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Zebrafish integrin-linked kinase is required in skeletal muscles for strengthening the integrin–ECM adhesion complex

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ABSTRACT

Mechanical instability of skeletal muscle cells is the major cause of congenital muscular dystrophy. Here we show that the zebrafish *lost-contact* mutant, that lacks a functional integrin-linked kinase (*ilk*) gene, suffers from mechanical instability of skeletal muscle fibres. With genetic and morpholino knock-down experiments we demonstrate that: 1) laminin, itgα7, Ilk and β-parvin are all critical for mechanical stability in skeletal muscles. 2) Ilk acts redundantly with the dystrophin/dystroglycan adhesion complex in maintaining mechanical stability of skeletal muscles. 3) Ilk protein is recruited to the myotendinous junctions, which requires the ECM component laminin and the presence of itgα7 in the sarcolemma. 4) Ilk, unexpectedly, is dispensable for formation of the adhesion complex. Ilk, however, is required for strengthening the adhesion of the muscle fibre with the ECM and this activity requires the presence of a functional kinase domain in Ilk. 5) We identified a novel interaction between Ilk and the mechanical stretch sensor protein MLP. Thus, Ilk is an essential intracellular component downstream of laminin and itgα7, providing strengthening of skeletal muscle fibre adhesion with the ECM and therefore qualified as a novel candidate gene for congenital muscular dystrophy.

Introduction

Human muscular dystrophy is an inherited myogenic disorder characterized by progressive muscle wasting (Emery, 2002). The disease can be subdivided into several groups, e.g. Duchenne muscular dystrophy and congenital muscular dystrophy. Duchenne muscular dystrophy is caused by mutations in the sarcolemmal protein, dystrophin (Hoffman et al., 1987), which is part of the dystrophin glycoprotein complex. The dystrophin glycoprotein complex also contains dystroglycan, a transmembrane protein complex that interacts via its α-subunit with laminin-α2 (lama2) in the extracellular matrix (ECM) and with its β-subunit to dystrophin in the cytoplasm (Lisi and Cohn, 2007). Congenital muscular dystrophy is a heterogeneous group of autosomal recessively inherited muscular disorders characterized with hypotonia and weakness at birth or within the first few months of life (Emery, 2002). These maladies can be caused by mutations in either collagen VI, lama2, integrin-α7 (itgα7) or in genes encoding for proteins involved in the glycosylation of α-dystroglycan (Lisi and Cohn, 2007).

Collagen and laminin are structural proteins present in the ECM forming the basement membranes to which muscle fibres will attach. Laminins are cross-shaped, heterotrimeric, extracellular proteins consisting of one α, one β and one γ laminin chain. Besides lama2, also lama4 and lama5 are present in the basement membrane of skeletal muscles (Sorokin et al., 2000). *Lama2*-deficient mouse and zebrafish models have been generated that display severe muscular dystrophy phenotypes, which, in the case of the zebrafish *lama2* mutant, was characterized by muscle fibre detachments from the basement membrane without sarcolemmal rupture (Hall et al., 2007; Miyagoe et al., 1997).

At the plasma membrane, dystroglycan and itgα7β1 are the major receptor for laminins in skeletal muscles (von der Mark et al., 1991). Integrins are a family of heterodimeric transmembrane proteins composed of α and β subunits that mediate interactions between the cell and the ECM (Danen and Sonnenberg, 2003). As expected, *itgα7*-deficient mice display symptoms of progressive muscular dystrophy starting soon after birth (Mayer et al., 1997). This was attributed to an impaired function of the myotendinous junction (MTJ), which provides structural stability between the muscle fibre and the ECM. Although initially independent functions for itgα7 and the dystrophin glycoprotein complex in muscle integrity was suggested (Mayer et al., 1997), a later study demonstrated a genetic interaction between itgα7 and dystrophin pointing to complementary roles in maintaining mus-

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Abbreviations: ECM, extracellular matrix; ILK, integrin-linked kinase; MLP, muscle LIM protein; MTJ, myotendinous junction.

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cle integrity (Rooney et al., 2006). Although a role for the itgα7β1 complex in muscle cell integrity has been recognized for some time now, there is little understanding of the intracellular components interacting with the integrin receptor complex, required to provide mechanical stability to the skeletal muscle.

The focal adhesion protein Integrin-linked kinase (ILK) was first identified because of its ability to bind to the intracellular domain of the β1-integrin subunit (Hannigan et al., 1996). ILK also interacts with paxillin, PINCH and β-parvin, connecting it to the actin cytoskeleton (reviewed in (Legate et al., 2006). Previous studies showed that ILK protein can localize to costameres in cardiac cells (Chen et al., 2005) and the sarcolemma in skeletal muscle cells (Yamaji et al., 2001). Furthermore, we and others showed that ILK deficiencies in mouse and zebrafish results in reduced cardiac contractility and altered cardiomyocyte cell shapes resulting in a dilated cardiomyopathy (Bendig et al., 2006; Knoll et al., 2007; White et al., 2006). In addition we identified functionally relevant mutations in the integrin-binding domain of lama4 and in ILK of patients with a severe dilated cardiomyopathy (Knoll et al., 2007). A recent study demonstrates that loss of ILK in skeletal muscle fibres of 9-month-old mice leads to a muscular dystrophy phenotype (Gheyara et al., 2007). The mechanism, however, by which ILK regulates skeletal muscle fibre stability in this model, remains unclear. Earlier studies in *C. elegans* and Drosophila demonstrated an essential role for ILK in respectively body wall muscle and flight muscle function and integrity (Mackinnon et al., 2002; Zervas et al., 2001). Furthermore, these studies demonstrated that ILK does not require any kinase activity for its function suggesting that ILK is mainly acting as a scaffold protein. Several in vitro studies however, have suggested that ILK mediates signalling via its kinase activity downstream of integrin receptors (Dedhar, 2000; Delcommenne et al., 1998; Persad et al., 2001).

We previously isolated from a forward genetic screen the zebrafish *lost-contact* (*loc*) mutant, which harbours a premature stop codon mutation in the *ilk* gene, resulting in a complete loss of Ilk function (Knoll et al., 2007). Here we show that the *loc/ilk* mutants are characterized by consistent skeletal muscle fibre detachments along the zebrafish body. Ilk protein is recruited to the MTJ, the sites at the somite boundaries where skeletal muscle fibres attach to the basement membrane. This recruitment of ILK protein requires the presence of lama4 and itgα7. Mechanistically, our data suggests that Ilk is dispensable for the assembly of the MTJ complex but is required for strengthening the adhesion of the muscle fibres with the basement membrane when mechanical forces increase, which is dependent on the presence of a functional kinase domain. Furthermore we show that Ilk act redundantly with the dystrophin glycoprotein complex during skeletal muscle fibre adhesion, highlighting the suitability of the zebrafish model to uncover the molecular mechanisms linking Ilk activity and congenital muscular dystrophy.

Results

Zebrafish Ilk is required for skeletal muscle integrity

Although zebrafish *loc/ilk* mutant embryos initially exhibit normal swimming behaviour, they become progressively paralysed by 4 days post fertilization (dpf), which was accompanied by retraction of skeletal muscle fibres at various locations in the trunk and the tail (Figs. 1A,B). Despite this, muscle differentiation markers such as *myoD* and *myoC*, were expressed normally in *loc/ilk* mutants (Supplementary Fig. 1). To investigate the integrity of the actin cytoskeleton in the skeletal muscle fibres, we used phalloidin-TRITC to stain filamentous actin in embryos at 4.5 dpf. In 50% of *loc/ilk* mutant embryos (n=69), we observed the retraction of the actin cytoskeleton in a proportion of skeletal muscle fibres (Figs. 1C,D). Using an antibody recognizing sarcomeric α-actinin we observed a similar retraction of the cytoskeleton in *loc/ilk* mutant embryos (Figs. 1E,F). The progressive paralysis of the *loc/ilk* mutant embryos suggests that the muscle detachments are induced by mechanical stress. Indeed, raising Ilk deficient embryos under anesthetizing conditions (see Materials and methods) rescues the muscle detachments completely (*ilk* MO injected embryos, 77% muscle detachments (n=70; *ilk* MO injected embryos raised under anesthetizing conditions, 0% muscle detachments (n=65)).

