

A central multifunctional role of integrin-linked kinase at muscle attachment sites

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Summary

Integrin-linked kinase (ILK) is an essential component of a multiprotein complex that links actin to the plasma membrane. Here, we have used a genetic approach to examine the molecular interactions that are essential for the assembly of this ILK-containing complex at *Drosophila* muscle attachment sites (MASs). We show that, downstream of integrins, talin plays a decisive role in the recruitment of three proteins: ILK, PINCH and paxillin. The accumulation of ILK at MASs appears to follow an amplification mechanism, suggesting that numerous binding sites are generated by minimal levels of the upstream integrin and talin effectors. This property suggests that ILK functions as an essential hub in the assembly of its partner proteins at sites of integrin adhesion. We found that PINCH stability, and its subcellular localization at MASs, depends upon ILK function, but that ILK stability and localization is not dependent upon PINCH. An *in vivo* structure–function analysis of ILK demonstrated that each ILK domain has sufficient information for its independent recruitment at embryonic MASs, whereas at later developmental stages only the kinase domain was effectively recruited. Our data strengthen the view that the ILK complex is assembled sequentially at sites of integrin adhesion by employing multiple molecular interactions, which collectively stabilize the integrin–actin link.

Key words: Cell adhesion, ILK, Talin, PINCH, Paxillin

Introduction

In multicellular organisms, tissue integrity depends upon the stable association of cells with the extracellular matrix (ECM). This requires a link between matrix ligands and the cytoskeleton, which is primarily mediated by the integrin family of surface receptors. Each integrin is composed of one α and one β subunit, and because these subunits have very short cytoplasmic tails it has been postulated that other scaffolding proteins assist integrins in the recruitment of downstream components that mediate the link to actin (Legate and Fassler, 2009). The cytoskeletal molecules involved in this link, and the hierarchy of their interactions, are currently a subject of intense investigation, and genetic approaches in model organisms have provided novel insights into the function of these proteins (Legate and Fassler, 2009). One such example is integrin-linked kinase (ILK), a protein initially identified by its ability to bind directly to the cytoplasmic tail of the $\beta 1$ integrin subunit (Hannigan et al., 1996). ILK is a modular protein containing five tandem ankyrin repeats (ANKRs), a putative phosphoinositide-binding site and a kinase-like domain (Chiswell et al., 2008). ILK is required to maintain the molecular link between integrins and the sarcomeric actin filaments in *Drosophila* embryonic muscles (Zervas et al., 2001) and to assemble the link between integrins and the contractile apparatus of developing *Caenorhabditis elegans* muscles (Mackinnon et al., 2002). Knockout of the single mouse

gene showed that ILK is required for diverse developmental processes, with its earliest function being in epithelial polarization and normal actin distribution of the developing epiblast (Lange et al., 2009; Sakai et al., 2003). In all three organisms, certain site-directed mutations within the kinase domain of ILK that should eliminate kinase activity did not show any defects, suggesting that the essential role of ILK is not the phosphorylation of target proteins (Lange et al., 2009; Mackinnon et al., 2002; Zervas and Brown, 2002). Instead, ILK binds to multiple proteins, suggesting that its primary function is as an adaptor. The recently published structure of the kinase domain of ILK supports the view that it is a binding adaptor rather than a kinase (Fukuda et al., 2009). Thus, genetic and structural data indicate that ILK is a pseudokinase (Boudeau et al., 2006; Wickstrom et al., 2010). However, we have yet to develop a clear picture of the essential adaptor function of ILK in assisting integrin function.

The large number of proteins that bind ILK suggests it is a central scaffolding molecule for the assembly of a protein complex (Zervas and Brown, 2002). Interacting proteins have been identified by yeast two-hybrid screening and biochemical methods (Boulter and Van Obberghen-Schilling, 2006). Among these proteins are: PINCH, which contains five LIM domains and binds to the ANKRs of ILK through its first LIM domain (Tu et al., 1999; Velyvis et al., 2001); paxillin, which has four LIM domains and five leucine-rich motifs (LD) and binds to the ILK kinase domain (Nikolopoulos and Turner, 2001); and parvin, which has two calponin homology domains and binds to the ILK kinase domain (Nikolopoulos and Turner, 2000; Olski et al., 2001; Tu et al., 2001). Both the ANKRs and kinase domains are essential for recruitment of ILK at focal

adhesions in vertebrate cells in culture (Boulter and Van Obberghen-Schilling, 2006; Li et al., 1999). Even before its association with integrins, ILK is in a cytoplasmic complex with PINCH and parvin (Wu, 2005) and a fourth protein, Ras suppressor protein 1 (Rsu1), which binds the fifth PINCH LIM domain (Dougherty et al., 2005; Kadrmas et al., 2004). So far the model of functional coordination between ILK, PINCH and parvin has not been fully supported by genetic studies. ILK is required for parvin (Pat-6) recruitment to sites of integrin adhesion in *C. elegans* but not the reverse (Lin et al., 2003), and PINCH is not required for ILK recruitment in either *Drosophila* or *C. elegans* (Clark et al., 2003; Norman et al., 2007).

