



## Review

## Recent advances in the study of zebrafish extracellular matrix proteins

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## ABSTRACT

The zebrafish extracellular matrix (ECM) is a dynamic and pleomorphic structure consisting of numerous proteins that together regulate a variety of cellular and morphogenetic events beginning as early as gastrulation. The zebrafish genome encodes a similar complement of ECM proteins as found in other vertebrate organisms including glycoproteins, fibrous proteins, proteoglycans, glycosaminoglycans, and interacting or modifying proteins such as integrins and matrix metalloproteinases. As a genetic model system combined with its amenability to high-resolution microscopic imaging, the zebrafish allows interrogation of ECM protein structure and function in both the embryo and adult. Accumulating data have identified important roles for zebrafish ECM proteins in processes as diverse as cell polarity, migration, tissue mechanics, organ laterality, muscle contraction, and regeneration. In this review, I highlight recently published data on these topics that demonstrate how the ECM proteins fibronectin, laminin, and collagen contribute to zebrafish development and adult homeostasis.

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## Introduction

The extracellular matrix (ECM) is composed of a complex set of macromolecules that is produced by cells and organized into a meshwork through interactions with specific cell surface matrix receptors. Fibronectin and laminin for example, bind transmembrane proteins of the integrin family. It is well established that matrix assembly into fibrils is a cell-mediated process involving integrin engagement of the actin cytoskeleton and activation of intracellular signaling pathways (Wierzbicka-Patynowski and Schwarzbauer, 2003). Integrin binding to matrix proteins also creates tension on the ECM and thus promotes further integrin binding and crosslinking of fibrils (Schwarzbauer and Sechler, 1999). In addition to integrin receptors, generation of fibrillar ECM matrices can require mechanical forces created by cell-cell adhesion (Schwarzbauer and DeSimone, 2011). In epithelial tissues, the ECM is assembled into a specialized structure called the basal lamina (or basement membrane) and consists of several proteins including laminin and type IV collagen. Laminin, with its multidomain stem-like structure, functions as an organizing center for basal laminae (Colognato and Yurchenco, 2000). Type IV collagen exists as long interacting fibers that form twisted structures that crisscross throughout the basal lamina and provide tensile strength (Yurchenco et al., 1986). Unlike thin basement membranes, the ECM associated with interstitial tissues forms a large macromolecular structure consisting of numerous types of ECM proteins including fibrous collagens, proteoglycans, glycosaminoglycans, and glycoproteins such as laminin and

fibronectin. Interstitial tissues also contain cells (e.g. fibroblasts, chondroblasts, and osteoblasts) that both secrete ECM proteins and also control ECM assembly by exerting tension on matrix proteins through non-covalent interactions. Fibrillar collagens are a major component of interstitial ECM and provide structural support for cells in part through interactions with fibronectin and integrins.

It has long been recognized that ECM proteins provide more than just structural support for cells and tissues and a scaffold or barrier for cell migration. For example, pioneering work demonstrated that epithelial cell gene expression could be regulated by changes in the surrounding ECM (Bissell et al., 1982). The model of dynamic reciprocity states that through interactions with transmembrane receptors, ECM proteins influence the cytoskeleton and subsequently changes in gene expression levels (Bissell and Barcellos-Hoff, 1987; Bissell et al., 1982). The nature of an ECM meshwork also has a strong impact on the movement and availability of secreted molecules and thus the activation of signal transduction pathways. Proteoglycans, with their covalently attached glycosaminoglycan side chain(s), are particularly well known for the ability to interact with a variety of growth factors. For example, the *Drosophila melanogaster* glycan family member Dally (encoded by *division abnormally delayed*) regulates both TGF $\beta$ /BMP and Wingless signaling at the cell surface (Jackson et al., 1997; Tsuda et al., 1999). Even in regards to cell motility, data suggest that interactions between cells and their surrounding ECM can regulate both ECM rigidity and cell migration/invasion (Bordeleau et al., 2010; Kim et al., 2014).

Much of our knowledge regarding the diverse functions of ECM proteins has come from model organisms such as fly, frog, fish,

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chicken, and mouse. While some of the first reports on cloning and expression of zebrafish ECM proteins appeared in the 1990s (Higashijima et al., 1997), publications on the analysis of ECM structure and function have increased dramatically over the last decade. For a comprehensive review on the role of multiple zebrafish ECM and related proteins during development (see Mundell and Jessen, 2013). Notably, as researchers delve more deeply into the molecular mechanisms underlying various cellular processes, ECM proteins and their regulators are repeatedly being recognized for their significant roles. The goal of this review is to discuss recent findings that highlight the diverse roles played by the ECM proteins fibronectin, laminin, and collagen in the embryonic and adult zebrafish. The topics of these papers are organized into six sections that together cover multiple developmental stages and processes (gastrulation, somitogenesis, organogenesis, formation of skeletal muscle, heart development, and adult heart regeneration). Each section includes a background for the topic discussed and provides, when necessary, general information regarding the genetic characterization and expression of zebrafish ECM genes and their encoded proteins.

### ECM and planar cell polarity during gastrulation

Gastrulation is the process whereby the three embryonic germ layers (ectoderm, mesoderm, and endoderm) are established and involves complex morphogenetic cell movements that shape the body plan into an embryo with anterior-posterior and dorsal-ventral axes (Jessen and Solnica-Krezel, 2005; Solnica-Krezel, 2005). Unlike zebrafish, it has been well established that ECM proteins influence amphibian gastrulation (Boucaut and Darribere, 1983; Lee et al., 1984) with fibronectin/Integrin  $\alpha 5\beta 1$  interactions being required for polarized membrane protrusive activity underlying the gastrulation cell movement termed convergent extension (Davidson et al., 2006; Marsden and DeSimone, 2003). Convergent extension is the narrowing of a tissue in one direction with simultaneous extension in the perpendicular direction and requires the cell behavior of mediolateral intercalation (Keller and Tibbets, 1989; Keller et al., 1985). Loss of fibronectin or integrin activity produces misshapen frog gastrula embryos with shortened and broadened body axes, hallmarks of disrupted convergent extension (Skoglund and Keller, 2010). By contrast, loss of fibronectin in the mouse is embryonic lethal producing numerous problems associated with mesoderm morphogenesis including somite and heart defects (George et al., 1993; Georges-Labouesse et al., 1996).

