCTTTAATTCACTTACTC

Data obtained with hmcn2-atg-1 MO are shown. However, for all studies, both hmcn2 MOs were used and gave identical results in at least three independent experiments. The same applies to the two fbln1-atg-1 and fbln1-atg-2 MOs.
Table 1
Phenotypes obtained upon single or double injections of different hmcn2 and fbln1 morpholinos.

<table>
<thead>
<tr>
<th>Stage</th>
<th>MO</th>
<th>Phenotype</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blisters trunk (%)</td>
</tr>
<tr>
<td>30hpf</td>
<td>hmcn2 0.5 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>fbln1 0.1 mM</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>fbln1c 0.125 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>fbln1d 0.125 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hmcn2 0.5 mM + fbln1 0.25 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hmcn2 0.5 mM + fbln1c 0.125 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hmcn2 0.5 mM + fbln1d 0.125 mM</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hmcn2 0.5 mM + fbln1d 0.125 mM + fbln1c 0.125 mM</td>
<td>61.3</td>
</tr>
</tbody>
</table>

Defects were analyzed via light microscopy at the indicated stages. The reduction in trunk blistering frequency from 30 hpf to 55 hpf is caused by progressive healing of this phenotypic trait (see Fig. 2 and Supplementary Fig. S5C and E for 72 hpf), whereas fin defects persist (see Supplementary Fig. S5D and F for 72 hpf). For testing of Fbln1 splice variants, note that fin defects were obtained both upon co-injection of fbln1c and hmcn2 MOs, and upon co-injection of fbln1d and hmcn2 MOs, whereas trunk blistering required triple injection of fbln1c, fbln1d and hmcn2 MOs. n, the number of scored embryos (three independent experiments).

For efficacy control of the fbln1-arg MOs, an Fbln1-GFP construct was generated as follows: cDNA of zebrafish embryos was synthesized with Superscript III first strand (Invitrogen) according to manufacturer’s protocol. The 5' region of the fbln1 cDNA was amplified by PCR with primers containing EcoRI or XbaI restriction sites. After restriction digest, the PCR fragment was cloned into XLT.GFP<sub>LT</sub> CS2+. The resulting fbln1-XLT.GFP<sub>LT</sub> CS2 plasmid contained the Sp6 promoter, the fbln1 5'UTR and 195bp of fbln1 coding region fused in frame to the Green Fluorescent Protein (GFP) coding region. Capped sense mRNAs from XLT.GFP<sub>LT</sub> CS2 (GFP control) and fbln1-XLT.GFP<sub>LT</sub> CS2 were synthesized using the MEGA script kit (Ambion).

For morphant rescue experiments, mouse Fbn1C plasmid IRAP-p968A0213D (Imagenes GmbH; GenBank number BC007140; 22.2 kb) was linearized with NotI and capped mRNA was synthesized with Sp6 RNA polymerase. For synthesis of capped mouse Fbn1D mRNA, plasmid 1420027B09 (Imagenes GmbH; GenBank AK159646; 2.69 kb) was linearized with KpnI and transcribed with T7 RNA polymerase, using the Message Machine kit (Ambion). mRNAs were co-injected with morpholinos after dilution in distilled water/phenol red at final concentrations of 25 ng/µl (Fbn1C) or 5 ng/µl (Fbn1D) (1.5 nl per embryo).

For efficacy control of fbln1c-slice and fbln1d-slice MOs, semiquantitative RT-PCR of RNA isolated from embryos (24 hpf) injected with either of the two MOs was carried out, using the following primers and PCR conditions: fbln1c forward: GACGTCTGACGCCGACTCA; fbln1c reverse: CATCCCGAAGAAGTACCTG; fbln1d forward: TCTGAAGTGGTGGAGGAGA. Loading control: ef1x forward: TCA-CCTCGGAGTGAACAGC; ef1x reverse: ACTTGCAAGCCGATGGACAG. Annealing temperature: 57 °C; 30 cycles.

Cell transplantations

Wild-type embryos were injected either with rhodamin dextran (RD; 10 kDa; 50 mg/ml) or with RD + hmcn2 MO + fbln1 MO. At the shield stage (6 hpf), cells were collected from the ventral side of injected donors and transplanted into the same side of either uninjected ET37 hosts of the same stage, or into ET37 hosts injected hmcn2 and fbln1MOs. Chimeric embryos were evaluated at 72 hpf via confocal microscopy.

In situ hybridization

Embryos were fixed in 4% PFA overnight at 4 °C, and in situ hybridizations were performed as previously described (Hammerschmidt et al., 1996) with RNA probes generated from linearized plasmids using the Roche digoxigenin RNA synthesis kit. For fbln1-5, plasmid pSpot-zffbln1 (PMMPGp609N1134) with a 1.7 kb fbln1 cDNA fragment was linearized with Sall and transcribed with Sp6 RNA polymerase, plasmid pBluescriptSK-zffbln2 (recloned from IMAGp998B086485Q) with a 1.8 kb fbln2 cDNA fragment was linearized with EcoRI and transcribed with T3 RNA polymerase, plasmid pExpress1-zffbln3 (IMAGp998D1614790Q) with a 3.5 kb fbln3 cDNA fragment was linearized with EcoRI and transcribed with T7 RNA polymerase, plasmid pBluescriptSK-zffbln4 (recloned from IRBOp991B0854D) with a 1.8 kb fbln4 cDNA fragment was linearized with EcoRI and transcribed with T3 RNA polymerase, plasmid pExpress1-zffbln5 (IMAGp998A0619304Q) with a 1.5 kb fbln5 cDNA fragment was linearized with EcoRI and transcribed with T7 RNA polymerase. For hmcn2, the number of scored embryos (three independent experiments).