Here, we combined genetic analysis and structure–function approaches to examine the mutual interactions of ILK and PINCH, as well as their interactions with talin and paxillin. We discovered that low levels of integrins were sufficient to recruit substantial levels of ILK to the major sites of integrin adhesion in the embryo and larva, namely the muscle attachment sites (MASs), suggesting an amplification mechanism. Complete removal of talin resulted in the loss of ILK, PINCH and paxillin from MASs, in agreement with its crucial contribution to integrin function. Neither ILK nor PINCH was required for paxillin recruitment. Unexpectedly, PINCH stability and recruitment required both domains of ILK (i.e. PINCH-binding ANKRs and kinase domains). In embryos each ILK domain was recruited to the MASs, but recruitment of the isolated ANKRs was lost during development, indicating a change in mechanism. Finally, we tested the function of a number of conserved ILK residues that have been shown to be important for interactions with other proteins, but found them dispensable. Collectively, our results indicate that ILK functions upstream of PINCH in the muscle and that the function of ILK is executed by simultaneous interactions of both the kinase and ANKR domains.

Results

ILK is recruited by a very low level of integrins

As *Drosophila* embryos develop, integrins and their associated proteins become most highly concentrated where muscle ends attach to epidermal tendon cells, through intervening ECM. The muscles are large multinucleate cells, so concentration at muscle ends reflects subcellular localization of these proteins into adhesive junctions capable of resisting muscle contraction. These muscle attachment sites (MASs) therefore provide a useful system for examining protein interactions necessary for recruitment of components of the complex that links integrins to the actin cytoskeleton, which we exploit here. We utilized two views of MASs, optical sections showing muscles and tendon cells, and a surface view of the embryo showing the muscle pattern (see Materials and Methods).

We previously reported that localization of ILK to MASs did not require integrin function, as it was still observed in embryos lacking the β PS integrin subunit (Fig. 1A,A') (Zervas et al., 2001). Similarly, genetic ablation of β 1 in mice also did not affect localization of ILK to the myotendinous junctions (Schwander et al., 2003). Furthermore, talin, a core component of the integrin–actin-linker complex, remains strongly localized at *Drosophila* MASs even when there is a dramatic reduction in integrin levels, caused by a hypomorphic mutation (*ij^{sef}*) in the *inflated* gene, which encodes α PS2, the major, if not only, α -integrin subunit expressed in *Drosophila* muscles (Devenport et al., 2007). Similarly, ILK–GFP was strongly localized to the MASs in the same mutant (Fig. 1B,B'). We were therefore surprised to find that complete removal of α PS2 resulted in a failure in the localization of ILK to

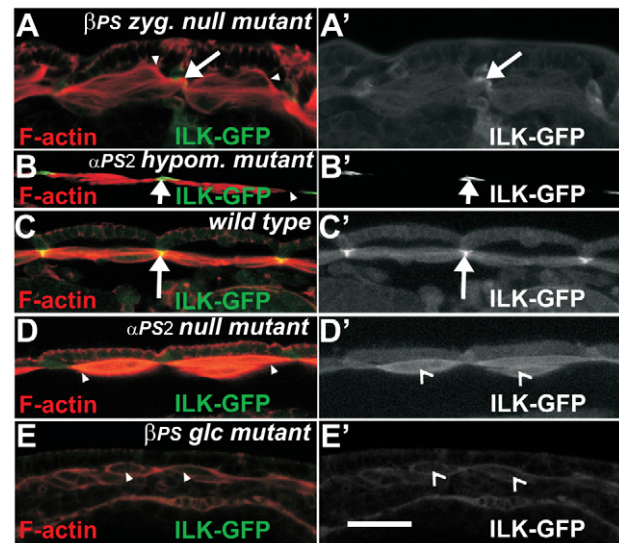


Fig. 1. Integrins are essential for ILK recruitment. Horizontal optical sections of muscles attaching to tendon cells in stage 17 embryos showing ILK–GFP (green, and right-hand panels) and phalloidin-stained F-actin (red). In mutant embryos lacking zygotic expression of the β PS integrin subunit (A), or expressing reduced levels of the α PS2 integrin subunit (*ij^{sef}*) (B), ILK–GFP was still localized at MASs (arrow), even as muscles detach (arrowhead). (C) The ILK–GFP localization in wild-type embryos is shown for comparison. By contrast, ILK–GFP was diffuse and cytoplasmic (angle) in embryos completely lacking α PS2 (*ij^{sef}*) (D) or both maternal and zygotic β PS (E). Scale bar: 25 μ m. hypom., hypomorph; glc, germline clone.