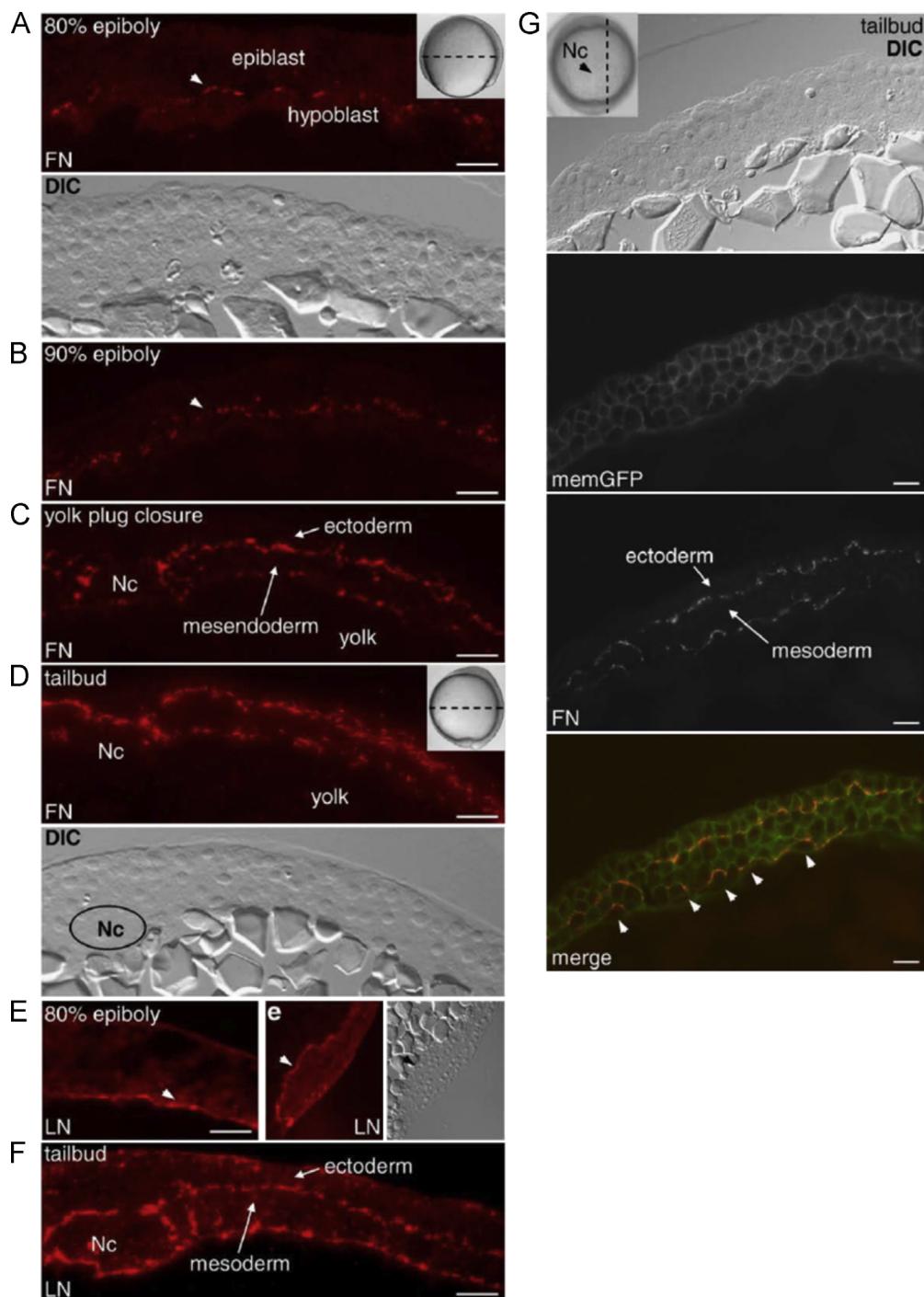
What then is the nature of the ECM in the early zebrafish embryo? The most current study of ECM dynamics in zebrafish gastrula-stage embryos (6–10 h post-fertilization) analyzed fibronectin and laminin protein expression (Fig. 1) (Latimer and Jessen, 2010). Fibronectin is a large multidomain dimeric glycoprotein typically expressed in both embryonic and adult tissues as either a soluble or insoluble form. Insoluble fibronectin constitutes a major ECM protein and is assembled from a dimer into a fibrillar meshwork (Schwarzbaumer and Sechler, 1999) and it is clear that the state of the fibrillar fibronectin matrix can have different effects on embryonic morphogenetic cell movements (Rozario et al., 2009). The zebrafish genome encodes two fibronectin isoforms, Fibronectin 1 and Fibronectin 1b (Sun et al., 2005; Zhao et al., 2001). The expression of *fibronectin1* mRNA (*natter* is the mutation) is restricted in the early embryo (prior to 24 h post-fertilization) localizing to mesoderm, posterior notochord, tailbud, and yolk syncytial layer (Thisse et al., 2004; Trinh and Stainier, 2004). *Fibronectin1b* mRNA expression is also restricted localizing to somitic and paraxial mesoderm at early embryonic stages (Thisse et al., 2004). Unlike fibronectin, laminins consist of

different combinations of alpha, beta, and gamma subunits and the zebrafish genome encodes at least 10 laminin genes with distinct and overlapping mRNA expression patterns. Notable expression domains include the eye, brain and spinal cord, pharyngeal arches, notochord, and trunk muscle (Sztal et al., 2011). Unlike *fibronectin* and *laminin* (Latimer and Jessen, 2010), few *collagen* family members are expressed during gastrulation and these are predominantly restricted to axial tissue (Rauch et al., 2003; Thisse et al., 2001, 2004).

Gastrulation-stage expression of fibronectin and laminin proteins is first detected by immunofluorescence at approximately 65% epiboly (7.3 h post-fertilization) and localizes to the epiblast-hypoblast boundary (Latimer and Jessen, 2010). As gastrulation proceeds, fibronectin and laminin expression and fibrillogenesis increases and a new domain appears between the mesendodermal cells and the underlying extra-embryonic yolk syncytial layer (Fig. 1). Fibronectin fibrils also extend between individual cells and associate with protrusions produced by migrating deep mesodermal cells (see Fig. 1 and Latimer and Jessen, 2010). These data indicate that the ECM in gastrula-stage zebrafish embryos forms a layer at the ectoderm–mesoderm tissue boundary and beneath and surrounding the deep mesodermal cells. The precise role of these matrices during gastrulation remains unclear but, as in frog embryos, ECM proteins likely influence polarized cell behaviors necessary for zebrafish convergence and extension cell movements. This notion is supported by data showing that antisense morpholino oligonucleotide-mediated knockdown of both *fibronectin1* and *fibronectin1b* produces misshapen zebrafish embryos with phenotypes characteristic of convergence and extension defects (Latimer and Jessen, 2010).

In zebrafish, it has become clear that gastrulation cell movements are regulated by homologs of *D. melanogaster* planar cell polarity genes including *vang-like 2* (*vangl2*), *prickle*, and *frizzled* (Carreira-Barbosa et al., 2003, 2009; Jessen et al., 2002; Lin et al., 2010). During zebrafish gastrulation, planar cell polarity is defined as the elongation and mediolateral alignment of both mesodermal and ectodermal cell populations (Jessen et al., 2002; Topczewski et al., 2001). Loss of function mutations in *vangl2* for example, produce misshapen embryos with defective convergence and extension (Jessen et al., 2002). Significantly, data have now identified relationships between zebrafish planar cell polarity proteins and assembly/organization of the ECM. For example, it was shown that *vangl2/trilobite* mutant embryos (Jessen et al., 2002; Solnica-Krezel et al., 1996) have increased matrix metalloproteinase activity and decreased fibronectin protein levels (Williams et al., 2012). Mechanistically, it is thought that the transmembrane protein Vangl2 regulates endocytosis and cell surface levels of membrane-type 1 matrix metalloproteinase (Mmp14) and that Mmp14 could function downstream of Vangl2 to influence convergence and extension cell movements (Coyle et al., 2008; Williams et al., 2012).