A cDNA construct encoding amino acid residues 34–425 of zebrafish Hemicentin 2 was generated by RT-PCR on total RNA from 24 hpf embryos and cloned with 5'-terminal Xhol restriction sites. The amplified PCR product was inserted into a modified pCEP-Pu vector containing an N-terminal BM-40 signal peptide and a C-terminal strepII-tag downstream of the restriction sites (Maertens et al., 2007). The recombinant plasmid was introduced into HEK293-EBNA cells (Invitrogen) using FuGENE 6 transfection reagents (Roche). Cells were selected with puromycin (1 μg/ml) and the recombinant protein was purified directly from serum-containing cell culture medium. After filtration and centrifugation (30 min, 10,000 × g), cell culture supernatants were applied to a Streptactin column (1.5 ml, IBA GmbH) and eluted with 2.5 mM desthiobiotin, 10 mM Tris–HCl, pH 8.0. The purified recombinant Hmcn2 fragment was used to immunize a rabbit (Pineda Antikörper Service, Berlin, Germany). The obtained antisera were purified by affinity chromatography on a column with purified Hmcn2 fragment coupled to CNBr-activated Sepharose (GE Healthcare). Specific antibodies were eluted with 3 M KSCN, and the eluate was dialysed against PBS, pH 7.4.

Immunohistochemistry

Whole mount immunostainings were done according to Schulte-Merker et al. (1992) with minor changes (washes in

PBS + 0.05% Tween20). The antibodies used and their dilutions were: rabbit anti-zebrafish Hmcn2 (1:50), rabbit anti-laminin Hmcn2 (1:200, Sigma L9393), mouse anti-p63 (1:200, Santa Cruz A4A), goat anti-rabbit Cy3 (1:200, Invitrogen A10520), AlexaFluor488 goat anti-rabbit (1:200, Invitrogen A11008). In the case of Phalloidin-cy3 (1:200) staining, samples were washed in PBST after fixation and immersed in water, followed by the regular immunostaining protocol. For counterstaining of nuclear DNA, immunostained embryos were incubated in DAPI solution for 10 min and post-fixed with 4% PFA in PBS. Afterwards, embryos were embedded in Durcupan and sectioned at 7 μm as described in (Feitosa et al., 2011) or mounted for microscopy analysis.

Microscopy

For imaging, live embryos were anesthetized with Tricaine and mounted in 1% methyl cellulose, and embryos stained by in situ hybridization were mounted in 2:1 solution of benzylbenzoate-benzylalcohol (Nüsslein-Volhard and Dahm, 2002). Fluorescent images and time-lapse movies were taken using a Zeiss Confocal microscope (LSM710 META). For the time-lapse recordings, anesthetized embryos were immobilized in 1.2% low melting agarose and pictures were taken every 5 min for 5 h. Bright-field or Nomarski microscopy was performed on a Zeiss Axiomager. Transmission electron microscopy (TEM) was carried out as previously described (Feitosa et al., 2011).

Results

hmcn2 and fbln1 are co-expressed in somites and fin mesenchymal cells

According to the zebrafish genome project (http://www.ensembl.org/Danio_rerio/Info/Index), the zebrafish has two hemicentin (hmcn) genes. One of them, hmcn1, located on chromosome 10 and mutated in nagel mutants (Carney et al., 2010), encodes a protein of 5615 amino acid residues and a domain composition identical to mammalian Hmcn1 proteins, with an N-terminal VWA domain, 44 Ig domains, 6 TSR domains, one G2F domain, 8 EGF-like domains and a C-terminal FC domain (Supplementary Fig. S1A). The predicted protein encoded by the second hmcn gene (GenBank accession number XM_001920466.2), located on chromosome 8, is slightly smaller (4211 aa), with a VWA domain followed by only 32 Ig domains, one TSR domain, one G2F domain, 8 EGF-like domains and one FC domain (GenBank accession number XP001920501.2). These numbers of Ig and TSR domains are different from those of both mammalian Hmcn1 (44x Ig, 6x TSR, 8x EGF-like domains) and mammalian Hmcn2 (42/43x Ig, no TSR, 6x EGF-like domains) (Supplementary Fig. S1A). However, phylogenetic tree analysis with Hmcn1 and Hmcn2 proteins from different species (Supplementary Fig. S1B) and conservation of synteny at the genomic level (Supplementary Fig. S2) clearly indicate that this second zebrafish hmcn gene is a hmcn2 orthologue, rather than a second hmcn1 gene. This phylogenetic analysis further indicates that the vertebrate Hmcn1 genes are the orthologues of the single invertebrate hmcn gene, while the vertebrate Hmcn2 genes are more diverged.

As reported previously (Carney et al., 2010), hmcn1 shows prominent expression in apical-most epidermal cells of the emanating median fin folds (Fig. 1B and C). hmcn2 is expressed in the same cells, however, at much lower levels and more transiently, with expression vanishing between 24 and 48 h post fertilization (hpf) (Carney et al., 2010), the time when strong hmcn2 expression becomes visible in mesenchymal cells migrating into the dermal space of the median fin folds (Fig. 1E and F).

In addition, in contrast to hmcn1 (Fig. 1A), hmcn2 shows strong expression in developing somites of trunk and tail (Fig. 1D).

To study Hmcn2 protein distribution, we generated polyclonal antibodies against zebrafish Hmcn2 (see Materials and Methods). Whole mount immunohistochemistry (IHC) and subsequent sectioning revealed Hmcn2 at myosepta between individual somites (Fig. 1J and L), and, at lower levels, surrounding the somites in the limits of the overlying skin and the neural tube (Fig. 1I). Furthermore, at later stages, Hmcn2 was present in subepidermal regions of the developing median fins (Fig. 1M), consistent with the expression pattern determined via in situ hybridization. This indicates that Hmcn2 protein is secreted from cells and associated with BMs or other components of the ECM.