MASs (Fig. 1C–D'). An explanation for this difference is that although the embryos examined previously almost completely lacked the β PS integrin subunit (as they were homozygous for a null mutation in the gene encoding β PS and therefore lacked zygotic β PS expressed from the embryonic genome) they did contain a small amount of β PS deposited in the egg by the heterozygous mother, which could have recruited ILK. This maternally provided β PS is not easily detected with antibodies, but its presence has been revealed genetically, as removing it enhances the mutant phenotype caused by the absence of zygotically expressed β PS (Roote and Zusman, 1995). When both the maternal and zygotic β PS integrin subunit was removed, we no longer detected localization of ILK–GFP at MASs (Fig. 1E,E'). Thus, ILK recruitment to MAS does require integrins, albeit in very small amounts.

Building a hierarchy: talin is essential for the assembly of the ILK-containing complex

To delineate further the hierarchy of genetic interactions in the assembly of the ILK-containing complex, we examined the requirement for talin, as this protein is necessary for all integrin adhesive functions (Brown et al., 2002; Calderwood, 2004). The recent identification of Wech, an adaptor protein that binds ILK and talin, provides a mechanism for ILK recruitment through talin (Löer et al., 2008). We tested whether talin was required for not only ILK but also PINCH and paxillin localization at MASs. Talin, as for integrins, was essential for the recruitment of the integrin-associated proteins ILK (Fig. 2A,A',B,B',C,C',D,E), PINCH (Fig. 2A,A'',B,B'',C,C''), paxillin (Fig. 2D,D',E,E') and tensin (Brown et al., 2002; Torgler et al., 2004). In the same manner as for β PS,

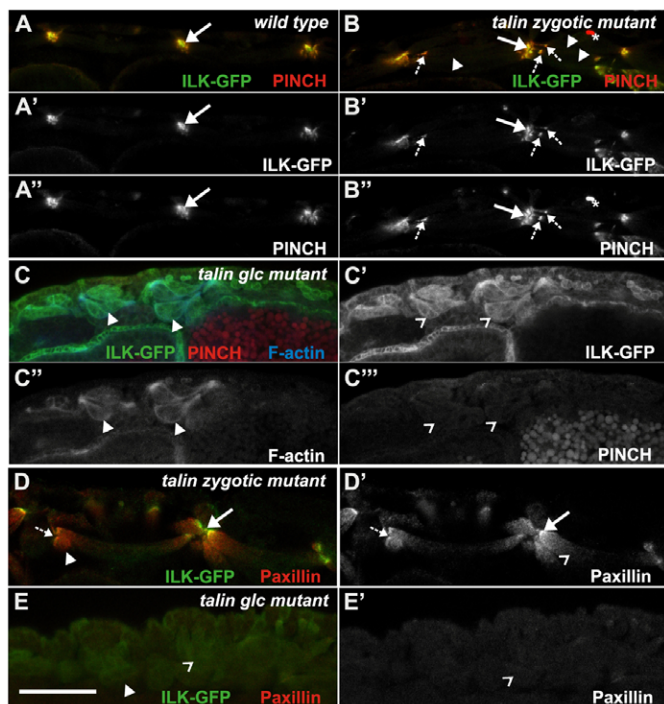


Fig. 2. Talin is essential for recruitment of ILK and associated proteins. Horizontal optical sections of stage 17 embryos showing ILK–GFP (A–E, green; A', B', C', white) antibody labelling of PINCH (A–C, red; A'', B'', C'', white) and paxillin (D, E, red and white), and phalloidin-stained F-actin (C, blue; C'', white). (A) In wild-type embryos ILK and PINCH are localized at MASs. (B) In embryos lacking the zygotic contribution of talin, ILK–GFP and PINCH were still localized at MASs and were retained at detached muscle ends. (C) When both maternal and zygotic talin was removed, ILK–GFP and PINCH were not localized. (D, E) Similarly, both maternal and zygotic talin needed to be removed to lose paxillin recruitment. Arrows, protein at MASs; angles, cytoplasmic distribution; arrowheads, detached muscles; dashed arrows, muscle ends; asterisk, antibody debris. Scale bar: 25 μ m.

the zygotic removal of talin did not eliminate ILK from the MAS, nor did it cause the complete loss of PINCH and paxillin (Fig. 2B', B'', D'). This result was not unexpected, as zygotic talin mutant embryos contain clearly detectable maternal talin (Brown et al., 2002). However, all three proteins were no longer present at MASs when we completely removed talin by generating germline clones (Fig. 2C–C'', E, E'). Thus, integrins need talin to recruit ILK, PINCH and paxillin, and a small amount of talin can recruit substantial amounts of these proteins.