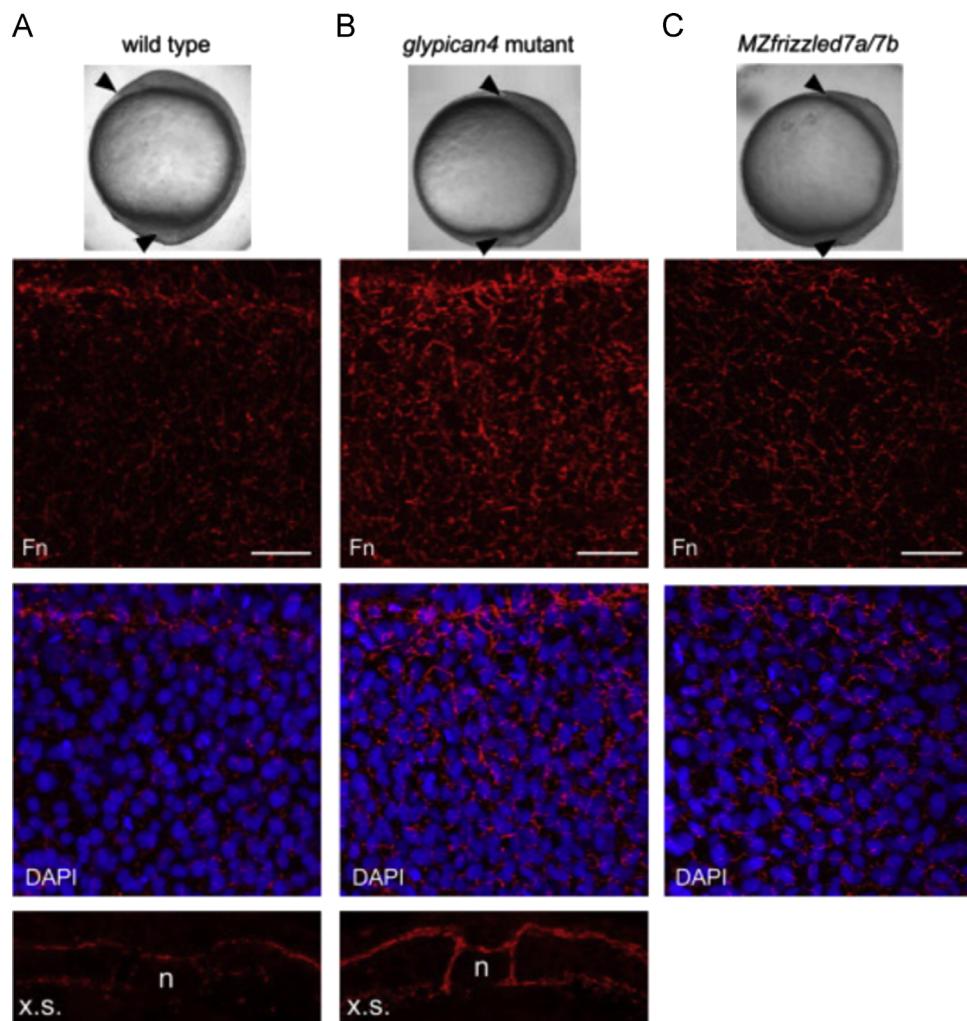
Vangl2 is a four-pass transmembrane planar cell polarity protein thought to regulate cell behaviors through a physical interaction with the cytosolic protein Prickle (Carreira-Barbosa et al., 2003). Recent work has now shown that, similar to *vangl2/trilobite* mutant embryos, wild-type embryos injected with *prickle1a* morpholino exhibit reduced fibronectin protein expression (Dohn et al., 2013). While it is unclear whether Vangl2 and Prickle1a interactions are required for Mmp14 trafficking to and from the cell surface, these data indicate that two so-called core planar cell polarity proteins regulate ECM dynamics during zebrafish gastrulation. Similar to loss of Vangl2 or Prickle1a function, reduced activity of other planar cell polarity proteins such as Frizzled7 and the heparan sulfate proteoglycan and Wnt co-receptor Glypican4 also produces strong convergence and extension phenotypes. However, gastrula-stage *frizzled7a/7b* and *glypican4*/



**Fig. 1.** Fibronectin (FN) and laminin (LN) expression during zebrafish gastrulation. (A) Fibronectin and laminin expression in cross-sectioned (A–F) and sagittally sectioned (E and G) gastrula-stage embryos. Insets in (A) and (D) show the cross-section plane (horizontal lines) while the inset in panel (G) depicts the sagittal-section plane (vertical line); arrowhead in (G) marks the embryonic midline or notochord (Nc). (A and B) At 80% and 90% epiboly fibronectin localizes to the epiblast–hypoblast boundary, arrowheads. (C) At yolk plug closure two fibronectin domains are visible, the ectoderm–mesoderm boundary (formally epiblast–hypoblast) and the deeper mesendoderm–yolk boundary. Fibronectin is also observed adjacent to the Nc. (D) By the end of gastrulation, fibronectin assembly becomes more fibrillar and continues to define germ layer tissue boundaries. (E) Laminin expression at 80% epiboly localizes to the deep mesendoderm–yolk boundary, arrowhead. (e) Notably at this stage, in sagittally sectioned embryos, laminin is observed underneath internalized hypoblast cells near the blastoderm margin (arrowheads). (F) By the end of gastrulation, laminin expression demarcates tissue boundaries similar but not identical to fibronectin. (G) Fibronectin expression in membrane-GFP injected wild-type embryos. Arrowheads denote deep membrane-GFP-labeled mesendodermal cells. Scale bars, 20  $\mu$ m. Adapted from Latimer and Jessen (2010) with permission.

*knyapek* mutant embryos exhibit increased fibronectin fibrillogenesis as indicated by immunofluorescence (Fig. 2) (Dohn et al., 2013). This increase is not associated with an increase in fibronectin protein expression supporting the notion that it is likely due to increased matrix assembly and not disrupted matrix metalloproteinase activity. Furthermore, it was shown that *glypican4/knyapek* mutant

embryos have increased cell surface cadherin expression and cell-cell adhesion (Dohn et al., 2013). These data raise several important questions regarding the relationship between planar cell polarity and ECM assembly. First, how can disruption of Vangl2 and Glypican4 function produce embryos with similar convergence and extension phenotypes (Marlow et al., 1998; Solnica-Krezel



**Fig. 2.** *glypican4* and *frizzled7* mutant embryos have increased fibronectin assembly. (A–C) Top panels show morphological convergence and extension phenotypes at tailbud stage (see arrowheads) of wild type, *glypican4* mutant, and maternal-zygotic *frizzled7a/7b* double mutant embryos. Middle two panels show confocal images of fibronectin (Fn) immunolabeling without and with nuclear DAPI staining. Scale bars = 20  $\mu$ m. The bottom images in (A) and (B) show Fn expression in cross-sections (x.s.) of tailbud stage embryos (n, notochord). Adapted from Dohn et al. (2013) with permission.

et al., 1996) yet cause such different effects on fibronectin matrix assembly? Second, what are the roles of pericellular proteolysis of ECM substrates and cell adhesion during zebrafish gastrulation? Third, how do cell–ECM interactions influence the establishment of planar cell polarity and directed cell migration? Fourth, does the ECM move with migrating gastrula cells, as shown in chick (Zamir et al., 2008), and is this required to maintain planar cell polarity?

#### ECM, somitogenesis, and trunk elongation

It is well known that fibronectin, laminin, and collagen-containing extracellular matrices function as substrates for cell migration events both during embryonic development and in disease processes such as tumor progression. However, cell–ECM interactions also regulate tissue level morphogenetic processes. Somites are transient epithelial segmental structures that form as bilateral pairs along the anterior–posterior body axis of the developing embryo (Holley, 2007). Somites are derived from mesenchymal presomitic mesoderm and a molecular oscillator (cycles of positive and negative feedback) termed the segmentation or somite clock regulates their formation (Julich et al., 2005, 2009). The expression of zebrafish fibronectin and laminin at somitic boundaries has been recognized for more than a decade

(Crawford et al., 2003). Furthermore, the expression of Integrin  $\alpha 5$ , paxillin, and focal adhesion kinase at intersomitic furrows strongly suggests that integrin–ECM adhesion plays a key role in somitic boundary formation (Crawford et al., 2003; Henry et al., 2001; Julich et al., 2005). This is supported by the observation that *fibronectin* and *Integrina5* knockout mice have defects in somitogenesis (George et al., 1993; Yang et al., 1999). The zebrafish genome contains at least 17 integrin or integrin-like genes including genes encoding 9 alpha and 8 beta subunits. A comparison of *fibronectin1* and *fibronectin1b* mRNA localization with integrin receptor subunits *integrina5* and *integrin $\beta 1$*  identifies distinct and overlapping expression domains including adaxial cells (muscle precursor cells adjacent to the notochord), posterior tailbud, pre-somitic mesoderm, notochord, and somites (Julich et al., 2005; Thisse et al., 2001, 2004).