The late defects in muscle fibre attachments to the ECM observed in the *loc/ilk* mutants (day 4.5) compared to the much earlier defects reported for the *lama2* mutant *candyfloss* (day 1.5) (Hall et al., 2007),

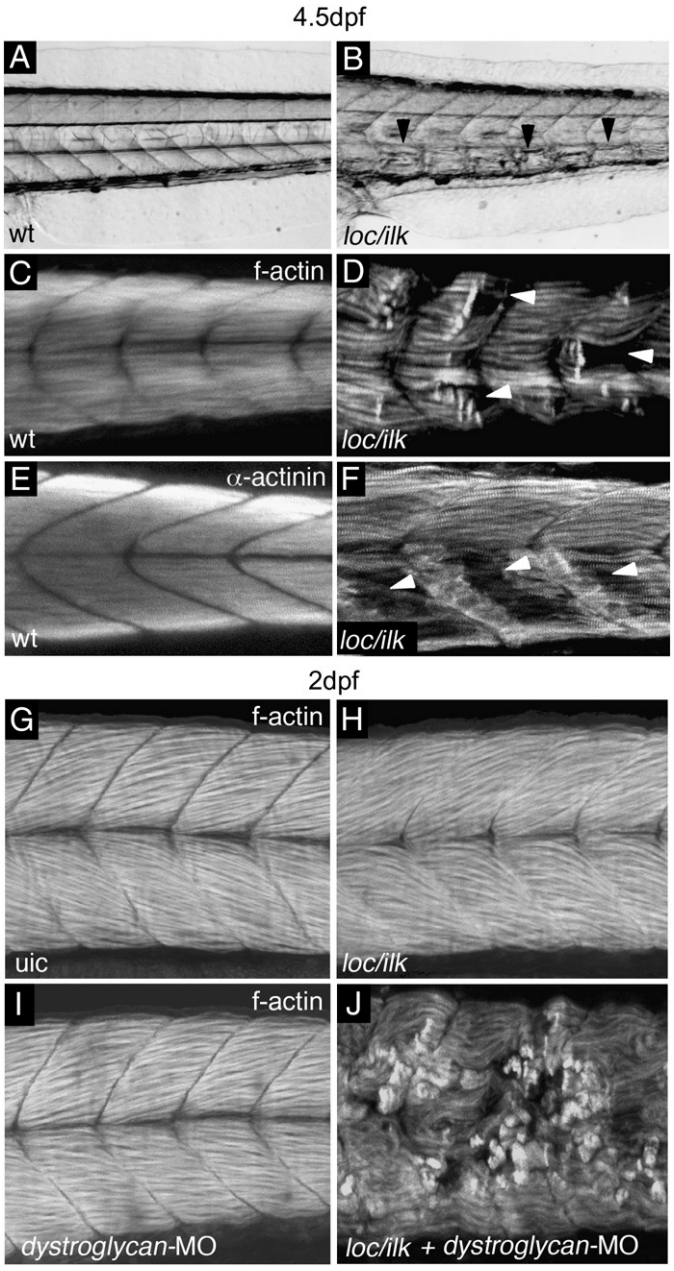

Fig. 1. Skeletal muscle detachments in *loc/ilk* mutant embryos. (A,B) Transmitted light images of tail regions in wt (A) and *loc/ilk* mutant embryos (B) at 4.5 dpf. Muscle fibre retractions are indicated with arrowheads. (C,D) Phalloidin staining and confocal images of wt sibling embryo (C) and of *loc/ilk* mutant embryo (D) at 4.5 dpf. Muscle fibre detachments are apparent in 50% of the *loc/ilk* mutant embryos (arrowheads). (E,F) α-actinin antibody staining of wt sibling (E) and *loc/ilk* mutant embryo (F) at 4.5 dpf. Regions of muscle fibre detachments are indicated by arrowheads. (G–J) Phalloidin staining and confocal images of uninjected control embryo (G), uninjected *loc/ilk* mutant embryo (H), *dystroglycan* MO injected wt embryo (I) and a *dystroglycan* MO injected *loc/ilk* mutant embryo (J). Tail regions of 2 dpf embryos are shown.

suggest that parallel pathways play redundant functions in adhesion of the muscle fibre to the ECM. Previous studies have shown that both the laminin–integrin interaction as well as the laminin–dystroglycan interaction are required to anchor the sarcolemma to the ECM and that both pathways act redundantly (reviewed in Jimenez-Mallebrera et al., 2005). To determine whether Ilk and dystroglycan have redundant functions we injected *dystroglycan* MOs in wild type and *loc/ilk* mutant embryos. Injection of a *dystroglycan* MO in wild type embryos resulted in skeletal muscle detachment starting at day 4 (Supplementary Fig. 2). In addition, we observed much earlier and severe muscle fibre detachments in embryos deficient for both dystroglycan and Ilk, which were not observed in single *dystroglycan* MO knock-down embryos or *loc/ilk* mutant embryos (Figs. 1G–J). In embryos deficient for both dystroglycan and Ilk, muscle fibre detachments were already apparent at day 2 (12/12) (Figs. 1J). These results demonstrate a cooperative function of Ilk and the dystrophin glycoprotein complex in muscle fibre attachment.

Ilk localizes to the myotendinous junctions

Ilk mRNA is abundantly present in the somites of the developing zebrafish embryo (Figs. 2A,B). To study how the observed phenotypes correlate with the presence of the Ilk protein in skeletal muscles, we generated a GFP–Ilk fusion construct. Injection of synthetic *ilk–gfp* mRNA in *loc/ilk* mutant embryos resulted in an efficient rescue of the *loc/ilk* mutant phenotypes (Table 1), demonstrating that the GFP–Ilk fusion protein is fully functional. Upon injection of *ilk–gfp* mRNA we observed GFP–Ilk in the cytoplasm of the myoblasts at the 10-somite stage (15 hpf) (Figs. 2C,D), which could be easily distinguished from a membrane–GFP protein localization (Figs. 2E,F). At 24 hpf, the localization of the GFP–Ilk protein changes from cytoplasmic into a very specific accumulation of the GFP–Ilk fusion protein at the somite boundaries (Figs. 2G,H). Using an Ilk-specific antibody we confirmed the localization of Ilk at the somite boundary (Supplementary Fig. 3). Ilk localization occurs after somite boundaries are established (Holley, 2006) and we did not observe any visual defects in somite boundary formation in the *loc/ilk* mutants (data not shown), suggesting that zygotic Ilk is not required for somite boundary formation. Laminin deposition occurs at the somite boundaries after boundary formation and at the time of Ilk recruitment from the cytoplasm to the somite

Table 1
Rescue of the skeletal muscle detachments upon injection of various concentrations of wt and mutant *ilk* RNA

Injection	*n*	Wildtype embryos[a] (%)	Embryos with muscle detachments[a] (%)
Uninjected *loc/ilk*	138	74	26
gfp–ilk (20 pg) in *loc/ilk*	69	90	10
gfp–ilk (80 pg) in *loc/ilk*	68	95	5
gfp–ilk (160 pg) in *loc/ilk*	47	96	4
gfp–ilk–K220M (80 pg) in *loc/ilk*	63	70	30
gfp–ilk–K220M (160 pg) in *loc/ilk*	66	73	27
gfp–ilk–K220A (160 pg) in *loc/ilk*	60	74	26

[a] Visualized by phalloidin staining.