Regions of ILK required for localization at MASs

Wech functions as an adaptor between talin and ILK, and contributes to ILK localization at MASs (Löer et al., 2008). However, this cannot be a universal mechanism, as other sites of integrin adhesion lack Wech (Delon and Brown, 2009). Studies in vertebrate cells have identified additional ILK interactions that provide alternative mechanisms to link ILK to talin. Thus, the ILK–paxillin interaction is required for localization of ILK to focal adhesions (Nikolopoulos and Turner, 2001), and we have shown above that recruitment of both ILK and paxillin requires talin. Talin recruits an isoform of phosphatidylinositol 4-phosphate 5-kinase (Di Paolo et al., 2002), potentially elevating phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂] levels,

which could recruit ILK through a region that has sequence similarity to phosphoinositide-binding pleckstrin homology (PH) domains (Delcommenne et al., 1998). Finally, undiscovered interactions with the ANKRs or kinase domain could contribute to ILK recruitment.

To address the mechanism of ILK recruitment to MASs, we examined how mutations in ILK interaction domains affected its localization. Wild-type and truncated ILK proteins were fused to GFP, so that their localization could be quantified by confocal microscopy (Figs 3, 4). ILK–GFP was expressed in two ways: first, using its endogenous regulatory elements to express the proteins at normal spatial and quantitative levels (Fig. 3), and second, by using *mef2Gal4* to drive expression of UAS::ILK–GFP, which results in the overexpression of ILK–GFP only within the muscle (Fig. 4), permitting assessment of its recruitment even if the altered protein has reduced stability. We examined ILK recruitment at two stages: in the newly hatched first-instar larvae (1stIL; at 22–28 hours) and in second-instar larvae (2ndIL; at 48–60 hours).

When wild-type ILK–GFP was expressed under its own promoter nearly 95% of the total fluorescent intensity was concentrated at MASs in 1stIL (Fig. 3A, H), whereas in 2ndIL it reached nearly 99% (Fig. 3I, P). When ILK–GFP was overexpressed only ~50% was recruited to MASs in 1stIL, with the rest diffuse in the cytoplasm or associated with sarcomeric Z-lines (Fig. 4A, I). However, in 2ndIL the fraction of overexpressed ILK–GFP at MASs was close to 95% (Fig. 4J, R), suggesting either that the number of ILK recruitment sites had increased or that unlocalized ILK had been degraded.

The paxillin-binding region in human ILK has been mapped to a motif encompassing residues V386 and T387, and mutagenesis in this region reduces the level of ILK recruitment to focal adhesions (Nikolopoulos and Turner, 2001). In *Drosophila*, only the corresponding T384 is conserved, so we replaced this residue with alanine (T384A), or with aspartic acid (T384D) to mimic its phosphorylation. Both mutant forms of ILK were localized equivalently to the wild-type protein, whether expressed at endogenous levels or overexpressed (Fig. 3F–H, N–P; data not shown), demonstrating that T384 is not involved in protein interactions that mediate localization of ILK. This suggests that paxillin does not contribute to the localization of ILK in MASs, in agreement with the recent finding that functional MASs are formed in the absence of paxillin (Bataillé et al., 2010).

We next examined the role of the ILK region with similarity to PH domains (180–215), which could recruit ILK by binding phosphoinositides in the plasma membrane (Persad et al., 2001). We replaced the conserved residues R208 and R210 with oppositely charged aspartic acid residues (R208D, R210D), but this did not alter ILK recruitment (Fig. 3B, H, J, P and Fig. 4B, I, K, R). This is consistent with the finding that mammalian ILK with part of the PH-like motif deleted did localize to focal adhesions (Zhang et al., 2002).

The localization of ILK to focal adhesions in mammalian cells requires an interaction between PINCH and the ANKRs of ILK (Li et al., 1999); however, PINCH is not required for recruitment of ILK to MASs (Clark et al., 2003) (see also Fig. 6). Consistent with this, expression of ILK with a deletion of ANKRs 2–5, which bind PINCH (Chiswell et al., 2008), leaving the kinase-like domain intact, reduced but did not eliminate the localization at MASs. In 1stIL, 30% of the ILK kinase domain was recruited to MASs, (Fig. 3C, H), whereas in 2ndIL only 20% was recruited (Fig. 3K, P).