For loss-of-function experiments, we designed two sequence-independent antisense morpholin oligonucleotides (MOs) directed against the translational start site (hmcn2-atg-1 MO, hmcn2-atg-2 MO). Embryos injected with either of the two hmcn2 MOs lacked specific anti-Hmcn2 immunolabeling (Fig. 1K; and data not shown), indicating that both work efficiently. However, neither of the two morpholinos yielded any discernable morphological defects (data not shown), suggesting that Hmcn2 function per se is dispensable for zebrafish development.

In C. elegans, Hmcn protein has been shown to move rather long distances within the ECM (Vogel and Hedgecock, 2001; Vogel et al., 2006). Therefore, we speculated that despite their differential expression pattern, hmcn2 might share functions with hmcn1. However, co-injection of hmcn1 and hmcn2 MOs, or injection of hmcn2 MOs into nagel/hmcn1 mutants (Carney et al., 2010) did not increase the phenotypic strength or penetrance of the fin blistering defects of hmcn1 morphants/nagel mutants (data not shown), suggesting that Hmcn2 does not act in partial redundancy with its direct parologue.

As a first step to study whether Hmcn2 might act redundantly with other members of the Fibulin family, we carried out comparative in situ hybridization analyses between hmcn2, fbln1, fbln2, fbln3, fbln4 and fbln5. Notably, of all tested five fibulin genes, only fbln1 showed co-expression with hmcn2 both in the somites and in the fin mesenchymal cells (Fig. 1G–I), whereas fbln1 expression was absent in apical epidermal cells of the fin folds, the site of transient hmcn1 and hmcn2 co-expression (data not shown). In contrast, none of the other fbln genes was expressed in fin mesenchymal cells, while only fbln4 was also expressed in somites (Supplementary Fig. S3; and data not shown).

Concomitant inactivation of hmcn2 and fbln1 leads to compromised epidermal–dermal junction formation in the trunk

Zebrafish fbln1 has been previously shown to be expressed in somites, fins and other embryonic structures, including the heart valves (Zhang et al., 1997), however, functional analyses have been missing thus far. We designed two sequence-independent fbln1 translation-blocking MOs, both of which fully suppressed translation of co-injected mRNA containing the 5’ region of fbln1, including the translation initiation sequence complementary to the MOs, fused to the GFP coding sequence (Supplementary Fig. S4A and B; and data not shown). fbln1 morphant embryos, while morphologically grossly normal, displayed alterations in the patterning and integrity of intersomitic vessels (Supplementary Fig. S4C and D), pointing to a role of Fbln1 generated by somitic cells in governing angiogenesis, and consistent with the described role of Fbln1 in blood vessel formation in the mouse (Cooley et al., 2008; Kostka et al., 2001). Distinct and more striking defects were obtained upon co-injection of hmcn2 (0.5 mM) and fbln1 (0.1 mM) MOs in all possible combinations (hmcn2-atg-1 + fbln1-atg-1; hmcn2-atg-2 + fbln1-atg-2; hmcn2-atg-1 + fbln1-atg-2; hmcn2-atg-2 + fbln1-atg-2), whereas none of these traits was obtained when
either of the MOs was co-injected with the standard control MO. At 24 hpf, hmcn2/fbln1 double morphants displayed massive blistering of the skin in the trunk and tail region covering the somites (Fig. 2A and B). Trunk blisters were also apparent at 48 hpf (Fig. 2C and D), but started to disappear during further development (Supplementary Fig. S5C and E). We do not know the reason for the later healing of the blisters, however, similar phenomena are observed for the transient embryonic skin blistering caused by mutations in other ECM proteins, such as Fras1, both in zebrafish (Carney et al., 2010) and in mouse (McGregor et al., 2003; Vrontou et al., 2003).

In light of data obtained in C. elegans pointing to both shared and isoform-specific roles of the alternative splicing variants Fbln1C and Fbln1D (see Introduction; Muriel et al., 2005), we carried out co-injections of hmcn2 MOs with MOs blocking Fbln1C or Fbln1D splicing. These morpholinos were designed according to the genomic sequences from the trace archives of the Danio rerio genome. The zebrafish Fbln1C and 1D proteins share the first 351 amino acid residues, whereas the C-terminal 98 (Fbln1C) or 140 (Fbln1D) aa residues, respectively, are distinct (Zhang et al., 1997). Specific and efficient abrogation of fbln1C or fbln1D splicing in embryos injected with fbln1C-splice MO or fbln1D-splice MO, respectively, was revealed by semi-quantitative RT-PCR analysis (Supplementary Fig. S6). Strikingly, embryos co-injected with hmcn2 MO and fbln1C MO, or co-injected with hmcn2 MO and fbln1D MO lacked trunk blisters, whereas embryos co-injected with all three MOs displayed trunk blisters at a strength and penetration as embryos co-injected with hmcn2 MO and a translation-blocking fbln1 MO (Fig. 2E and F; Table 1). Consistently, the blistering defects of fbln1-atg/hmcn2 double morphant embryos (47/75; 63%) could be significantly alleviated upon co-injection with synthetic mRNA encoding either mouse Fbln1C (20/96; 21%) or mouse Fbln1D (12/76; 16%). Together, this indicates that during epidermal–dermal junction formation, Fbln1C and Fbln1D act in functional redundancy to Hmcn2 and to each other.