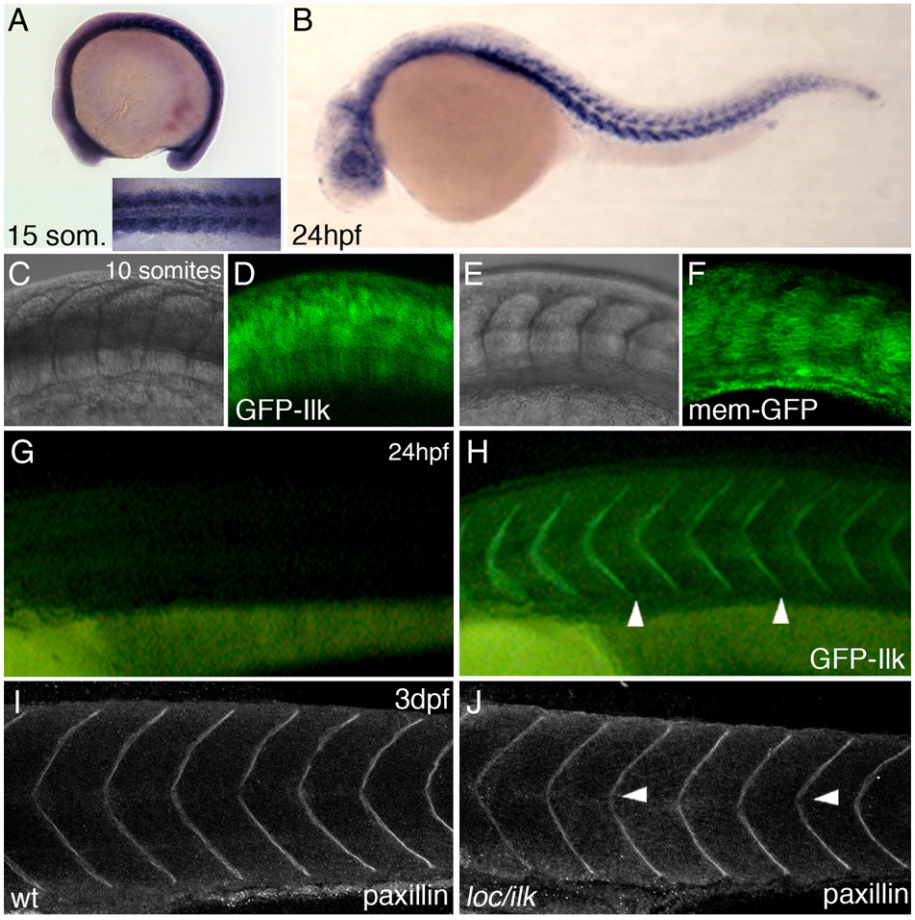

Fig. 2. Ilk–GFP cellular localization. (A,B) Whole mount ISH with dig-labelled antisense *ilk* mRNA at 15-somite stage (A and inset) and 24 hpf (B). Wt embryos injected with synthetic mRNA encoding GFP–ILK (C and D) or memGFP (E and F). Images were taken at the 10-somite stage (15 hpf) at the region of the forming somites. The GFP–Ilk protein shows a predominant cytoplasmic localization at this stage. (G,H) Uninjected wt embryo (G) and a wt embryo injected with synthetic mRNA encoding GFP–Ilk (H) at 24 hpf. GFP–Ilk protein localization at the somite boundaries is indicated by arrowheads. (I,J) Anti-paxillin antibody staining and confocal images of a wt embryo (I) or *loc/ilk* mutant embryo (J) at 3 dpf.

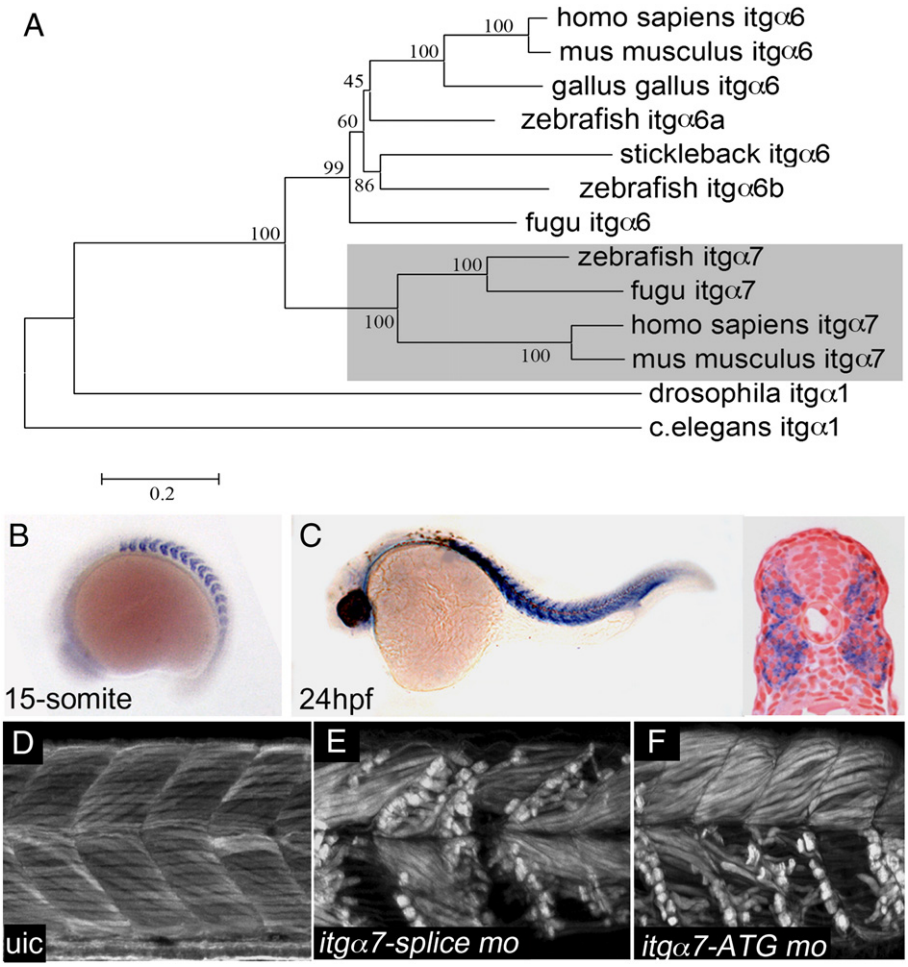

Fig. 3. Knock-down of zebrafish *itgα7* results in muscle fibre detachments. (A) Phylogenetic tree of the *itgα6* and *itgα7* sequence of several species. *itgα1* of *C. elegans* and Drosophila was used as an outer group. (B,C) Whole mount ISH with dig-labelled antisense *itgα7* mRNA at 15-somite stage (B) and 24 hpf with cross section (C). (D–F) Compared to wt embryos at 4 dpf (D) injections of *itgα7*-splice MO (E) or *itgα7*-ATG MO (F) result in muscle fibre detachments.

boundary (Crawford et al., 2003). Laminins at the somite boundary form the basement membrane to which the skeletal muscle fibres are attached by their MTJs (Hall et al., 2007). Paxillin and vinculin are focal adhesion proteins present in MTJs. Their localization to the MTJ is not dependent on Ilk, since they localize normally in *loc/ilk* mutant embryos (Figs. 2I and J and data not shown). Together these data