Genomic ILK-GFP constructs

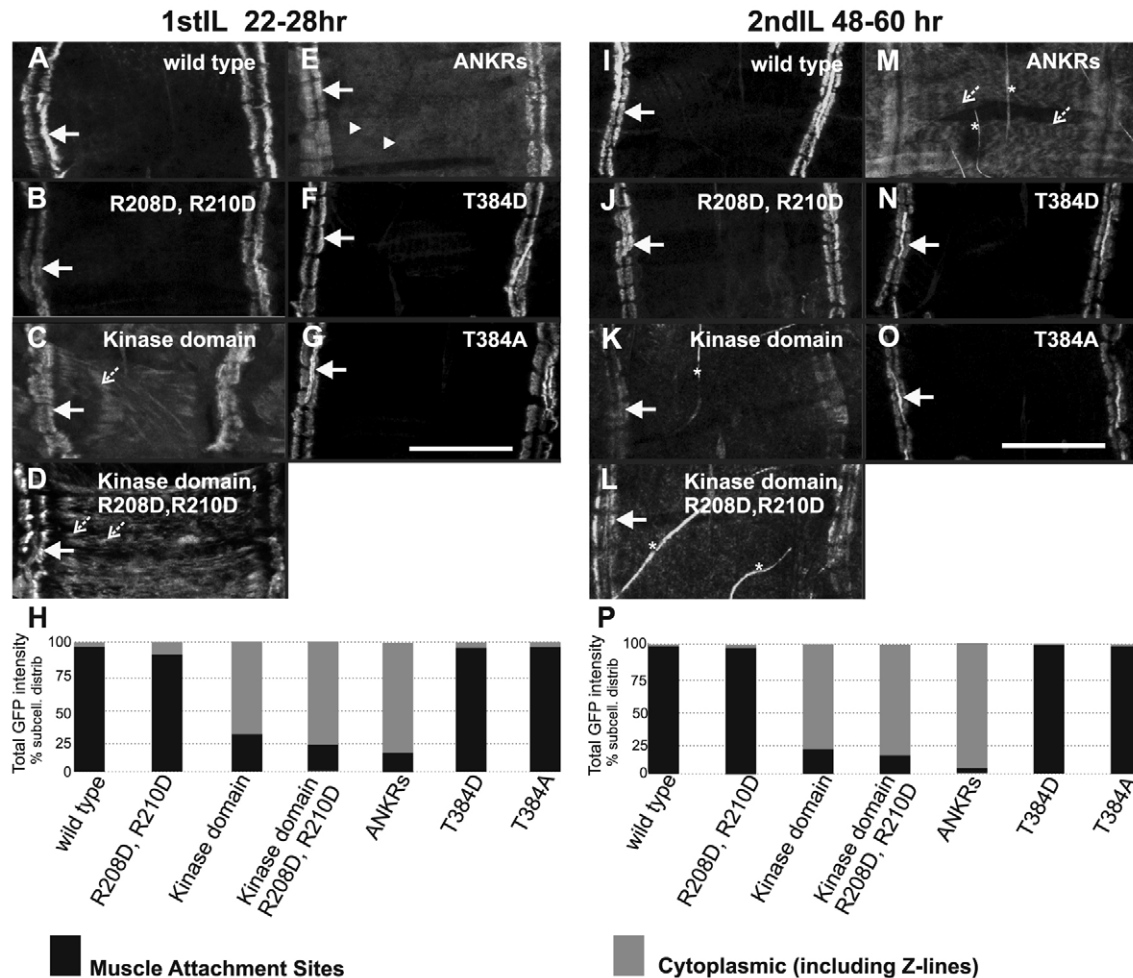


Fig. 3. MAS recruitment of mutant forms of ILK-GFP expressed at endogenous levels. Stacks of confocal sections of dorsal oblique muscles showing fluorescence of wild-type and mutant forms of ILK-GFP, as indicated, expressed from its own promoter in first-instar larvae (A–G; 1stIL) or second-instar larvae (I–O; 2ndIL). Arrows, ILK-GFP at MASs; dashed arrows, Z-lines; arrowheads, perinuclear regions; asterisks, tracheal fibres. Scale bars: 25 μ m (A–G), 50 μ m (I–O). (H,P) Quantification of the fluorescent intensity distribution at MASs compared with that in the cytoplasm.

Similarly, the recruitment of the overexpressed ILK kinase domain to MASs was reduced (Fig. 4C,L). In 1stIL, 25% was recruited but this increased to 60% at 2ndIL (Fig. 4I,R). Therefore, the ANKRs are important for recruiting normal levels of ILK, and more so in 1stIL than 2ndIL, but are not absolutely required for recruitment. Combining the point mutations in the PH domain with the deletion of the ANKRs did not further impair recruitment (Fig. 3D,H,I,L,P and Fig. 4D,I,M,R).

Another residue required for ILK localization to focal adhesions is F436, which is located in the last α -helix of the kinase C-lobe (Fukuda et al., 2009; Zhang et al., 2002). An F436A mutation reduced recruitment of overexpressed ILK; 25% was recruited to 1stIL MASs (Fig. 4F,I), increasing to 70% in 2ndIL (Fig. 4O,R). Thus, as with the ANKRs, this residue contributes to MAS recruitment but is not essential.