It was proposed that, independent of the Notch-mediated oscillator clock, Integrin  $\alpha 5$  adhesion to fibronectin is required for the assembly of intersomitic ECM and necessary for both epithelialization and maintenance of somite boundaries (Julich et al., 2005; Koshida et al., 2005). Notably, *integrina5* mutant zebrafish embryos have undetectable levels of Y397 phosphorylated focal adhesion kinase perhaps suggesting that the formation of cell–ECM adhesions is disrupted (Koshida et al., 2005). However, the phosphorylation of focal adhesion kinase at other amino acids

in *integrina5* mutant embryos warrants investigation. Presomitic mesodermal cells undergo morphological changes to become polarized epithelial cells that border nascent somite boundaries (Barrios et al., 2003; Henry et al., 2000) and this organization is lost in *integrina5* and *fibronectin1* mutant embryos (Koshida et al., 2005). Because fibronectin fibrillogenesis is a cell-mediated process (Mao and Schwarzbauer, 2005), interactions between Integrin  $\alpha 5\beta 1$  expressed by adaxial cells and secreted fibronectin dimers likely trigger cross-linking and further integrin–fibronectin binding (Koshida et al., 2005). However, it is unknown how cell–ECM interactions and integrin signaling promote epithelialization of somitic boundary cells. Ephrin receptor tyrosine kinase (Eph)-mediated signaling regulates Integrin  $\alpha 5$  clustering and this event precedes ECM formation and is independent of fibronectin binding (Julich et al., 2009). Interestingly, cell surface Integrin  $\alpha 5$  on adjacent paraxial mesodermal cells non-cell autonomously inhibits Integrin  $\alpha 5$  clustering, fibronectin binding, and fibrillogenesis along somitic boundaries (Julich et al., 2009). Derepression induced by Ephrin B2a reverse signaling initiates Integrin  $\alpha 5$  clustering and subsequent fibronectin binding thus providing a mechanism to restrict ECM assembly to specific tissue surfaces (Julich et al., 2009).

Recent work has now implicated the small monomeric GTPase Rap1b as a potential regulator of Integrin  $\alpha 5$  and inside-out signaling and fibronectin matrix assembly (Lackner et al., 2013). Knockdown of *rap1b* function in an *integrina5* mutant background causes a large reduction in fibronectin matrix assembly surrounding the somites. This synergistic interaction subsequently leads to a failure in somite border formation along the anterior–posterior body axis but does not affect somite patterning. Significantly, no genetic interaction was identified between *rap1b* and *ephrinB2a* as indicated by normal fibronectin assembly around somites though the trunk extension defect was enhanced. Thus while it cannot yet be concluded, the data by Lackner et al. support the notion that Rabp1b does not provide a link between EphrinB2a and integrin activation. However, it is clear that Rap1b is a component of a signaling pathway that promotes integrin/fibronectin protein interactions and subsequent fibronectin matrix assembly necessary for formation and stabilization of somite boundaries (Lackner et al., 2013).

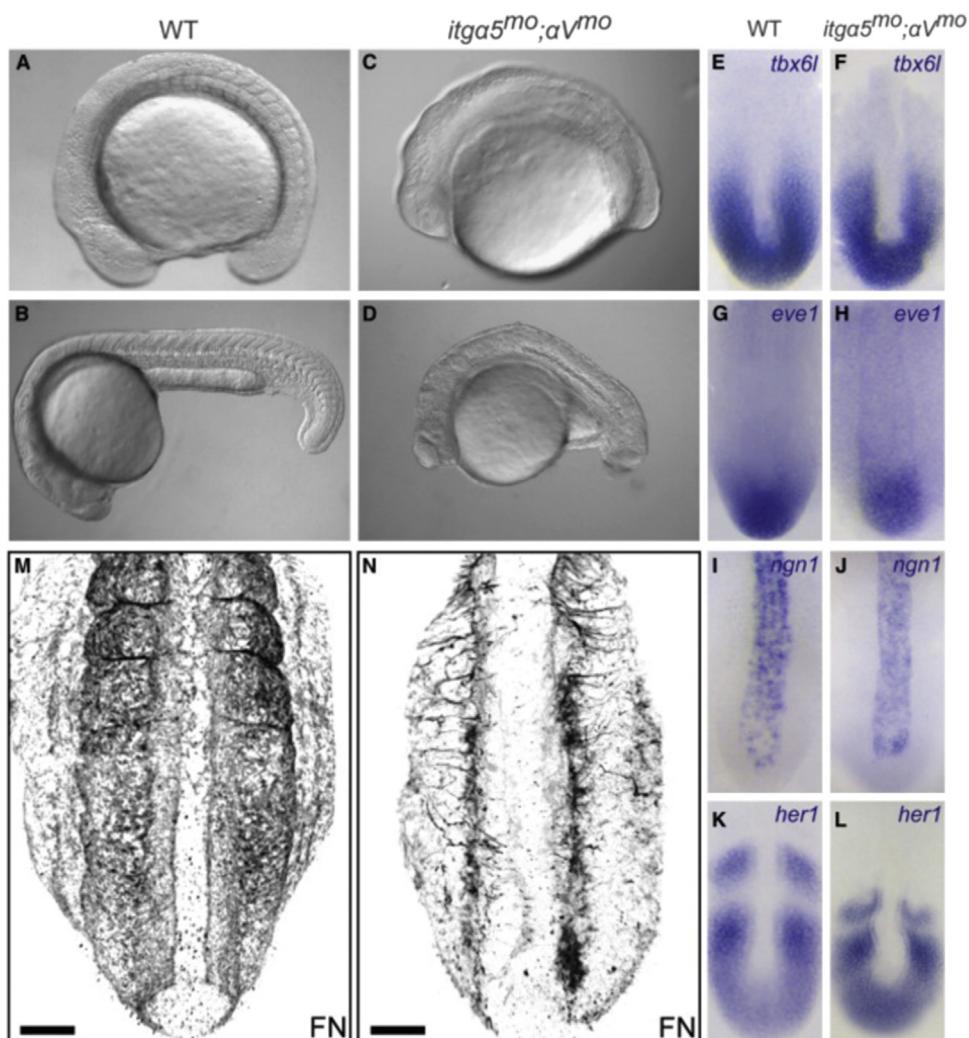
The above discussion demonstrates how cell–ECM interactions are utilized to promote fibronectin matrix assembly at local tissue boundaries, in this case, those between developing somites and the adjacent notochord and mesoderm. In an elegant study by Dray et al. (2013), the role of cell–fibronectin interactions during zebrafish trunk elongation was addressed. Here, a combination of *integrina5/integrinaV* mutant and/or morpholinos was utilized to assess trunk phenotypes after loss of both fibronectin receptors. Double knockdown produced embryos with severe body truncation that was not due to convergence and extension, cell proliferation, patterning, or cell migration defects (Dray et al., 2013). Fig. 3 shows analyses of *integrina5/integrinaV* morpholino injected embryos including the trunk elongation defect in live embryos, normal cell fate specification as indicated by whole-mount *in situ* hybridization, and abnormal fibronectin matrix assembly. One notable aspect of the *integrina5/integrinaV* loss of function phenotype is the reduced fibronectin fibrillogenesis and abnormal alignment of fibronectin fibers in relation to the embryonic body axis. It was hypothesized that a disruption of mechanical force in the paraxial mesoderm and abnormal ECM tension caused the defect in matrix assembly resulting in a shortening of the embryonic trunk. To address this, a *tbx6l* enhancer-containing *integrina5-RFP* transgene was utilized to restore integrin–fibronectin interactions specifically within the paraxial mesoderm (Dray et al., 2013). Significantly, injection of this transgene partially rescued the *integrina5/integrinaV* knockdown body elongation defect and

restored fibronectin matrix assembly on the paraxial mesoderm and adhesion between the paraxial mesoderm and the notochord. All together, this paper demonstrates how integrin binding of fibronectin provides mechanical strength and adhesion that can be integrated across trunk tissues during the process of tail morphogenesis in zebrafish.