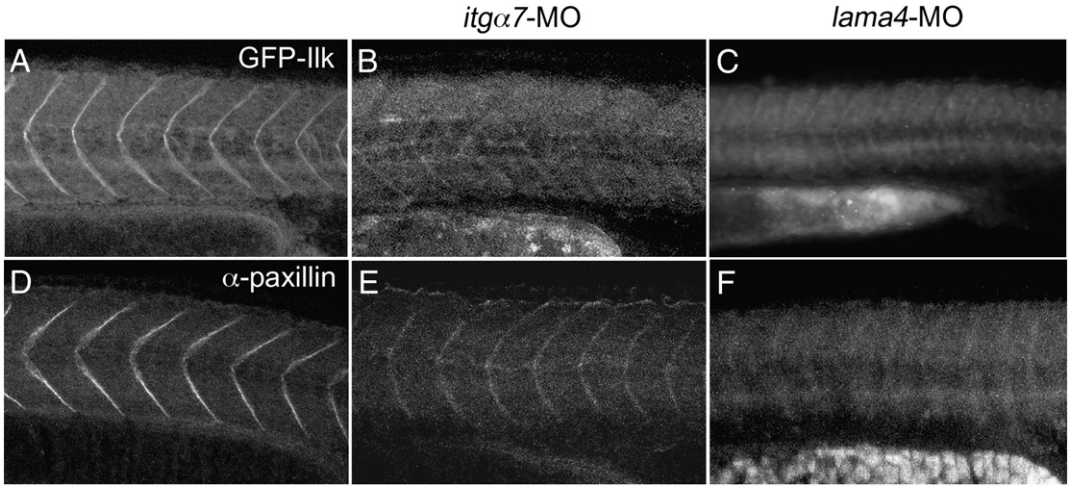

Fig. 4. GFP–Ilk and paxillin protein localizations in *itgα7* and lama4 morphant embryos. (A–C) Injection of *gfp–ilk* mRNA in wt embryos results in GFP–Ilk protein localization to the MTJ at the somite boundaries in 24 hpf embryos (A). Reduced GFP–Ilk protein localization in embryos co-injected with an *itgα7*–ATG MO (B) or *lama4*–ATG MO (C). (D–F) Anti-paxillin antibody staining on wt embryos (D). Reduced paxillin localization to the MTJ at the somite boundary in *itgα7*–ATG MO (E) or *lama4*–ATG MO (F) injected embryos at 24 hpf.

demonstrate that Ilk is recruited from the cytoplasm to sarcolemma at the MTJ during early stages of muscle fibre formation but is not required for the assembly of the MTJ protein complex.

Ilk recruitment to the MTJ requires lama4 and the itgα7 receptor

To address the question whether integrin receptors are required for the recruitment of Ilk to the MTJ, we first identified a zebrafish skeletal muscle specific *itgα7* gene (Figs. 3A–C). To perform loss of function analysis on the zebrafish skeletal muscle-specific itgα7 we designed an *itgα7*-splice MO, which prevented the splicing of intron 1 upon injection of the MO at the 1-cell stage (Supplementary Figs. 4A, B). Injection of the *itgα7* MO resulted in normal embryos that were indistinguishable from uninjected control embryos during the first 2 days of development. Starting on day 3, *itgα7* morphant embryos became paralysed with an accompanying retraction of the actin cytoskeleton in skeletal muscle fibres (32 out of 44 embryos, Figs. 3D, E). Similar results were obtained with a second independent *itgα7* ATG MO (Fig. 3F). To address whether a reduction in itgα7 levels would affect localization of Ilk to the MTJs, the *itgα7* MO was co-injected with the *ilk–gfp* mRNA. While localization of ILK to the MTJ was observed in control embryos (Fig. 4A), a cytoplasmic localization of Ilk with reduced Ilk protein at the MTJ was observed in the *itgα7* MO knock-down embryos (Fig. 4B). Additionally, we observed a strong reduction in paxillin localization to the MTJs in *itgα7* embryos (Figs. 4D,E). Since lama4 is present in the basement membrane of skeletal muscles and we previously showed a strong genetic interaction between lama4 and Ilk (Knoll et al., 2007), we studied Ilk localization

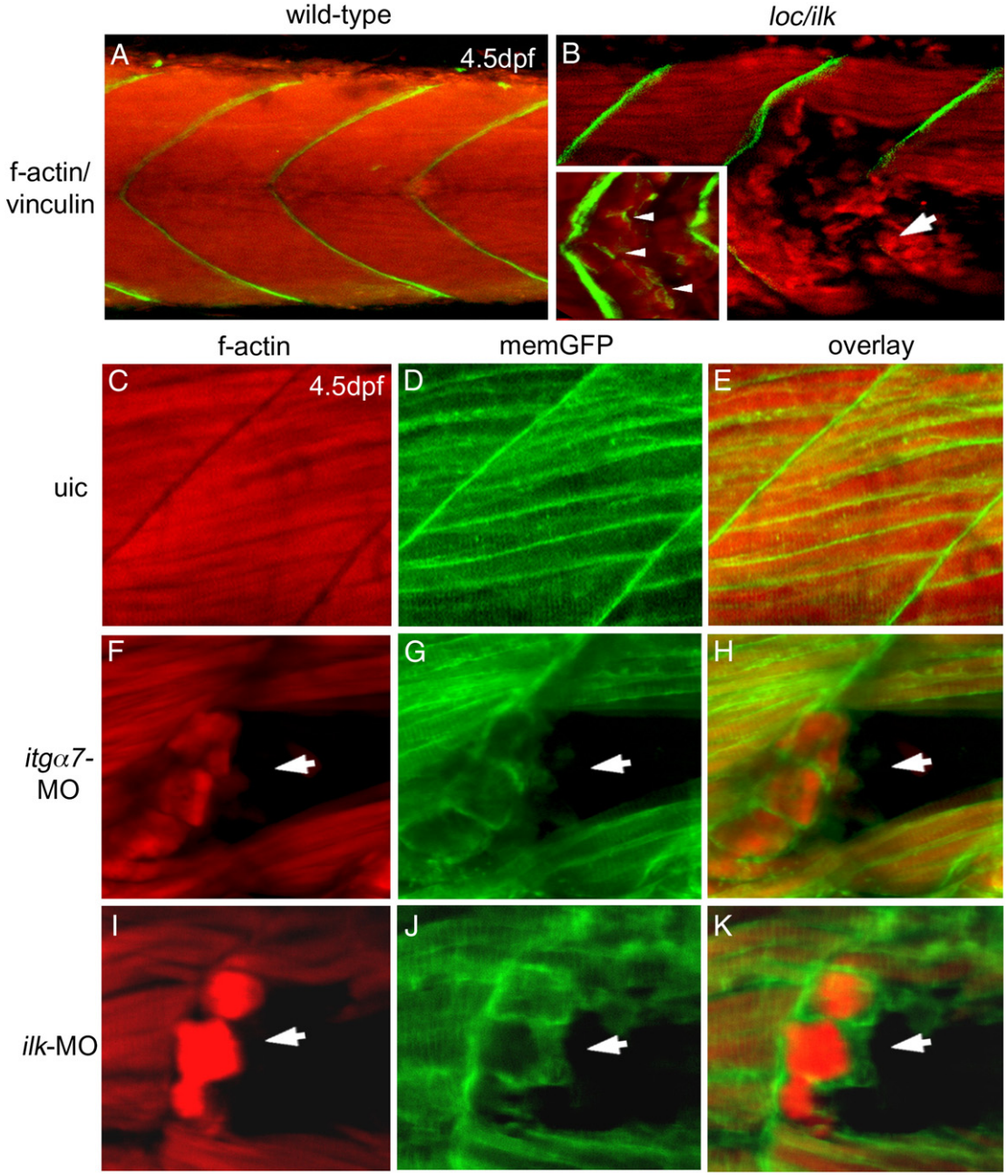

Fig. 5. Plasma membrane retractions of skeletal muscle fibres. (A,B) Double labelling with anti-vinculin antibody (green) and phalloidin (red) in wt sibling embryos (A) and *loc/ilk* mutant embryos (B) at 4.5 dpf when muscle fibres detach (arrow). Inset shows vinculin staining at the tip of the f-actin filaments (inset in panel B; white arrowheads). (C–K) Double staining for phalloidin (red) and memGFP (green) as separate images or as an overlay taken from a wt embryo (C–E), an *itgα7* MO injected embryo (F–H) and an *ilk* MO injected embryo (I–K) in a region where muscle fibres detached (arrow) at 4.5 dpf.

in *lama4* MO knock-down embryos. We observed a loss of Ilk at the MTJ in *lama4* MO knock-down embryos (Fig. 4C). Also paxillin is no longer recruited to the MTJs in *lama4* MO knock-down embryos (Fig. 4F). Together these results demonstrate that Ilk recruitment to the MTJs requires the presence of itgα7 at the sarcolemma and lama4 in the basement membrane.