We next tested whether the isolated N-terminal region (1–177), including the ANKRs (1–128), contained a localization signal. Some of the truncated protein (ILK^{ANKRs}-GFP) was localized to the MASs, with ~18% recruited in 1stIL (Fig. 3E,H), and 10% recruited when overexpressed (Fig. 4E,I). The reduced recruitment

of the ILK truncated protein containing the ANKRs became more evident in 2ndIL (Fig. 3M,P; Fig. 4N,R). This suggested that, in contrast with full-length ILK or the ILK kinase domain, where more of these forms of ILK became localized as the larva grows, there is a reduction in the ability to recruit ILK containing ANKRs. We tested whether this was caused by a limited amount of PINCH, by cooverexpressing untagged PINCH, but this failed to increase ILK^{ANKRs}-GFP recruitment (data not shown). Therefore, the improved recruitment of overexpressed ILK during larval development is mediated by interactions with the kinase domain.

Finally, we investigated whether phosphorylation regulates ILK localization and function. Mammalian ILK contains four putative p21-activated kinase 1 (PAK1) phosphorylation sites, containing the consensus K/RxxT/S motif (Acconcia et al., 2007); PAK1-dependent phosphorylation of ILK enhances its subcellular distribution within the cytoplasm, whereas depletion of PAK1 promotes translocation of ILK to the nucleus (Acconcia et al., 2007). The T180 PAK1 site is well-conserved in ILK, whereas the conservation of other sites is more variable (supplementary material Fig. S1). T180 and S176, which also form a putative PAK1