## ECM and trunk neural crest cell migration

Zebrafish trunk neural crest cells delaminate from the neural tube and migrate along specific tracts towards their final destination (Erickson, 1985). In the region of the somites, these cells transition from a so-called sheet-like migration pattern into individual streams of migrating cells. While it is known that signals from the somites regulate neural crest cell migration, for example Ephrin/Eph receptor dependent signaling (Krull et al., 1997) and Wnt signaling (Banerjee et al., 2011), the in vivo role of ECM molecules is less clear. ECM proteins known to be involved in neural crest cell migration include the integrin receptor ligands fibronectin and laminin (Perris and Perissinotto, 2000). In a recent study the enzyme lysyl hydroxylase 3 was shown to regulate trunk neural crest cell migration in zebrafish potentially through post-translational modification of the non-fibrillar collagen, Collagen 18A1 (Banerjee et al., 2013). It is notable that while the zebrafish genome encodes at least 22 collagen isoforms including 10 representative collagen family members (types I, II, IV, V, VI, VII, VIII, IX, X, and XI), few collagen germ-line mutants have been described. Zebrafish collagen mRNAs are expressed in numerous tissues including the somites (*collagen1a1a* and *5a1*) and notochord (*collagen4a5*, *5a3*, *11a1*, and *11a2*) (Fang et al., 2010; Thisse et al., 2001, 2004) where they might affect neural crest cell migration. Notably, it was previously shown that *collagen18a1* and *lysyl hydroxylase 3* are co-expressed in adaxial muscle precursor cells adjacent to the notochord (Schneider and Granato, 2006). Lysyl hydroxylase 3 is a glycosyltransferase known to post-translationally modify collagens through the addition of galactosyl or glucosyl sugars (Myllyla et al., 2007). Banerjee et al. hypothesized that enzymatic modification of Collagen 18A1 might regulate neural crest cell migration. Antisense morpholino oligonucleotides were utilized to knockdown translation of *collagen18a1*. Injected embryos exhibited a neural crest cell migration defect very similar to that in *lysyl hydroxylase 3* mutant embryos. Specifically, knockdown of *collagen18a1* caused cells to either stall their migration or migrate through the somites along ectopic trajectories (Fig. 4). Notably, loss of Collagen 18A1 function did not disrupt adaxial cell morphology, differentiation, or number suggesting a specific requirement for this ECM protein during neural crest cell migration (Fig. 4). How might Collagen 18A1 function to regulate cell migration? Banerjee et al. suggest that modification of Collagen 18A1 could create tissue regions that are either permissive or non-permissive for neural crest cell migration. This scenario would suggest that cell–ECM interactions, perhaps mediated by integrins, provide a substrate for cell migration. Alternatively, Collagen 18A1 may utilize its cysteine rich domain (also found in frizzled receptors) to influence the availability of Wnt migratory cues or its thrombospondin domain that is common to semaphorin guidance cues. Whatever the mechanism, it is clear that lysyl hydroxylase 3 and Collagen 18A1 function are required for neural crest cell motility likely acting to impact cell–ECM interactions and downstream cytoskeletal remodeling events necessary for cell motility.

Collagens also function as cleavage substrates for metalloproteases. In another recent study, the function of matrix metalloproteinase 17b (Mmp17b) in migrating trunk neural crest cells was

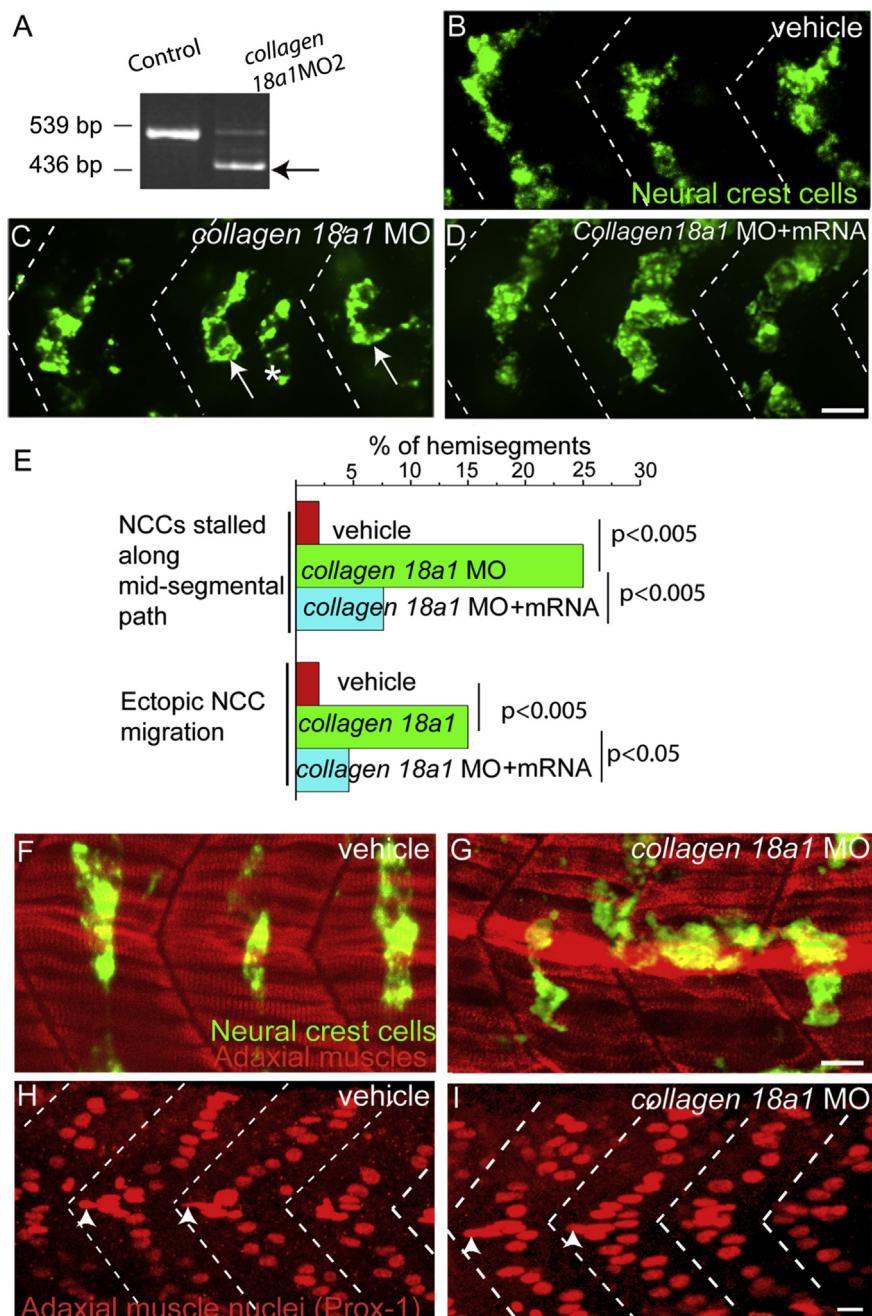


**Fig. 3.** Axis Elongation Defects after Loss of Both *integrinα5* and *integrinαV*. (A–D) Wild-type (WT) (A and B) and truncated (C and D) *integrinα5<sup>mo</sup>*; *integrinαV<sup>mo</sup>* embryos at the end of trunk elongation; i.e., 16 somite-stage embryos (A and C) and 24 h post-fertilization (B and D). At the 16 somite stage, we find that distance from the otic vesicle to the anterior of the head in *integrinα5<sup>mo</sup>*; *integrinαV<sup>mo</sup>* embryos is 74% of that in WT embryos and that the distance from the otic vesicle to the tip of the tail is 71% of that in WT embryos. (E–L) In situ hybridization of tail bud gene expression in 13 somite-stage embryos. (M and N) Fibronectin immunolocalization in 16 somite-stage WT (M) and *integrinα5<sup>mo</sup>*; *integrinαV<sup>mo</sup>* embryos (N). Note the reduction in fibronectin matrix as well as the prominent medial-lateral fiber orientation in (N). Scale bars are 50 μm. In (A–D), anterior is the left. In (E–N), anterior is up. Adapted from Dray et al. (2013) with permission.