Ilk is required for strengthening of the MTJ

To address whether it is exclusively the actin cytoskeleton retracting in *loc/ilk* mutant embryos or whether other components of the cell adhesion complex also retract, we stained 4.5-day-old *loc/ilk* mutant zebrafish larvae with an antibody recognizing vinculin. Before muscle retractions are visible in *loc/ilk* mutant embryos, vinculin localizes normally to the MTJ. We only observed a loss of vinculin from the myotendinous junctions at regions where the actin filaments had retracted (Figs. 5A,B). Higher magnification of the retracting fibre revealed that the vinculin remained attached to the f-actin fibres (inset in Fig. 5B). This suggested that Ilk is involved in more than simply anchoring the actin cytoskeleton with the integrin receptors in the membrane. It is conceivable that Ilk may be required for extracellular adhesion of the muscle fibre with the ECM by, for example, modifying integrin adhesion to the ECM. To investigate this hypothesis we made use of a transgenic line expressing membranous GFP in all tissues including the sarcolemma of muscle fibres. In wt embryos, the f-actin filaments of skeletal muscle cells are properly surrounded by the sarcolemma, visualized by the memGFP (Figs. 5C–E). In *itgα7* MO knock-down embryos, however, we observed a complete retraction of both the actin cytoskeleton and the sarcolemma of the affected muscle fibres (Figs. 5F–H and Supplementary Fig. 5A). Interestingly, upon loss of Ilk we also observed a similar and complete retraction of the sarcolemma from the ECM (Figs. 5I–K and Supplementary Fig. 5B), demonstrating a requirement for Ilk in strengthening adhesion of the MTJ complex with the ECM.

Ilk K220M cannot rescue loc/ilk mutant phenotypes

Although in vitro studies have suggested that ILK can phosphorylate GSK-3β and Akt/PKB (Dedhar, 2000; Delcommenne et al., 1998; Persad et al., 2000), rescue experiments in Drosophila and *C. elegans* demonstrated that ILK in vivo kinase activity is dispensable for proper development in these organisms (Mackinnon et al., 2002; Zervas et al., 2001). Therefore, we examined the phosphorylation of Akt/PKB and GSK-3β in *loc/ilk* mutant embryos. We observed, however, no difference in the phosphorylation levels of Akt/PKB on ser 473 and GSK-3β on ser 9 in *loc/ilk* mutant embryos compared to their wt siblings (Fig. 6A). Next we addressed the question whether ILK variants, which have been associated with reduced in vitro kinase activity, are still able to rescue the *loc/ilk* phenotypes in vivo. We replaced glutamic acid 359, a conserved residue located in sub domain VIII (Hanks and Hunter, 1995), to a lysine (E359K). We also mutated Lys 220, which is located in the ATP binding site of the kinase subdomain II. This lysine residue is conserved among all other serine/threonine kinases and is essential for kinase activity (Hanks and Hunter, 1995; Snyder et al., 1985). To investigate in vivo functionality we introduced these point mutations in the GFP–Ilk fusion protein and expressed the protein in zebrafish embryos. Introduction of the E359K, K220M and K220A missense mutations had no effect on protein levels (Fig. 6B). Furthermore, we observed that these mutant GFP–Ilk proteins all localized to the MTJ comparable to the wt form of the protein (Figs. 6C–F). To address whether the E359K, K220M and the K220A variants are functional in vivo, we expressed the mutant Ilk proteins in *loc/ilk* mutant embryos. *Loc/ilk* mutant embryos were injected with synthetic mRNAs encoding the different Ilk variants and scored for phenotypic rescues, including skeletal muscle detachments

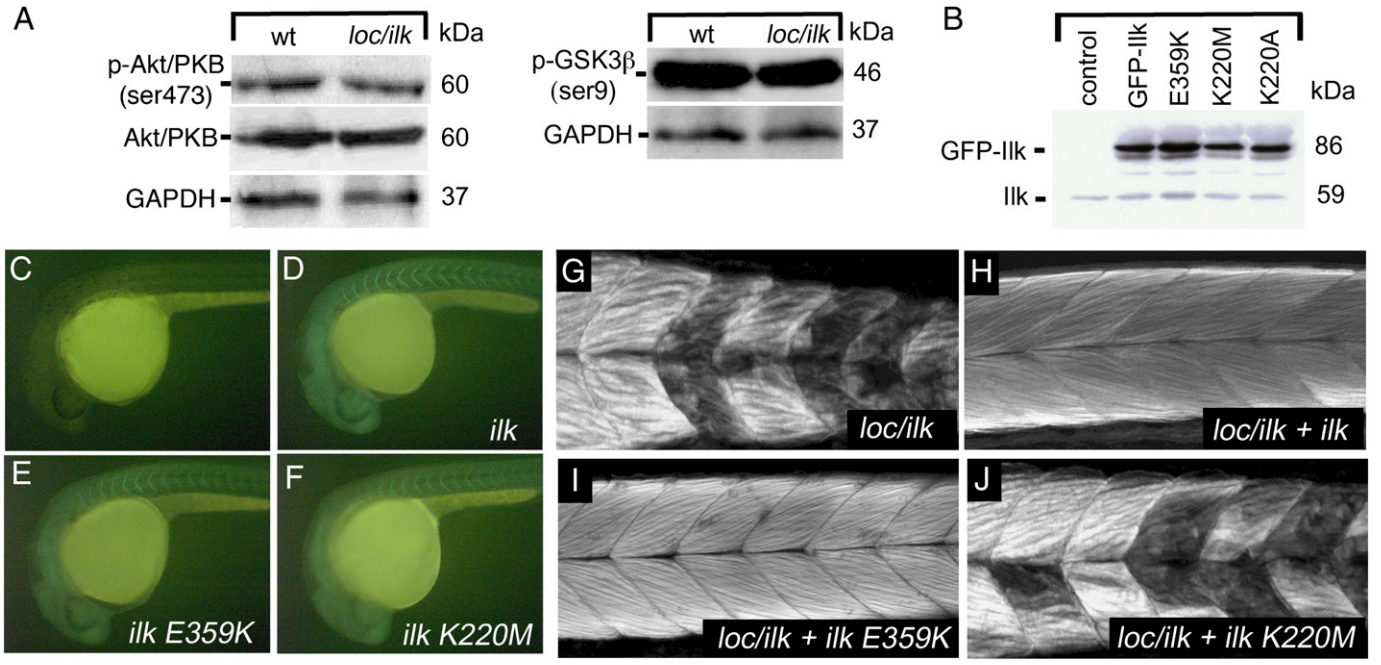

Fig. 6. Lysine 220 is essential for in vivo Ilk function. (A) Phosphorylation of Akt/PKB at ser 473 and GSK-3β at ser 9 is not altered in *loc/ilk* mutant embryos. Whole embryo lysates of wt sibling embryos and *loc/ilk* mutant embryos at 3 dpf show identical amounts of phosphorylated Akt/PKB and GSK-3β by Western blot analysis using an anti-phospho Akt/PKB ser 473 or an anti-phospho GSK-3β ser 9 antibody. Western blotting with a non-phospho specific Akt/PKB antibody using the same lysates shows identical levels of Akt/PKB protein present in both samples. Anti-GAPDH was used as an additional loading control. (B) Relative expression levels of Ilk in embryonic extracts of uninjected (control) or *gfp–ilk*, *gfp–ilkK220M* and *gfp–ilkK220A* mRNA injected embryos. (C–F) An uninjected control embryo with a weak and non-specific autofluorescence (C). Embryos injected with synthetic mRNA encoding wt GFP–Ilk (D), GFP–Ilk E359K (E) or GFP–Ilk K220M (F) all show a normal localization of the GFP–Ilk protein to the MTJs located at the somite boundary. (G–J) Muscle detachments are obvious in *loc/ilk* mutant embryos (G). The phenotypes including muscle detachments of *loc/ilk* mutant embryos were rescued by injection with synthetic mRNA encoding wt Ilk (H) or Ilk E359K (I). No rescue was observed in *loc/ilk* mutant embryos injected with mRNA encoding Ilk K220M (J).