UAS:ILK-GFP constructs

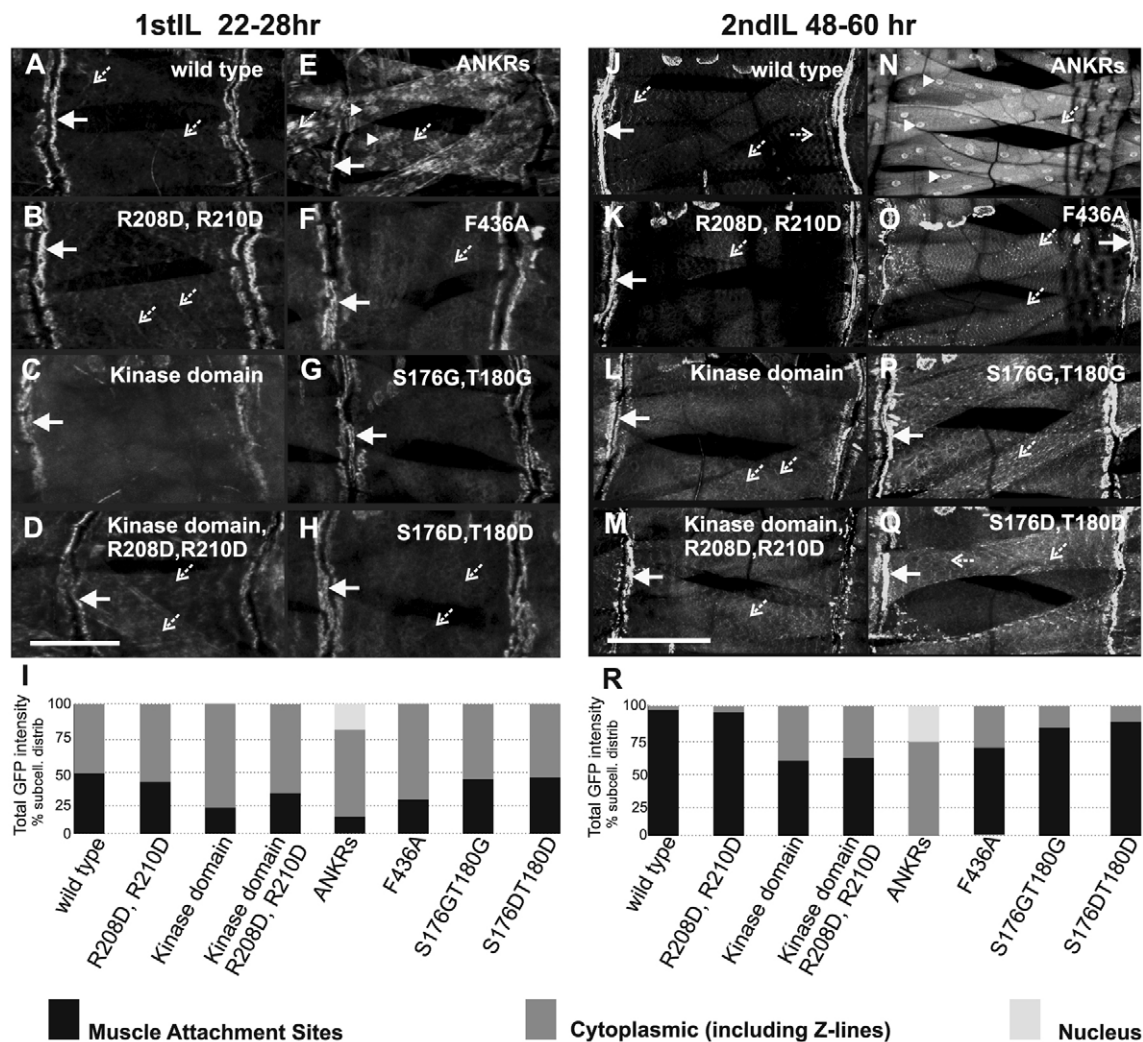


Fig. 4. MAS recruitment of overexpressed mutant forms of ILK-GFP. Stacks of confocal sections of dorsal oblique muscles showing fluorescence of wild-type and mutant forms of ILK-GFP, as indicated, expressed by *mef2Gal4* driven UAS:ILK-GFP in first-instar larvae (A-H; 1stIL) or second-instar larvae (J-Q; 2ndIL). Arrows, ILK-GFP at MASs; dashed arrows, Z-lines; arrowheads, perinuclear regions. Scale bars: 25 μ m (A-H), 50 μ m (J-Q). (I,R) Quantification of the fluorescent intensity distribution at MASs compared with that in cytoplasm and nucleus.

phosphorylation site, are phosphorylated in *Drosophila* cells (Bodenmiller et al., 2007). T180 is also within a match to the protein kinase B (PKB) phosphorylation consensus RxRxxTL (Alessi et al., 1996). These results suggest that ILK S176 and T180 are phosphorylated by PAK, PKB or another kinase. *Drosophila* PAK1 is concentrated at MASs in an integrin-independent manner (Harden et al., 1996). We mutated both potential phosphorylation sites, generating phosphorylation-null (S176G, T180G) and phosphomimetic (S176D, T180D) ILK mutants. When overexpressed, both of these mutant proteins showed only mildly reduced MAS recruitment [in 1stIL 40% was recruited, increasing to 85–90% in 2ndIL (Fig. 4G–I,P–R)] and neither mutant protein showed nuclear translocation. Therefore, in contrast with the findings in mammalian cells, phosphorylation of these residues does not regulate the distribution of ILK in muscles.

In summary, our results show that cutting ILK into two halves results in proteins that are recruited to MASs, albeit less efficiently

than the full-length protein, demonstrating that there are at least two protein interactions that contribute to ILK recruitment. By examining recruitment at two stages of larval life, we discovered that the mechanism that recruits the isolated ANKRs of ILK is lost in older larvae.

Functional analysis of ILK mutants

We next tested how the ILK mutations described above affect the function of the proteins, analysing multiple transgenic lines to control for variability in expression (supplementary material Fig. S2). The C-terminal GFP tag does not interfere with the function of ILK, as ILK-GFP expressed from its own promoter fully rescues the null *ilk¹* embryonic lethal mutation (Zervas et al., 2001). Overexpression of untagged UAS:ILK, through the *24B Gal4* line (which drives expression primarily in muscles and tendon cells) rescues *ilk* lethal alleles to give viable adults, although such flies have wing blisters, presumably due to insufficient expression in