investigated utilizing both morpholino-mediated *mmp17b* knock-down and chemical inhibition of MMP function (Leigh et al., 2013). *Mmp17b* is a glycosylphosphatidylinositol anchored membrane-tethered metalloproteinase. Unlike *collagen18a1*, *mmp17b* mRNA expression is detected within the neural crest cells themselves. Knockdown of *mmp17b* or broad spectrum MMP inhibition caused a neural crest cell migration defect with cells localizing to the posterior trunk as opposed to being dispersed along the anterior-posterior embryonic axis. The authors provide evidence that the matrix metalloproteinase inhibitor RECK (reversion-inducing-cysteine-rich protein with Kazal motifs) physically binds *Mmp17b* raising the possibility that these proteins functionally interact to regulate neural crest cell migration (Leigh et al., 2013). A key question raised by this study is the identity of the *Mmp17b* cleavage substrate present in the neural crest cell microenvironment. Is it an ECM protein such as collagen or is it a non-ECM protein such as ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin-like motif-4) (Gao et al., 2004). ECM cleavage by *Mmp17b* could function to promote cell migration in multiple ways including by the release of guidance cues sequestered in the ECM.

#### ECM and organ laterality

ECM remodeling influences several aspects of organogenesis including migration, cell–cell interactions, and branching morphogenesis. For example, regulation of ECM remodeling is required for the asymmetric migration of lateral plate mesoderm necessary for gut-looping and correct spatial organization of digestive organs (Yin et al., 2010). By examining the expression of the transcription factor *hand2*, a novel cell rearrangement was identified that occurs in the lateral plate mesoderm and is regulated by left-right gene expression. Significantly, whereas in wild-type embryos laminin expression diminishes along the path of lateral plate mesoderm migration, in *hand2* mutant embryos laminin deposition or expression persists, an effect not due to altered *laminin* or *integrin* gene expression (Yin et al., 2010). Unlike the heart primordium where *hand2* mutants exhibit disorganized fibronectin matrix assembly, loss of *hand2* does not disrupt fibronectin distribution in the gut-looping region (Trinh et al., 2005; Yin et al., 2010). Notably, partial loss of *laminin* in a *hand2* mutant background suppressed the lateral plate mesoderm migration defects while broad inhibition of matrix metalloproteinase activity recapitulated



**Fig. 4.** Knockdown of *collagen18a1* results in neural crest cell migration defects. (A) RT PCR analysis showing efficiency of *collagen18a1* knockdown following morpholino treatment. Arrow marks the expected band following morpholino treatment sized at 439 base pairs. Lateral views of 28 h post-fertilization vehicle (B) *collagen18a1* MO (C) and *collagen18a1* MO plus *collagen18a1* mRNA injected embryo (D), stained with *crestin* to visualize neural crest cells. Arrows indicate neural crest cells stalled along the mid-segmental path (C) and asterisks indicate neural crest cells along the ectopic path (C and E). (E) Quantification of neural crest cell migration defects in *collagen18a1* MO injected embryos. p values were calculated using one tailed Fisher Exact Probability test. Vehicle injected and *collagen18a1* (G) MO injected embryos, stained with P59 to visualize adaxial cells (red), and *crestin* to visualize neural crest cells (green) (F–G) and with prox-1 antibody (H–I). Arrowheads mark adaxial cell nuclei located anteriorly near horizontal myoseptum region (H–I). Scale bar=10 μm. Adapted from Banerjee et al. (2013) with permission.

aspects of the *hand2* mutant phenotype (Yin et al., 2010). Membrane-tethered *mmp14a* was decreased in *hand2* mutants while expression of the Mmp inhibitors *tmp2a* and *tmp2b* was increased. Knockdown of *mmp14a* using morpholinos produced a gut-looping phenotype in wild-type embryos (Yin et al., 2010). Taken together, these data implicate Hand2 as a regulator of Mmp proteolytic activity and subsequently ECM remodeling and cell migration during the early stages of organogenesis in zebrafish.

Certain organs including liver, pancreas, and heart do not exhibit bilateral symmetry, rather these organs localize asymmetrically in

relation to the central body axis. While it is known that left-right asymmetry initiates during zebrafish gastrulation when motile cilia regulate directional fluid flow within Kupffer's vesicle (Amack and Yost, 2004), molecular details connecting early events with later stages of morphogenesis are lacking. Recently, the ECM protein Laminin 1 was shown to regulate establishment of left-right asymmetry for both the liver and pancreas (Hochgreb-Hagele et al., 2013). This is a previously unrecognized phenotype attributable to *lamininb1a* loss of function. Mutations in Laminin 1 and Laminin 2 subunits were previously shown to produce strong developmental

defects. Loss of either *laminin $\beta$ 1* (*grumpy*) or *laminin $\gamma$ 1* (*sleepy*) prevents proper formation of both the intersegmental blood vessels and the basement membrane adjacent to the notochord resulting in a failure in cell differentiation (Parsons et al., 2002). Mutation of the *laminina1* gene (*bashful*) causes a milder notochord phenotype that is enhanced by simultaneous disruption of *laminina4* (Pollard et al., 2006). Mutation of *laminina1* also disrupts the directed migration of hindbrain motor neurons but not axonal guidance (Paulus and Halloran, 2006). By contrast, mutations in *laminina2* (Hall et al., 2007) and *laminin $\beta$ 2* (Jacoby et al., 2009) genes produce dystrophic phenotypes characterized by degeneration of embryonic skeletal muscle (see below).

In the study by Hochgreb-Hagele et al. a novel *lamininb1a* mutant (*s804*) was identified during a forward genetic screen that utilized transgenic embryos with GFP-labeled endoderm (Field et al., 2003). This mutant has defects in the left-right asymmetric positioning of both liver and pancreas with the liver spanning the embryonic midline and the ventral pancreatic bud split into bilateral structures. Zebrafish Laminin 1 protein expression is observed in several basement membranes including those associated with the neural tube and somites at the 16 somite-stage (Hochgreb-Hagele et al., 2013). At later developmental stages (30 h post-fertilization), Laminin 1 localizes to the basement membrane of the dorsal lateral plate mesoderm and at the boundary of lateral plate mesoderm and endoderm of the gut (Hochgreb-Hagele et al., 2013; Yin et al., 2010). At earlier stages (7–8 somites), *lamininb1a* (*s804*) mutant embryos were shown to have a reduction in cilia length but not number in Kupffer's vesicle compared to controls. These mutant embryos also had slightly reduced fluid flow within Kupffer's vesicle and reduced expression of *T. brachyury homolog a* (formerly *no tail*) within the notochord near the region that gives rise to the digestive organs (Hochgreb-Hagele et al., 2013). Of note, *lamininb1a* does not affect *T. brachyury homolog a* expression in the area of heart formation nor is it required for left-right asymmetry of the heart. The migration of lateral plate mesodermal cells is also compromised in *lamininb1a* mutant embryos (Hochgreb-Hagele et al., 2013). It was shown that the epithelial-like character of lateral plate mesoderm was abnormal with altered *hand2* expression and protrusion into the gut. It was hypothesized by these authors that the lack of a basement membrane at the lateral plate mesoderm-gut boundary in *lamininb1a* mutant embryos disrupts the sequestration or action of secreted cell migration cues. Indeed, it is becoming increasingly recognized that during embryonic development the ECM regulates the accumulation and/or transport of signaling molecules including Nodal, Lefty, BMPs, and FGF (Belenkaya et al., 2004; Garcia-Garcia and Anderson, 2003; Marjoram and Wright, 2011; Oki et al., 2007). In one report, laminin  $\gamma$ 1 was shown to modulate cellular responsiveness to secreted BMP by affecting heparan sulfate proteoglycans present in the ECM (Dolez et al., 2011). Identification of a specific secreted signaling molecule regulated by laminin at the boundary between mesoderm and gut will be a key area of future research.