(Figs. 6G–J and Table 1). While injecting wt *ilk* and *ilk E359K* mRNA rescued the *loc/ilk* mutant phenotypes including the skeletal muscle detachments, injection of 4× the concentration of either the *ilk K220M* or *ilk K220A* mRNA was ineffective (Table 1). In conclusion, these results demonstrate that lysine 220 of zebrafish Ilk is required for its in vivo function during skeletal muscle adhesion.

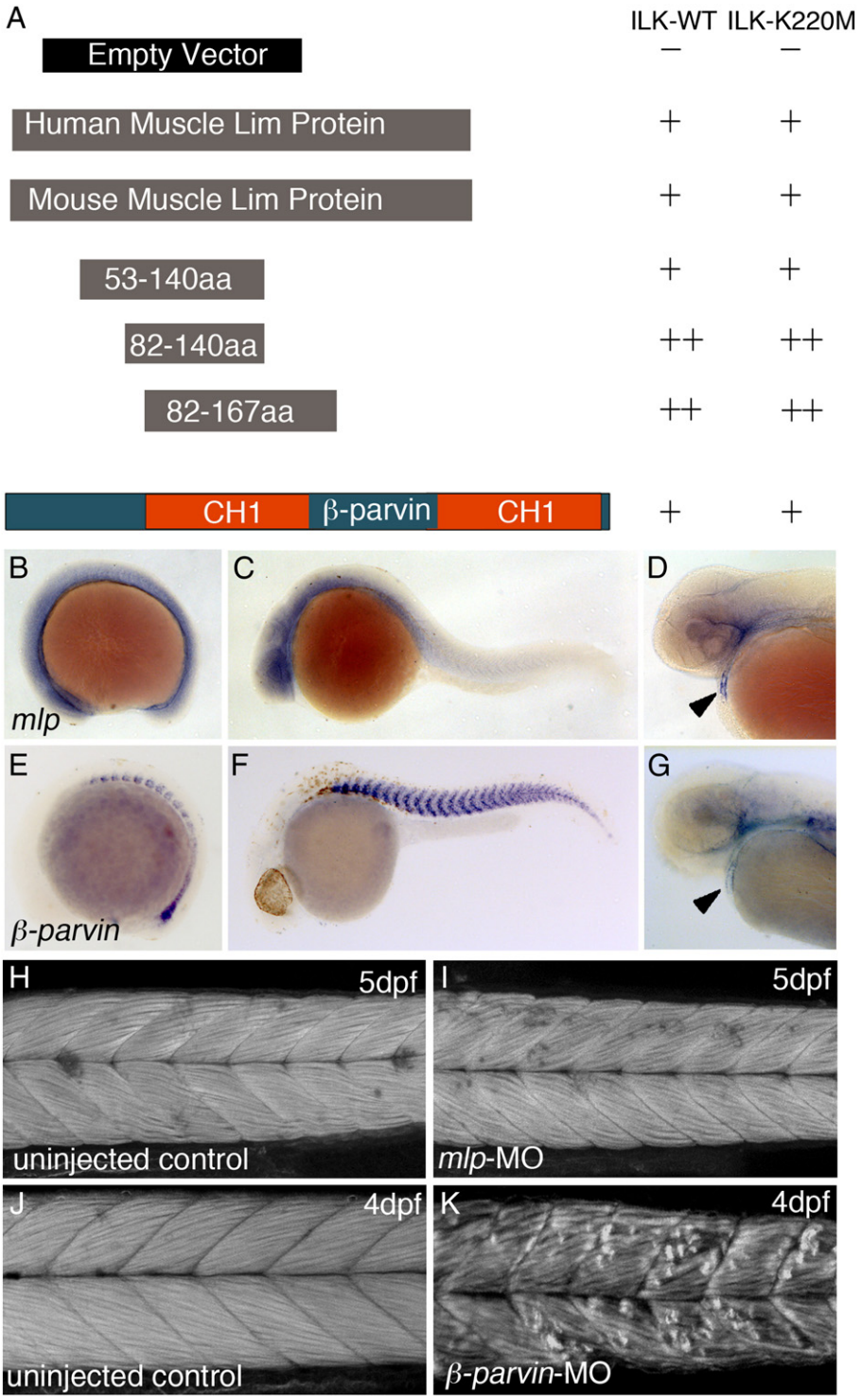

Fig. 7. In vitro interaction of ILK with MLP and β-parvin. (A) Yeast two hybrid analysis of ILK interaction with Muscle lim protein (MLP) and *β-parvin*: Schematic diagram illustrates the recombinant expressed MLP and the full-length β-parvin. Below the human MLP are the full-length and the deletion mutants of mouse origin that were generated to assess the interaction between ILK and MLP. Schematic representations are as follows Control empty plasmid pGBKT7, hMLP (full-length), mMLP (full-length) and various regions of mMLP, and full-length β-parvin. Quantification of the interaction was based on beta-galactosidase activity with growth on medium (+) and stringent (++) selection media (see also Materials and methods section and Supplementary Table 1). (B–D) In situ hybridization with dig-labelled antisense *mlp* probe at 15 somite stage (B), 24 hpf (C) and 48 hpf (D). Arrowhead marks *mlp* mRNA expression in the heart. (E–G) In situ hybridization with dig-labelled antisense *β-parvin* probe at 12 somite stage (E), 30 hpf (F) and 48 hpf. Arrowheads marks *β-parvin* expression in the heart (G). (H,I) Phalloidin staining of f-actin in 5 dpf wt non-injected embryo (H) compared to *mlp/crp3* MO injected embryo (I). No muscle fibre defects were observed in *mlp/crp3* MO injected embryos. (J,K) Phalloidin staining of f-actin in 4 dpf wt non-injected embryos (J) compared to *β-parvin*-MO injected embryos (K). Severe muscle fibre detachments were observed in *β-parvin* morphants.

Ilk interacts with β-parvin and MLP

Muscle cells can sense their mechanical load and upon mechanical stress they can respond with changes in gene expression. One of the key regulators for the stretch sense response in cardiac muscles is the sarcomeric muscle LIM protein CRP3 (MLP/CRP3) (Knöll et al., 2002). It is believed that upon the stretch sense response MLP/CRP3 is able to activate the pro-hypertrophic gene program, thereby stabilizing striated muscle cells during mechanical stress (reviewed in (Ehler and Perriard, 2000). Since MLP/CRP3 and Ilk can both be located at the Z-disc in cardiomyocytes and both have been suggested to play a role in the cardiac mechanical stretch sensor (Bendig et al., 2006; Knöll et al., 2002), we analyzed whether both proteins can interact in vitro. By yeast-two hybrid analysis we indeed observed a strong interaction between the Ilk and MLP/CRP3 proteins. We found that human wildtype ILK as well as K220M–ILK interacts with human MLP/CRP3 and mouse MLP/CRP3 (Fig. 7A). Using deletion constructs, we observed that ILK interacts with MLP/CRP3 amino acids 82–140. This interaction was found while the yeast colonies were grown on stringent selection plates, pointing to strong interaction of both proteins (Supplementary Table 2). We next identified a zebrafish homologue of the mouse MLP/CRP3 gene (ENSDART00000053404). We found that in zebrafish *mlp/crp3* is expressed ubiquitously including the somites at early stages and more specifically in the heart at 48 hpf (Figs. 7B–D). To address whether MLP/CRP3 is required to maintain skeletal muscle integrity we injected a splice-MO targeting the boundary of exon 3 to intron 3 in *mlp/crp3*. Injection of the *mlp/crp3* splice MO efficiently blocked splicing of intron 3, introducing a premature stop codon upstream of the second LIM domain (Supplementary Fig. 4). Embryos injected with the *mlp/crp3* MO and stained with phalloidin, did however not show any skeletal muscle detachments (Figs. 7H,I).