To analyze the trunk blisters of hmcn2/fbln1 double morphants in more detail, we carried out IHC and transmission electron microscopy (TEM) studies. Anti-Laminin IHC at 28 hpf (Fig. 3A and B) revealed that blisters occurred within the space between the forming BM underlying the epidermis and the BM surrounding the somites. Consistent results were obtained via TEM. During the first 3 days of development of wild-type fish, the dermal space is largely acellular, with rather scarce fibroblast-like cells called dermal endothelial cells and some melanocytes (Le Guellec et al., 2004). During these stages, dermal ECM components such as type 1 Collagens are mainly generated by epidermal cells, while dermal fibroblasts only immigrate later (Le Guellec et al., 2004). At 30 hpf, first collagen fibrils are visible underneath the epidermal...

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**Fig. 1.** hmcn2 and fbln1 are co-expressed in somites and fin mesenchymal cells. (A–I) In situ hybridizations with probes indicated in lower right corners; (A, D, G) transverse sections through trunk at 26 hpf (14 somite stage); (B, E, H) lateral view on tail at 24 hpf (B) or 48 hpf (E, H); (C, F, I) transverse sections through tail of embryos as shown in (B, E, H), respectively; view on dorsal median fin fold. (A, D, G) hmcn2 (D) and fbln1 (G) are co-expressed in somites, whereas hmcn1 (A) is not. (B, C, E, F, H, I) hmcn1 (B, C) is expressed in epidermal cells at the apical ridge of the fin fold, while hmcn2 (E, F) and fbln1 (H, I) are expressed in fin mesenchymal cells within the dermal (interepidermal) space of the fin fold. (J–M) Anti-Hmcn2 immunostainings. (J, K) Lateral view on trunk at 24 hpf, revealing Hmcn2 protein at myosepta of uninjected control (J), whereas myoseptal staining is absent in hmcn2 morphant (K). (L) Transverse section through 24 hpf wild-type embryo at level indicated in (J); counterstained with anti-p63 antibody to stain nuclei of basal keratinocytes, and DAPI to visualize all nuclei. Arrowhead points to Hmcn2 protein in dermal space between epidermis and somites, arrow to Hmcn2 protein between neural tube and somites. (M) Transverse section through dorsal fin at 55 hpf embryo, showing Hmcn2 protein in red, mesenchymal cells in green (anti-GFP of ET37 transgene product), and DAPI-positive nuclei in blue. Hmcn2 protein is present in continuous lines below the epidermal sheets (blue nuclei). Strikingly, Hmcn2 protein is not restricted to regions that contain fin mesenchymal cells and their protrusions (in green), but also found in more apical regions (indicated by arrow), in line with its non-pericellular function during the guidance of mesenchymal cell migration described below (Fig. 4). However, inner regions of the dermal space of the fin lack Hmcn2 signals (indicated by stars). Abbreviations: wt, wild type; hmcn2 MO, hmcn2 morphant; hpf, hours post fertilization; ms, horizontal myoseptum; nt, neural tube; s, somite.
BM (Fig. 3D), which later obtain a multi-layered plywood-like organization (Fig. 3F).  

**hmcn2/fbln1** double morphants displayed a rupture of dermal integrity within the first layers of collagen fibers below the BM (Fig. 3E and G). The resulting blisters initially appeared rather clear, suggesting that they are filled with fluid (Fig. 3E), whereas later on almost all of them were filled with more electron-dense, amorphic material (Fig. 3C and I), intermingled with patches of disorganized collagen fibers (Fig. 3I). In wild-type fish, such amorphic material was rarely observed and at sites with wide subepidermal spaces, such as at the base of the fins. At these locations, collagen fibers remained organized and positioned distal of the amorphic material (Fig. 3H), in contrast to the double morphant (Fig. 3I).

Together this suggests that Hmecn2 and Fbn1, generated by somites are secreted into the dermal space between somites and surrounding epidermis and contribute to proper attachment of epidermal-derived collagen fibers below the epidermal BM, and thereby proper epidermal–dermal junction formation.

In contrast to the trunk, formation of epidermal–dermal junction in the fin folds of **hmcn2/fbln1** double morphants was rather normal. As the dermis of the trunk, the dermal (inter-epidermal) space of the fin fold is initially cell-free, with fibroblast-like mesenchymal cells only immigrating toward the end of the second day of development (see below). Fibers consisting of collagen I and Collagen II (Duran et al., 2011) form large parallel bundles called actinotrichia, which run along the epidermal BM (Fig. 3J). **hmcn2/fbln1** displayed rather moderate, late and locally restricted dissociation of actinotrichia from the epidermal BM (Fig. 3K).

This is in striking contrast to the situation in **hmcn1** mutants, in which the epidermal–dermal junction is severely compromised from much earlier stages onwards and along the entire length of the fin fold (Carney et al., 2010). This points to a predominant role of Hmecn1 over Hmecn2 during epidermal–dermal junction formation in the fins, consistent with the stronger and more persistent expression of **hmcn1** in the apical epidermal cells (see above).