## ECM and skeletal muscle

Our knowledge of cell-ECM protein interactions and their importance for zebrafish cell and tissue function has increased with identification of embryos with null mutations in the dystrophin gene and realization that dystrophin-deficient embryos can be used to model human Duchenne muscular dystrophy. Thus zebrafish can be utilized to analyze the function of integrins and the collagen- and laminin-containing basement membrane required during muscle contraction (Hall et al., 2007; Jacoby et al., 2009; Kim and Ingham, 2009; Postel et al., 2008). For more details, see the following review on zebrafish models of muscular

dystrophies (Berger and Currie, 2012). In the skeletal basement membrane, laminin forms two important linkages with transmembrane proteins, one to the dystrophin-associated glycoprotein complex (composed of Laminin 2, dystroglycan, sarcoglycan, and dystrophin among other proteins) and the other to integrins of the subsarcolemmal focal adhesion complexes. These cytoskeletal linkages are important for transmitting the force generated by muscle contraction across the sarcolemma (or myolemma) and their disruption is thought to play a role in the pathogenesis of muscular dystrophy (Carmignac and Durbeej, 2012). Mutations in human laminin subunits contribute to a variety of diseases including cardiomyopathy and muscular dystrophy (Knoll et al., 2007; Mostacciolo et al., 1996). In zebrafish, *candyfloss* (*laminina2*) mutant has a degenerative muscle phenotype first detected at 36 h post-fertilization that is characterized by detachment and retraction of muscle fibers from the myoseptum adjacent to each somite (Hall et al., 2007). Mechanistically, Laminin  $\alpha$ 2 might function within the ECM to promote the stability of muscle attachments. Without Laminin  $\alpha$ 2, and upon mechanical load-induced stress, muscle fiber detachment occurs followed by apoptosis (Hall et al., 2007). Mutation of the zebrafish *softy* locus (*laminin $\beta$ 2*) produces a similar muscle detachment phenotype at 3 days post-fertilization (Jacoby et al., 2009). However, while homozygous *laminina2/candyfloss* mutant embryos often fail to survive, *laminin $\beta$ 2/softy* homozygotes survive to maturity (Jacoby et al., 2009). This is attributed to the formation of ectopic fiber terminations in *softy* mutants characterized as myoseptum-like structures able to support the attachment of fibers (Jacoby et al., 2009). Analysis of *candyfloss/softy* double mutant embryos demonstrated that *laminina2* is epistatic to *laminin $\beta$ 2* with double mutants having a phenotype most similar to *laminina2/candyfloss* (Jacoby et al., 2009). While *laminina2* and *laminin $\beta$ 2* co-localize in myotendinous junctions, identification of other laminins and ECM proteins expressed during muscle development is necessary to further our understanding of muscular dystrophy (Sztal et al., 2011). Collagens are major ECM proteins present in connective tissues and have been linked to muscular dystrophies (Charvet et al., 2013; Telfer et al., 2010).

In a recent paper by Charvet et al. (2013) Collagen XXII (a member of the fibrillar-associated collagens with interrupted triple helices subgroup) was shown to be required for the structure of the myotendinous junction, a major site of force transmission between muscle and tendons. Here, interactions between the actin cytoskeleton and ECM proteins of the basement membrane occur across the sarcolemma membrane of the striated muscle fiber cell. The zebrafish *collagen22a1* gene is expressed beginning at 22 h post-fertilization and is observed within the somites (Charvet et al., 2013). At later stages beyond 24 h post-fertilization *collagen22a1* localizes to the somite/myotome boundaries. Collagen XXII protein co-localizes with dystrophin at these boundaries and specifically marks the myotendinous junctions at day 5 post-fertilization and beyond to adulthood (Charvet et al., 2013). Morpholino-mediated knockdown of *collagen22a1* produces a phenotype reminiscent of dystrophic zebrafish with mutations in genes such as *laminina2* and *integrin $\alpha$ 7* (Charvet et al., 2013). These embryos exhibit impaired swimming movements and reduced muscle contraction with associated muscle fiber detachment. Together with the dystrophin-associated glycoprotein complex, Integrin  $\alpha$ 7 $\beta$ 1 is the major trans-sarcolemmal membrane protein complex linking muscle fibers to the ECM (Berger and Currie, 2012). Notably, a synergistic genetic relationship was identified between *collagen22a1* and *integrin $\alpha$ 7* supporting the notion that Collagen XXII interacts with Integrin  $\alpha$ 7 to stabilize muscle cell attachment to the ECM (Charvet et al., 2013). However, it is unclear whether Collagen XXII binds integrin directly or indirectly through another protein. These important

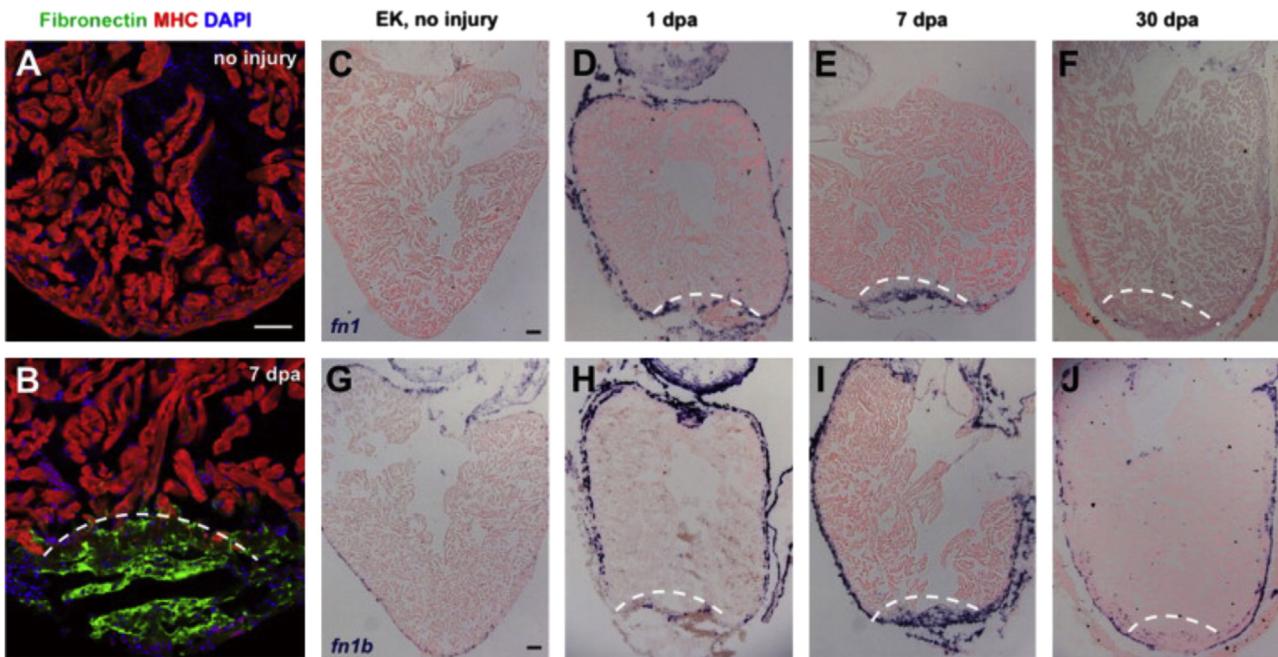
data now add Collagen XXII to the list of proteins required for stabilization of myotendinous junctions and the strengthening of muscle cell attachment to the ECM during contraction.