β-parvin/affixin is also located at the Z-disc and binds to Ilk in vitro (Yamaji et al., 2001). The reported interaction between Ilk and β-parvin is of particular interest since the phosphorylation of β-parvin by Ilk is required for β-parvin binding to α-actinin in vitro (Yamaji et al., 2004). Using the yeast two-hybrid system, we confirmed the interaction of β-parvin with Ilk. Zebrafish *β-parvin* is strongly expressed in the somites and only weakly in the heart (Figs. 7E–G). To address whether β-parvin is important for maintaining skeletal muscle identity we injected a MO targeting *β-parvin*. The *β-parvin* morphant embryos look normal up to day 4. Starting at 4.5 dpf the *β-parvin* morphant embryos become paralyzed, which is accompanied by severe muscle fibre detachments (Figs. 7J,K). Together these results demonstrate the interaction of Ilk with both MLP and β-parvin. While MLP seems to be dispensable for skeletal muscle adhesion, β-parvin is essential for this process.

Discussion

Although the role of integrins in the formation of adhesion complexes at the MTJ has been recognized for a long time, there is only little understanding of the intracellular components of this specialized adhesion complex in skeletal muscles. A very recent report demonstrated that Ilk deficiency in skeletal muscles results in muscular dystrophy phenotypes in mice resembling those observed in case of itgα7 deficiencies (Gheyara et al., 2007). It remained, however, unclear how ILK can be placed in the previously identified laminin, integrin and dystroglycan pathways and whether vertebrate ILK has similar functions in skeletal muscles as ILK has in muscle fibres of invertebrates. Here we show that zebrafish *loc/ilk* mutants, with a loss of function allele in the *ilk* gene, develop with severe skeletal muscle detachments similar to what was observed in *lama2* (Hall et al., 2007) or *lama4* and *itgα7*-deficient embryos (this study). The muscle detachments in *loc/ilk* mutants appear however rather late when compared to those found in *lama2*-deficient embryos due to a redundancy of the integrin–Ilk pathway with the dystrophin/dystroglycan complex acting in parallel. In addition, we have demonstrated that Ilk is recruited to the MTJ during early embryonic development, which requires laminins in the ECM and the presence of itgα7 receptor in the sarcolemma. Unexpectedly, Ilk is dispensable for recruiting other components of the adhesion complex, such as paxillin and vinculin, but required for strengthening the adhesion of the MTJ with the ECM. For this, the K220 residue within the Ilk kinase domain is required. Finally we identified a novel interaction between Ilk and the mechanical stretch sensor protein MLP and demonstrated that β-parvin, a known Ilk interacting protein, is required for muscle adhesion similar to Ilk.

Ilk recruitment to MTJs

Initially Ilk protein resides in the cytoplasm of myoblasts to become recruited to the somite boundaries at the time when myoblasts elongate and form the MTJ. Vertebrate Ilk can interact with the cytoplasmic domain of itgβ1 (Hannigan et al., 1996). In *C. elegans*, itgβ1 is required for the recruitment of Ilk to the adhesion sites in body wall muscles (Mackinnon et al., 2002). Surprisingly in Drosophila, itgβ1 does not interact with Ilk and Ilk localization to adhesion sites is not affected in *itgβ1* mutants (Zervas et al., 2001). Since we anticipated that analyzing the role of itgβ1 in skeletal muscle attachment would be difficult due to an early requirement for itgβ1 in various processes (Fassler and Meyer, 1995; Stephens et al., 1995), we instead studied itgα7, the major integrin alpha subunit in skeletal muscles. Here we now demonstrated that itgα7 is also required for Ilk recruitment to the MTJ. Surprisingly, although *itgα7* is abundantly expressed in myoblasts at early stages (Fig. 3), Ilk localization is predominantly cytoplasmic (Fig. 2). This is suggestive that Ilk may only interact with itgα7β1 in the plasma membrane following activation of integrin receptors by binding to the ECM. Itgα7β1 has been characterized as a laminin receptor and is activated upon interaction with laminins in the basement membrane (Givant-Horwitz et al., 2005) and references therein). Indeed, the redistribution of Ilk protein in the myoblasts coincides with the deposition of laminin at the somite boundaries (Crawford et al., 2003) and laminin in the ECM is required for Ilk recruitment to the MTJ (Fig. 4). Ilk recruitment to cell adhesion sites by laminins might also occur in other cell types, since we previously observed a strong genetic interaction between zebrafish Lama4 and Ilk in endothelial cells and cardiomyocytes (Knoll et al., 2007).

Ilk and muscle fibre adhesion

Our current study in skeletal muscle demonstrates an essential role for Ilk in adhesion of the muscle fibre with the basement membrane. Others have suggested that Ilk predominantly acts as an adaptor protein physically linking integrin receptors with the actin cytoskeleton. These conclusions are supported by experiments in *C. elegans* and Drosophila. In *C. elegans*, PAT4/ILK localizes to dense bodies in body wall muscles and is required for the recruitment of other components of the adhesion complex such as vinculin and UNC-89 (Mackinnon et al., 2002). From our data presented here, however, we conclude that in zebrafish, Ilk is required to strengthen adhesion between the muscle fibre and the basement membrane and that Ilk is dispensable for building the MTJ adhesion complex. We made several observations to support our conclusion. First, although Ilk is recruited to the MTJ, it is not required for building the adhesion complex since paxillin and vinculin localize normally to the MTJ in *loc/ilk* mutants. Second, muscle fibre detachments were observed rather late (day 4.5) long after MTJs had formed (day 1). On the contrary, muscle fibre detachments were observed much earlier in laminin-deficient embryos (day 1.5) (Hall et al., 2007) or in

itgα7-deficient embryos (day 3). Injecting *ilk*–ATG MOs targeting *ilk* mRNA of maternal origin did not affect the timing of muscle detachments, excluding the possibility that maternal Ilk is compensating for the loss of zygotic Ilk in the MTJ. Third, in *loc/ilk* mutant embryos the entire plasma membrane retracts from the basement membrane (Fig. 5). If Ilk is only required to link the cytoskeleton to the integrins in the membrane bound to the ECM, one would expect only the cytoskeleton to retract when Ilk is removed. This indeed occurs in Ilk-deficient muscle fibres in Drosophila, again suggesting a very different role for Ilk in Drosophila and zebrafish during muscle fibre adhesion. In addition these observations, together with previous observations made by others (Bendig et al., 2006), suggest that Ilk is involved in mechanosensing and signalling in muscle cells. Muscle LIM-domain only proteins are very well known for their role in mechanosensing and loss of MLP/CRP3 function in both human and mice results in a dilated cardiomyopathy (Knöll et al., 2002). Loss of Ilk in the cardiomyocytes also results in a dilated cardiomyopathy both in zebrafish as in mice (Knoll et al., 2007; White et al., 2006). The interaction between Ilk and MLP/CRP3 that we found makes ILK mechanosensor function now very likely and might be able to link extracellular mediated signalling with intrinsic (MLP/CRP3 mediated) signal transduction pathways important for muscle function and performance. The fact that we did not observe any skeletal muscle detachments in *mlp/crp3* knock-down embryos could be explained by its very low embryonic expression and by a possible redundancy with other LIM-domain only proteins present in zebrafish skeletal muscles, which will be investigated in a future study.