**hmcn2/fbln1 double morphants display compromised fin mesenchymal cell migration**

Actinotrichia of the fin folds have structural functions, as keeping the fin fold straight (Duran et al., 2011; Zhang et al., 2010). In addition, they guide the migration of invading mesenchymal cells within the fold (Wood and Thorogood, 1984). The exact function of these mesenchymal cells is still a matter of debate, but they seem to stabilize the larval fins as well as contributing to
actinotrichia and to the later formation of lepidotrichia, the bony fin rays (Duran et al., 2011; Zhang et al., 2010). During their immigration, zebrafish fin mesenchymal cells display strong expression of hmc2 and fbln1 (see above), while hmc2/fbln1 double morphants had striking defects in fin mesenchymal cell morphology and migration behavior (Figs. 4 and 5). In contrast to trunk blistering, these defects were not only obtained upon co-injection of hmc2 MO and translation-blocking fbln1 MO
targeting both Fbln1C and Fbln1D, but also upon co-injection of hmcn2 MO with either the fbln1C or the fbln1D-specific MO (Table 1), indicating that here, both Fbln1 isoforms are essential Hmcn2 partners. Although defects were visible via light microscopy (Supplementary Fig. S5D and F), for direct in vivo imaging of fin mesenchymal cells (Fig. 4), we took advantage of the transgenic enhancer trap line ET37, in which fin mesenchymal cells express GFP (Choo et al., 2006). At 55 hpf, fin mesenchymal
cells of wild-type embryos radiated from basal to apical and were highly arborized, with many projections extending apically (Fig. 4A and B). In contrast, fin mesenchymal cells of hmcn2/fbln1 double morphants agglomerated in basal regions of the fin and had a more roundish shape, with fewer and less extended projections (Fig. 4C,D).
The shape of the mesenchymal cells in hmcn2/fbln1 double morphants resembled that of wild-type cells at an earlier developmental stage and in basal-most positions of the fin, when they initiate their migratory process (Wood and Thorogood, 1984). Two sets of results indicate that altered cell shape and failed mesenchymal migration in hmcn2/fbln1 double morphants are not due to the intrinsic deficits of mesenchymal cells themselves (e.g. the incapability to form cellular protrusions via rearrangement of the actin cytoskeleton), but due to the problems in the extra-cellular environment. First, time-lapse studies from 35 to 40 hpf in the ET37 background revealed that fin mesenchymal cells of hmcn2/fbln1 double morphants were able to form cellular protrusions of reasonable lengths (Fig. 3F). However, compared to wild-type embryos (Fig. 3E), these protrusions were less persistent, and often retracted without a concomitant apical displacement of the cell body. Second, the analysis of chimeric embryos generated by transplantation of wild-type cells into hmcn2/fbln1 double morphant hosts during gastrula stages revealed that wild-type cells in a hmcn2/fbln1 double morphant environment failed to migrate and behave like hmcn2/fbln1 double morphant neighbors (Fig. 4G). Conversely, hmcn2/fbln1 double morphant mesenchymal cells in a wild-type environment showed normal cell morphology and migration behavior (Fig. 4H). This indicates that Hmcn2 and Fbln1 act in a non-cell autonomous manner, suggesting that mesenchymal cells secrete the proteins to “condition” the ECM for proper cell migration not just in a pericellular fashion, but also affecting at least adjacent fin mesenchymal cells.

hmcn2/fbln1 double morphants display disorganization of the fin fold ECM

To elucidate possible changes within the fin fold ECM that may cause compromised mesenchymal cell migration, we performed TEM. Consistent with the aforementioned ET37 transgenic analyses, cell bodies of mesenchymal cells were only found in basal fin fold regions of hmcn2/fbln1 double morphants (Fig. 5B). Furthermore, while in control embryos, cellular protrusions of fin mesenchymal cells were aligned to the actinotrichia (inset of Fig. 5A and G), protrusions in hmcn2/fbln1 double morphants were usually found in more central regions of the dermal space and remote from the actinotrichia (Fig. 5F), although actinotrichia themselves appeared normal (compare Fig. 5D and F with Fig. 5E and G). In addition, we observed striking changes in the organization of the cross fibers. It was shown earlier that these cross fibers are present in apical-most regions of the fin fold, spanning the subepidermal space at right angles to the actinotrichia, whereas they could not be detected in more medial and basal regions of the fin fold, leading to the speculation that they need to be eliminated to leave a clear path for the invasion of fin mesenchymal cells (Dane and Tucker, 1985). In line with these studies, we found cross fibers spanning the dermal space in apical regions of wild-type fin folds (Fig. 5C). However, cross fibers were also found in medial regions of wild-type fins, where they displayed a different spatial organization, forming bouquet-like structures in regions close to the BM and actinotrichia, while being covered with dense granular material in more interior regions of the dermal space (Fig. 5E and G). They did not seem to hinder protrusions of invading fin mesenchymal cells, which wove between them (Fig. 5G). In contrast, cross fibers of the apical organization, spanning the entire space in an unembellished and less decorated manner, were also present in medial and basal positions of hmcn2/fbln1 double morphant fin folds (Fig. 5D, F and H), in line with the strongly reduced width of the dermal space (Fig. 5B). Furthermore, cellular protrusions of fin mesenchymal cells seemed incapable of avoiding cross fibers, but were tightly attached to them both in medial-basal regions (Fig. 5F; thin protrusion) and in basal regions (Fig. 5H; thick protrusion) of the fin fold. Together, this suggests that Hmcn2 and Fbln1 have a positive effect on the association between fin mesenchymal cells and actinotrichia, but a negative effect on the association between fin mesenchymal cells and cross fibers, so that in their absence, fin mesenchymal cells cannot follow their normal track along the actinotrichia, but get trapped within the cross fibers. Their non-cell autonomous mode of action (see above) further suggests that this is not achieved by modulating cell surface properties of fin mesenchymal cells and their protrusions, but by modifying the ECM. In agreement with this latter notion, alterations in cross fiber organization were also seen in medial regions of wild-type fin folds, which were not reached by fin mesenchymal cell protrusions as yet (Fig. 5E).