### ECM and heart development and regeneration

The zebrafish ECM, in particular fibronectin, is well known for its ability to influence embryonic heart development (Trinh and Stainier, 2004). In the early zebrafish embryo, cardiomyocyte precursors undergo collective migration between the endoderm and extra-embryonic yolk syncytial layer and require interactions with these cell populations and the surrounding ECM for proper migration and heart tube formation (Alexander et al., 1999; Arrington and Yost, 2009; Dickmeis et al., 2001; Kikuchi et al., 2000; Trinh and Stainier, 2004). The requirement for cell–ECM interactions during zebrafish heart formation is evidenced by the appearance of cardiac malformations after loss of either *fibronectin1/natter*, the proteoglycan *syndecan 2*, or the lipid mediator *sphingosine 1-phosphate* (Arrington and Yost, 2009; Kawahara et al., 2009; Kupperman et al., 2000; Matsui et al., 2007; Osborne et al., 2008; Sakaguchi et al., 2006; Trinh and Stainier, 2004). Fibronectin protein exhibits a dynamic expression pattern during cardiac progenitor migration initially being deposited in the lateral plate mesoderm and later, during cardiac cone formation, surrounding the migrating bilateral myocardial progenitors at the ventral midline between the endoderm and endocardial cell layers (Trinh and Stainier, 2004). The characteristic cardia bifida phenotype in *fibronectin1/natter* mutant embryos results from loss of cell polarity and epithelial integrity during myocardial progenitor migration (Trinh and Stainier, 2004). During embryogenesis *fibronectin1/natter* mutants fail to undergo mediolateral expansion of the anterior lateral plate mesoderm while posterior lateral plate mesoderm remains unaffected (Trinh and Stainier, 2004), suggesting that Fibronectin 1 has a broad role during morphogenesis of anterior lateral plate mesoderm in addition to regulation of myocardial progenitor cell migration.

Interestingly, the basic helix-loop-helix transcription factor Hand2 (discussed above for its role in determining organ laterality) was shown to regulate cardiac fusion by impacting the deposition of fibronectin (Garavito-Aguilar et al., 2010; Trinh et al., 2005; Yelon et al., 2000). In *hand2* mutant embryos, *fibronectin1* gene expression is increased while ectopic overexpression of *hand2* mRNA decreases fibronectin protein deposition (Garavito-Aguilar et al., 2010). Cell transplantation experiments demonstrated that *hand2* functions non-cell autonomously to promote cardiac fusion (Garavito-Aguilar et al., 2010). What is the role of fibronectin in this process? The data from Garavito-Aguilar et al. indicate that both excess and reduced fibronectin deposition causes a disruption in cardiomyocyte cell movement and thus cardiac fusion. This is reminiscent of the situation described above where *vangl2/trilobite* and *glypican4/knypek* mutant embryos exhibit opposite effects on fibronectin matrix assembly but have similar defects in gastrulation cell movements (Dohn et al., 2013). For both cardiomyocytes and gastrula cells, it is possible that altered fibronectin matrix assembly affects cell–ECM interactions necessary for proper cell migration, perhaps by altering integrin signaling and/or cell adhesiveness to the extracellular environment. Notably, both *hand2* and *vangl2/trilobite* mutant embryos have disrupted matrix metalloproteinase activity (Williams et al., 2012; Yin et al., 2010).

Unlike the adult mammalian heart, zebrafish maintain the ability to regenerate injured heart tissue as they mature (Poss et al., 2002). Microarray profiling of regenerating zebrafish and newt tissues identified genes associated with the ECM as the most enriched (Lien et al., 2006; Mercer et al., 2013). These included a matrix metalloproteinase, tissue inhibitors of metalloproteinases, and the extracellular glycoprotein Tenascin C. In another recent report, fibronectin was shown to be a required component of the molecular program necessary for zebrafish heart regeneration (Wang et al., 2013). Here, a proteomics approach was utilized to identify proteins whose expression increases after ablation of more than half of all cardiomyocytes. Fibronectin was found to be significantly upregulated in ventricular epicardial cells during



**Fig. 5.** Fibronectin is dynamically expressed during heart regeneration. (A and B) Fibronectin expression by immunostaining in uninjured (A) and 7 days post-ablation (B) ventricles, localizing to the injury site. MHC, Myosin heavy chain. (C–J) In situ hybridization for fibronectin1 and fibronectin1b in uninjured, 1, 7 and 30 days post-ablation ventricles. In each section, violet indicates a positive signal. Dashed line indicates approximate resection plane. Scale bars: 50 μm. Adapted from Wang et al. (2013) with permission.

the heart regeneration process as demonstrated by both immunofluorescence and *in situ* hybridization (Fig. 5). Notably, both zebrafish *fibronectin* genes (*1* and *1b*) were induced after heart injury as were *integrinb3* and *integrinaV* (Wang et al., 2013). When heart injury was performed on animals either transgenic for a heat-shock inducible dominant-negative fibronectin or homozygous for a null *fibronectin* mutation, regeneration was incomplete and included a reduction in muscle at the site of injury (Wang et al., 2013). It was further found that loss of fibronectin does not impact cardiomyocyte proliferation rather fibronectin appears to be required for accumulation of these cells at the site of regeneration (Wang et al., 2013). As suggested by these authors, fibronectin interactions with Integrin b3 might regulate cardiomyocyte cell migration to the injury site. Interestingly, a recent report has shown that *hand2* mRNA overexpression in the zebrafish embryo promotes cardiomyocyte proliferation and can promote cardiac regeneration in injured adult hearts (Schindler et al., 2014). Considering the data from Wang et al., the effect of Hand2 on heart regeneration should be independent of its influence on fibronectin deposition during embryonic cardiac fusion. It will be important to determine the role of both fibronectin deposition and matrix remodeling by metalloproteinases during heart regeneration.

## Concluding remarks

The zebrafish ECM is a complex macromolecular assembly of diverse proteins whose composition depends on localization, developmental stage, and role. The utility of zebrafish as a vertebrate model organism for analysis of ECM assembly and function has increased significantly with the advent of genetic and confocal imaging methods. In addition, as researchers probe more deeply the underlying molecular underpinnings of specific cellular and morphogenetic processes, ECM proteins are frequently being identified as critical players. The continued establishment of transgenic and fluorescent fusion protein tools is thus critical for the analysis of ECM protein dynamics in live embryos. There remains much to be learned regarding cell–ECM adhesive interactions and their contribution to cell behaviors and matrix assembly/remodeling. One important area for future zebrafish ECM research is the analysis of integrin signaling and the roles of outside-in versus inside-out signaling pathways. In addition, deciphering the role of ECM proteins at the cellular versus tissue level will likely be relevant to most fields of ECM research that utilize zebrafish. Lastly, the function of metalloproteases and their inhibitors has been and should continue to be a significant and fruitful area of ECM analysis in zebrafish. Given the large number of metalloprotease family members (adamalysins, astacins, matrix metalloproteinases, and serralysins) and their capacity to cleave many ECM and non-ECM substrates, investigation of these enzymes is likely to yield novel information for a variety of embryological and adult processes.

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