Ilk kinase activity

Integrins are transmembrane proteins that are believed to lack any endogenous enzyme activity. For intracellular signalling, integrins depend on the association with the non-receptor kinases, FAK (focal adhesion kinase), ILK and PYK2 (proline rich tyrosine kinase 2). Although ILK was identified as a serine/threonine kinase based on some conservation with other kinases and its in vitro kinase activity, a conundrum remained about the significance of this kinase activity in vivo. Rescue experiments in Ilk-deficient Drosophila and *C. elegans* demonstrated that in these organisms, Ilk kinase activity is dispensable for its in vivo function (Mackinnon et al., 2002; Zervas et al., 2001). Our data presented here demonstrate that the same Ilk K220M variant that efficiently and completely rescues Ilk-deficient Drosophila embryos, does not rescue any of the phenotypes observed in the zebrafish *loc/ilk* mutant. These results would be in agreement with the previous observations that mutations affecting Ilk kinase activity correlate with reduced contractility of cardiac muscles in zebrafish and human (Bendig et al., 2006; Knoll et al., 2007). Alternatively, it could be that the K220 residue is not only required for kinase activity, but in addition also has an essential structural function in an Ilk–protein interaction. Recently it has been suggested that the functionally related FAK is not required for assembling the focal adhesion complex upon integrin clustering (Schober et al., 2007). These authors show that FAK is required for focal adhesion dynamics by regulating disassembly and actin polymerization by phosphorylation of downstream targets. Our data demonstrate that in skeletal muscles, Ilk is also dispensable for focal adhesion assembly but is required for strengthening of the focal adhesion complex. In addition, our data suggests that phosphorylation of downstream targets could be the underlying mechanism. Interestingly, others have shown previously that the binding of β-parvin with α-actinin requires the presence of Ilk with a functional lysine 220. These authors suggested that phosphorylation of β-parvin by Ilk is required for the binding of β-parvin to α-actinin (Yamaji et al., 2004). We have now shown that besides Ilk also β-parvin is required for skeletal muscle adhesion.

Materials and methods

Zebrafish lines

Embryos and adult fish were raised and maintained under standard laboratory conditions. We used the following lines: *Tg(memb:GFP)* (Cooper et al., 2005) and *loc/ilk^{hu801}* (Knoll et al., 2007).

Muscle relaxation assay

Embryos were raised in 0.02% tricaine methanesulfonate in E3 medium with 0.02% tricaine methanesulfonate from 48 hpf onwards to prevent muscle contractions. Treated and untreated embryos were fixed at 4.5 dpf and stained with phalloidin to visualize skeletal muscles.

Construct and primers

The full-length Ilk sequence was derived from the zebrafish EST Open Biosystems clone 6796955 (sequence identical to GenBank Acc# BC056593) was described before (Knoll et al., 2007). Ilk mutations were prepared by site-directed mutagenesis (Stratagene) in Ilk–pCS2+ vector. For the GFP–Ilk fusion construct, EGFP of the pEGFP-C2 construct (BD Biosciences) was subcloned (using PCR primers: cgggatccaccatggtgagcaagggcgaggagc; gcgaattccttgtacagctcgtccatgccg) in the Ilk–PCS2+ vector by *Eco*R1/*Bam*H1 digestion. An *itgα7* EST clone containing the entire ORF (Open Biosystems EXELIXIS2490190 and GenBank Acc# EB781151) was sequenced. An *itgα7* dig-labelled anti-sense probe was synthesized from this EST clone. Part of the *itgα7* genomic sequence is annotated on contig Zv7_NA1974 of the zebrafish ensemble. We used the following RT-PCR primers. For *itgα7* we used, F: 5′-cttctccgtggctctacac-3′ (exon 1) and R: 5′-cgagcagcaggaagttg-3′ (exon 4) and for *ef1α* we used, F: 5′-ggccacgtcgactccggaaagtcc-3′ and R: 5′-ctcaaaacgagcctggctgtaagg-3′.

The following primers were used to clone the partial zebrafish *mlp/crp3* gene (Ensemble: ENSDART00000053404) atggtttgtcgtaaaggtttgga; ctttaatctccaatggaaagtcgtt, or *β-parvin* (ENSDARG00000019117): 5′-CGTGAAAGACCTTGAGGAAG-3′ 5′-TCCGATCATCATTTCTGTTG-3′ in pTOPO. We used the following *mlp* RT-PCR primers 5′-GTCGTAAAGGTTTGGACAGC-3′ (exon 3); R: 5′-AGGAGGCAAATACTGACAGC-3′ (exon 6).

Injections and morpholinos

All injections in this study were performed with 1–2 nl in 1-cell stage embryos. Morpholino antisense oligonucleotides (MOs; Gene Tools, Philomath, OR) were designed: *integrin α7*-ATG MO, (5′-gacccacagagacatgaccagctcc-3′), *integrin α7*-splice MO (5′-ctcagatcagtgcagactcaccagc-3′) and *mlp-splice* MO (5′-gtattttgaggacgtactcttgagg-3′). *ilk* splice-MO (Knoll et al., 2007), *laminin-α2*-MO and *laminin-α4*-MO (Pollard et al., 2006), *dystroglycan*-MO (Parsons et al., 2002), *β-parvin* MO (Bendig et al., 2006), were used as described previously. A standard control MO was purchased from Gene Tools, LLC. All MOs were diluted in Danieu's buffer for injection.

In situ hybridization, immunohistology and staining

Whole mount in situ hybridization (ISH) was performed as described previously (Thisse et al., 1993). F-actin was stained with phalloidin-TRITC (Sigma; 1:100). The following antibodies were used: ILK (Upstate; 1:100), vinculin (Sigma; 1:200), α-actinin (sarcomeric) (Sigma; 1:400), laminin (Sigma; 1:400), paxillin (Transduction Laboratories; 1:200). As secondary antibody we used Cy™2/Cy™3 conjugated anti-mouse/rabbit IgG (Jackson Immuno Research laboratories, 1:1000).

Western blot analyses

Protein extracts generated from 100 embryos were prepared using 1 μl/embryo extraction buffer (250 mM sucrose, 100 mM NaCl, 10 mM EGTA, 20 mM HEPES, 1% triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 10 mM NaF, 1 mM Na_3VO_4 mM) at 4 °C, centrifuged and 2× SDS-sample buffer was added (1 μl/embryo). Samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). Chemiluminescent detection was illuminated with Vistra ECL (Amersham Pharmacia Biotech). Antibodies used: anti-ILK (Sigma; 1:500), anti-Akt (Cell Signalling; 1:1000), anti-phospho-Akt (Cell Signalling; 1:1000), anti-phospho-GSK-3β (Cell Signalling; 1:1000), anti-GAPDH (Chemicon; 1:1000).

Yeast two hybrid interaction

The Muscle lim protein (MLP/CRP3) and β-parvin were cloned into pGBKT7 bait vector and pretransformed into the *Saccharomyces cerevisiae* AH109 strain. All the pretransformed bait including the deletion mutants of mouse MLP/CRP3 were allowed to grow in the tryptophan drop out selection plates. The grown colonies were analyzed for self-activation of reporter gene and were found to be negative by the filter lift assay. Further, the pretransformed colonies were cotransformed with human ILK–WT and ILK–K220M cloned in the prey vector pGADT7. The cotransformants were subjected to medium selection (triple dropout medium, SD/-His/-Leu/-Trp) and high selection (quadruple dropout medium, SD/-Ade/-His/-Leu/-Trp) growth conditions to determine

the strength of the interaction. To confirm the specificity of the interaction beta-galactosidase activity of each cotransformants was determined according to the manufacturer's protocol (Clontech, Mountain View, CA, USA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.024.

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