Discussion

Hemicentin was initially discovered and functionally characterized in the nematode C. elegans, based on hmcn loss-of-function mutants in the him-4 (high incidence of male progeny-4) locus (for review, see Vogel et al., 2006). Sex distortion among the self-progeny of hermaphrodites is caused by incorrect chromosome segregation in the C. elegans germline, in the case of him-4 (hmcn) mutants due to the cytokinesis failures in germ cells (Vogel et al., 2011; Xu and Vogel, 2011). Interestingly, similar cytokinesis problems were observed in Hmcn1 mutant mice during the preimplantation stages, pointing to an evolutionary conserved role of these extracellular proteins to promote cleavage furrow maturation and contractile ring formation during cytokinesis (Xu and Vogel, 2011). However, C. elegans him-4/hmcn mutants have other developmental defects, mainly affecting adhesion and cell migration processes (Vogel and Hedgecock, 2001; Vogel et al., 2006). This work reveals comparable defects caused by concomitant loss of Hmcn2 and Fbln1 function during vertebrate development.

Hemicentinins and adhesion

In C. elegans, Hmcn regulates adhesion across BMs separating two adjacent tissues, with distances between the two opposing BMs ranging from 100 nm to up to several micrometers (Vogel and Hedgecock, 2001; Vogel et al., 2006). Immunohistochemistry further revealed that Hmcn is positioned between the BMs (Vogel and Hedgecock, 2001; Vogel et al., 2006), suggesting that it promotes tissue–tissue adhesion by regulating the anchorage between the BMs associated with each of the tissues/cells. We demonstrated a similar function for Hmcn1 during median fin formation in the zebrafish (Carney et al., 2010). At the relevant stages, the fin fold consists of two opposing epidermal sheets, both with underlying BMs, while the interepidermal space is cell-free (see Fig. 6). hmcn1 mutants display normal attachment between the basal epidermal layers and their BMs, whereas the connection between the BMs and the subepidermal space (or the connection between the two opposing epidermal BMs) is disrupted, leading to massive blistering below the BMs (Carney et al., 2010). Data presented in this work point to a similar function for Hmcn2, the more diverged Hcmn parologue only present in vertebrates, mediating the attachment between the trunk epidermis and the somites (Figs. 2 and 3). Also here, both the epidermis and the somites are surrounded by BMs, with a largely acellular dermal space in between, while Hmcn2 plays an important role in properly connecting epidermis and somites across the dermal space. Unlike Hmcn1 in the fin fold, however, Hmcn2 is not made by epidermal cells, but by somitic cells (Fig. 1), indicating that Hmcn proteins can come from either epidermal or inner cells to establish proper tissue linkage via their respective BMs.
Fig. 6. Simplified diagram illustrating the organization of the dorsal median fin fold in wild-type and hmcn2/fbn1 double morphant embryos at 55 hpf. The cartoon shows the bilayered epidermal sheets, the epidermal BM in gray, actinotrichia in yellow, cross fibers in black, and fin mesenchymal cells in green. In red are the sites of hmcn2/fbn1 action, based on immunohistochemistry and anti-Hmcn2 immunohistochemistry. For simplicity, only some cross fibers are shown. Left panel: in wild-type embryos, the cross fibers in the apical-most regions of the fin fold run in parallel and perpendicular to actinotrichia, spanning the entire interepidermal space, while in medial regions, they display a bouquet-like organization, possibly mediated by Hmcn2 located in subepidermal regions. Fin mesenchymal cells migrate on the inner surface of actinotrichia, weaving between the cross fibers. It is currently unclear whether this is directly mediated by Hmcn2 bound to actinotrichia or due to indirect effects (see Discussion). Right panel: in the absence of Hmcn2 and Fbn1, cross fibers throughout the entire length of the fin fold remain in an organization more reminiscent of apical-most regions in wild type. The mesenchymal cells fail to migrate along the actinotrichia and remain at the base of the fin, “trapped” within cross fibers.

In the fin fold, anchorage between the two epidermal sheets and their respective BMs has been proposed to be accomplished by cross fibers, which at least in apical-most regions of the fold span the entire interepidermal space, penetrate the BMs and terminate in direct contact with the epidermal cell membranes (Dane and Tucker, 1985) (Fig. 5C and G). The molecular composition of these cross fibers is unknown. According to our TEM analyses, they have a diameter of 10–15 nm, similar to mammalian microfibrils, which consist of polymerized Fibrillin proteins organized in a head-to-tail arrangement (Ramirez and Sakai, 2010). Intriguingly, loss of fibrillin2 (fbn2) function in zebrafish leads to fin blistering similar to that of hmcn1 mutants (Carney et al., 2010; Gansner et al., 2008b), while partial loss of Fbn2 and Hmcn1 synergistically enhance each other (Carney et al., 2010). Furthermore, cross fibers are not properly attached to the BMs in hmcn1 mutants (Carney et al., 2010). Hence, it is tempting to speculate that zebrafish Hmcn1 is involved in the anchorage of Fibrillin 2-containing cross fibers. Cross fiber-like structures might also be present in the dermal space between epidermis and somites (see Fig. 3 and its legend). Furthermore, hmcn2, fbn1 and fbn2 are co-expressed in somites, and fbn2 mutants display blisters in the trunk (Gansner et al., 2008b), as do hmcn2/fbn1 double morphants, suggesting that similar molecular systems might be at play in the subepidermal space of both the fin fold and the trunk to account for proper epidermal–dermal junction formation. Of note, trunk blisters of hmcn2/fbn1 double morphants occur shortly after melanocytes have migrated into the dermal space (see Fig. 3D and F), suggesting that blister formation is reinforced by mechanical stress caused by cell immigration. In addition, fbn1, but not hmcn2, is also expressed in premigratory neural crest (Zhang et al., 1997), the embryonic source of melanocytes, raising the possibility that a combinatorial supply of these ECM molecules by somites and melanocytes might contribute to proper dermal ECM reorganization during and after cell immigration.

**Hemicentins and cell migration**

An essential positive function of Hmcn during migratory processes has thus far only been described in the nematode C. elegans (Sherwood, 2006; Sherwood et al., 2005; Vogel et al., 2006), while a human cell model for epithelial breast cell invasion revealed HMCN2 as one of the genes most strongly up-regulated in invasive cells (Moon et al., 2008). Furthermore, for Fibulin 1, both positive and negative effects on cell migration have been reported in mammals and worms (Twal et al., 2001; Kubota et al., 2004; Cooley et al., 2008).

Here, we demonstrate an essential and positive role of Hmcn2 and Fbn1 on the immigration of fin mesenchymal cells into the zebrafish fin fold (Figs. 4 and 5; for schematic summary, see Fig. 6). Invading mesenchymal cells display strong hmcn2 expression (Fig. 1F) and secrete Hmcn2 protein into the interepidermal space where it becomes localized between epidermis and mesenchymal cells/protrusions (Fig. 1M). This is the region that contains the BMs and the actinotrichia, thick collagenous bundles positioned directly underneath the BMs, which span the fin folds in a proximodistal orientation. During normal development, the protrusions of migrating fin mesenchymal cells tightly attach to the inner surface of these actinotrichia (Figs. 5A, G and 6), suggesting that the actinotrichia act as their migration substrate. In hmcn2/fbn1 double morphants, mesenchymal cells display reduced association with actinotrichia (Fig. 5F), reduced cell protrusion persistency (Fig. 4F) and reduced cell migration. Our cell autonomy (Fig. 4G and H) and Hmcn2 localization analyses (Fig. 1M) further indicate that this conditioning of the migratory substrate does not occur in a pericellular fashion, but spreads beyond individual Hmcn2/Fbn1-generating fin mesenchymal cells. This is consistent with findings in C. elegans, according to which Hmcn can move rather long distances within the embryo (Vogel and Hedgecock, 2001; Vogel et al., 2006).

In addition to the attachment between actinotrichia and mesenchymal cell protrusions, we found zebrafish Hmcn2 to be involved in the reorganization of the interepidermal cross fibers, which run perpendicular to the actinotrichia and are supposed to keep the two epidermal sheets together, involving the function of Hmcn1 (see above). In wild-type embryos, continuous cross fibers spanning the entire interepidermal space are only present in apical/distal regions (Fig. 5C), while more proximally—but still distal of the first mesenchymal cell protrusions—they start to rearrange into bouquet-like bundles (Figs. 5E and H). Hmcn2 seems to be involved in this rearrangement, as cross fibers maintain a more distal-like organization throughout the entire proximodistal length of the fin fold (Fig. 5D). In this respect, Hmcn2 secreted by mesenchymal cells appears to modulate the spatial organization of ECM structures that had been set up with the help of its close relative Hmcn1 secreted by apical epidermal cells. In light of the tight attachment between mesenchymal cells protrusions and cross fibers observed in hmcn2/fbn1 double morphants (Fig. 5F and H), it is tempting to speculate that similar to the proposed function of Hmcn during male gonad development, allowing adjacent tissues to glide past each other (Vogel and Hedgecock, 2001; Vogel et al., 2006), zebrafish Hmcn2 might act as a kind of lubricant between cross fibers and mesenchymal cell protrusions to allow proper navigation of mesenchymal cell protrusion between the cross fibers and through the subepidermal space.

**Hemicentin 2 acts in functional redundancy with Fibulin 1**

In contrast to Hmcn1, even complete knockdown of Hmcn2, as judged by the absence of detectable protein via IHC, does not lead to any discernible phenotype, indicating that Hmcn2 per se is dispensable for zebrafish development. hmcn2 MOs also failed to...
strengthen the defects of hmcn1 mutants, suggesting that Hmcn2 does not act in functional redundancy with its direct paralog, and consistent with their largely complementary expression patterns (Fig. 1). However, Hmcn2 shares functions with Fibulin 1 (Fbln1), such that hmcn2/fbln1 double morphant embryos display defects (see above) neither observed in hmcn2 nor in fbln1 single morphants, consistent with hmcn2 and fbln1 co-expression in affected embryonic structures (Fig. 1).

Fibulin 1 is the founding member of the Fibulin family of ECM proteins (Argraves et al., 1989). In mammals, seven members of this family have been described, with Hmcn1 referred to as Fibulin 6 (Argraves et al., 2003; de Vega et al., 2009). However, we strongly believe that Hmcn2 should also be included, which would make a total of eight members. All of them share a stretch of EGF motifs followed by a globular FC domain in the C-terminal region, whereas the N-terminal regions of Fibulin 1 and Hemicentins are highly diverse (de Vega et al., 2009).

Compared to Hemicentins, the roles of Fibulin 1 during vertebrate development have been well studied. It is present in some BMs, in blood vessel walls and in multiple connective tissues, where it is often associated with microfibrils and elastic fibers (de Vega et al., 2009). Fibulin 1 knockout mice have multiple defects, including compromised formation of renal glomeruli and lung alveoli, and irregularly shaped endothelial compartments (Cooley et al., 2008; Kostka et al., 2001), consistent with the irregularly patterned intersegmental vessels observed in our fbln1 morphant zebrafish (Supplementary Fig. S4). Of note, neither mouse nor zebrafish embryos display endothelial fbln1 expression during normal development (Zhang et al., 1996, 1997), raising a question about the source of Fibulin 1 contributing to vascular development and integrity. In mouse, vascular defects could be related to the earlier expression of Fbln1 in cephalic neural crest cells that contribute to the pericytes and vascular smooth muscle cells (Cooley et al., 2008). However, in zebrafish, intersomitic vessel defects of fbln1 morphants can be detected before smooth muscle cells (called mural cells) appear during normal development (Santoro et al., 2009). Future studies have to reveal whether fbln1 expression in the somitic (Fig. 1) or ventral mesoderm (Zhang et al., 1997) might contribute to vessel development, and to which extent this might also apply to mice and other vertebrates.

Interestingly, of all vertebrate fibrillins, only Hemicentin and Fibulin 1 are present in C. elegans. For Fibulin 1, structural conservation even extends to the Fbln1C and Fbln1D splice isoforms (Barth et al., 1998). Most strikingly, Hmcn and the two Fbln1 isoforms display specific interactions during multiple processes of nematode development (Muriel et al., 2005; Vogel et al., 2006). Immunohistochemistry revealed that Fbln1 and Hmcn co-localize at many embryonic structures, and that Fbln1 is required for proper localization of Hmcn and vice versa, whereas other associated ECM proteins are not affected, pointing to a specific interaction between Hmcn and Fbln1 (Muriel et al., 2005). Furthermore, shared as well as isoform-specific functions and interactions with Hmcn have been reported for C. elegans fbln1C and Fbln1D (Hesselson and Kimberly, 2006; Muriel et al., 2005; Vogel et al., 2006), similar to our findings of shared functions of zebrafish Fbln1C and Fbln1D in epidermal–dermal junction formation in the trunk, but their distinct functions during fin morphogenesis (Table 1). To our knowledge, no C. elegans fbln1/hmcn double mutants have been described as yet. In light of our data obtained in zebrafish, we would expect them to show additional or stronger defects in processes with redundant, rather than closely interactive functions of Hmcn and Fbln1. It is also noteworthy that no skin defects comparable to those of fbln1/hmcn2 double morphant zebrafish were observed in Fbln1 mutant mice, although Fbln1 is a prominent component of the mouse skin, possibly also pointing to functional redundancy with other fibrillins present in the skin, such as Hemicentins (Xu et al., 2007).

In sum, data obtained in C. elegans and zebrafish suggest that the roles of Hemicentins and their interactions with Fibulin 1 and its splice variants have been highly conserved throughout evolution, while future studies have to reveal to which extent they play redundant roles in organisms other than the zebrafish.

Possible molecular mechanisms of Hemicentin function

Thus far, no physical binding partners of Hemicentin proteins have been described (de Vega et al., 2009). Therefore, we can only speculate about the molecular basis of their functions during adhesiveness and cell migration. In C. elegans, Hmcn protein can assemble into linear tracks connecting adjacent cells, tissues or their respective BMs. Expression studies of different Hmcn domains as GFP-tagged fusion proteins have revealed that the N-terminal VWA domain targets the protein to multipleassembly sites, while the C-terminal EGF–FC modules mediates direct homophilic interaction between Hmcn momomers during track formation (Dong et al., 2006). It has been speculated that these tracks might constitute elastic fiber-like structures, which are the precursors of the elastic fibers found in vertebrates (Vogel et al., 2006). Vertebrate elastic fibers consist of a core of polymerized fibrillin molecules, modified by the binding of elastin and several other associated proteins, including Fibulins (Ramirez and Sakai, 2010). Indeed, Fibulin 2, 4 and 5 have been shown to physically bind the microfiber core protein Fibrillin-1 (El-Hallouy et al., 2007; Reinhardt et al., 1996). Since these fibrin members only have the C-terminal EGF–FC modules in common, it seems likely that binding occurs via this region, which is also present in Hemicentins (de Vega et al., 2009). Therefore, it is tempting to speculate that zebrafish Fbln1 and Hmcn2 might also bind to Fibrillins, the possible constituents of the fin fold cross fibers (see above). However, according to our anti-Hmcn2 immunohistochemical analyses, Hmcn2 protein is most prominent in the immediate subepidermal regions (Fig. 1M). Therefore, Hmcn2 and Fbln1 might not necessarily be global regulators of cross fiber integrity, but might rather modulate the (Hmcn1-dependent; see above) anchorage of cross fibers either to the BM or to the basal surface of basal keratinocytes. In addition to cross fiber rearrangements, such interactions with BM components could also account for the observed compromised epidermal–dermal junction formation observed in the trunk (Fig. 2) and, to a lower extent, in the fin folds (Fig. 3K) of hmcn2/fbln1 double morphant embryos. However, it seems unlikely that BM interactions also account for the effect of Hmcn2/Fbln1 on the attachment of fin mesenchymal cells to their migratory substrate, the actinotrichia, as this attachment takes place on the side of the actinotrichia facing away from the BM (see Fig. 6). Actinotrichia–mesenchymal cell attachments could be mediated by direct binding between Collagen type I/II proteins, the major constituents of the actinotrichia (Duran et al., 2011), and integrin α2β1 receptors (Jokinen et al., 2004) at the surface of invading fin mesenchymal cells. However, it remains unclear how Hmcn2/Fbln1 could interfere with this binding, since apart from Collagen type XVIII, which is a non-fibrillar molecule found in BMs (Sasaki et al., 1998), no physical binding between Fibulin 1 and any collagens has been reported (de Vega et al., 2009).

In conclusion, this work identifies the thus far functionally uncharacterized vertebrate ECM protein Hmcn2 as a close and evolutionary conserved partner of Fibulin 1 during the regulation of different aspects of tissue adhesion and cell migration, thereby also extending current knowledge of Fibulin 1 functions (de Vega et al., 2009). In addition, it sheds first light on the ultrastructural basis of these in vivo functions, while further studies will be necessary to unravel the underlying molecular mechanisms.
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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.06.023.

References