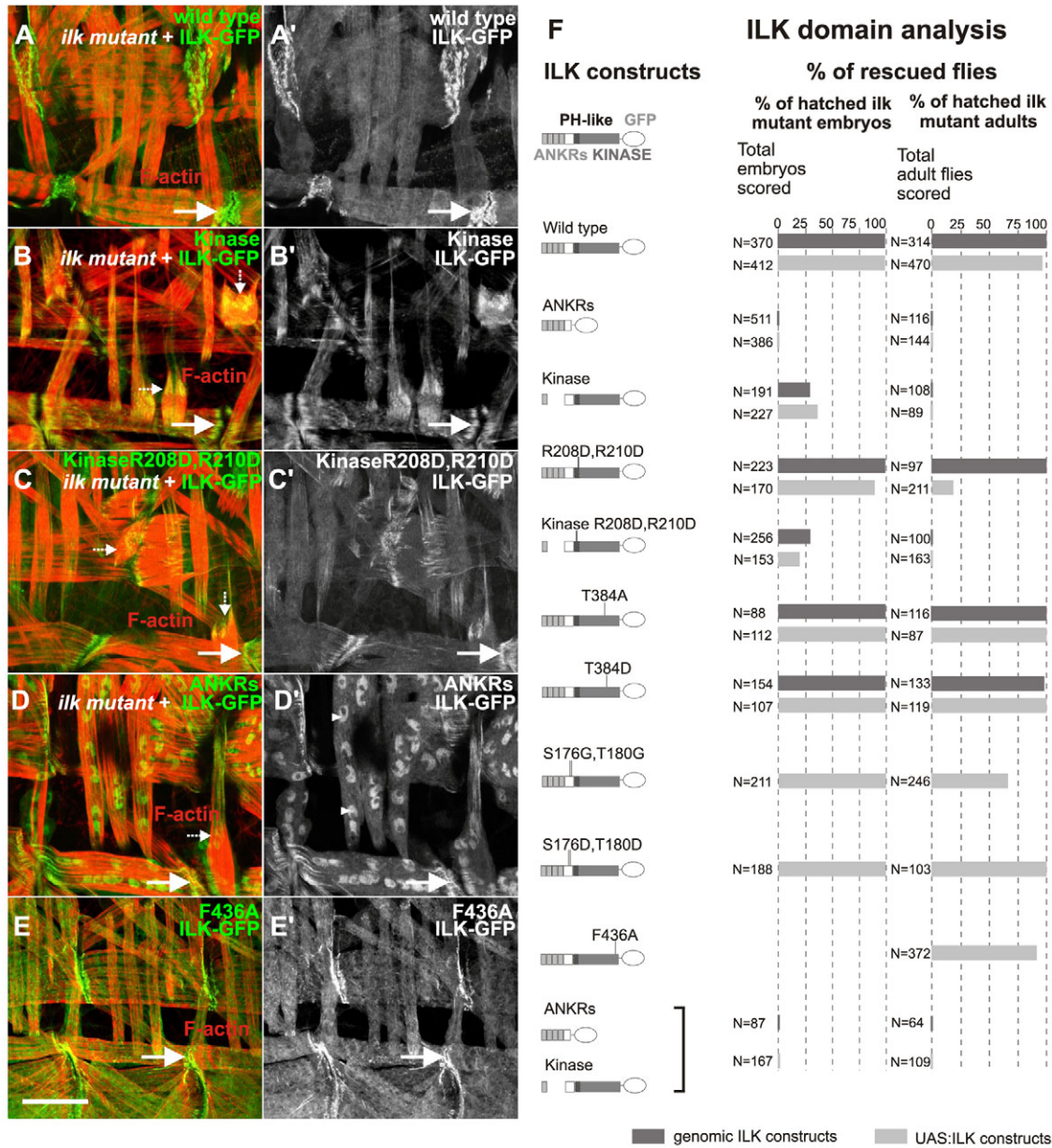


Fig. 5. Functional analysis of ILK domains in mediating the integrin–actin link. (A–E) Stacks of confocal sections of lateral muscles of stage 17 *ilk* mutant embryos (*ilk¹/Df*) rescued with wild-type or mutant ILK–GFP, as indicated, overexpressed by *24BGal4*. ILK–GFP fluorescence is in green and white, and phalloidin-stained F-actin in red. Arrows, ILK–GFP at MASSs; dashed arrows, detached muscles. Scale bar: 25 μ m. (F) The extent of rescue for all constructs tested is quantified in the right-hand panel.

the wing (Zervas et al., 2001). UAS::ILK–GFP gave equivalent rescue (Fig. 5A,F).

Deletion of ANKRs 2–5 did not completely eliminate ILK activity, but its function was severely reduced; it only partially rescued the actin-detachment phenotype of *ilk* mutant embryos (Fig. 5B); ~30% of the mutant embryos hatched into larvae, regardless of whether the construct was expressed at normal or high levels, compared with 0% in the absence of any rescue construct (Fig. 5F). The rescued 1stIL were unable to move normally and died during larvae development. This demonstrates that ANKRs are important for ILK function, and act to maintain the stable association of actin filaments with the plasma membrane, permitting development to the adult. However, these results also

show that the ILK kinase domain maintains some ILK function on its own, sufficient to delay muscle defects until larval stages in the 30% of the mutant embryos that escaped lethality.

Deletion of the kinase domain completely abolished ILK function (Fig. 5D,F). This result was expected given that the molecular lesion in *ilk¹* is a stop codon at residue W211, which causes a deletion of most of the kinase domain (Δ 211–448), and this allele behaves as an amorph (Zervas et al., 2001). We next examined the effect of mutating known interaction sites within the ILK kinase domain. Mutation of the phosphoinositide-binding site (ILK^{R208D,R210D}–GFP) did not reduce the rescue of lethality when expressed from the endogenous *ilk* promoter (Fig. 5F), but ~35% ($n=32$) of the rescued adults ($n=97$) had blisters in one or both

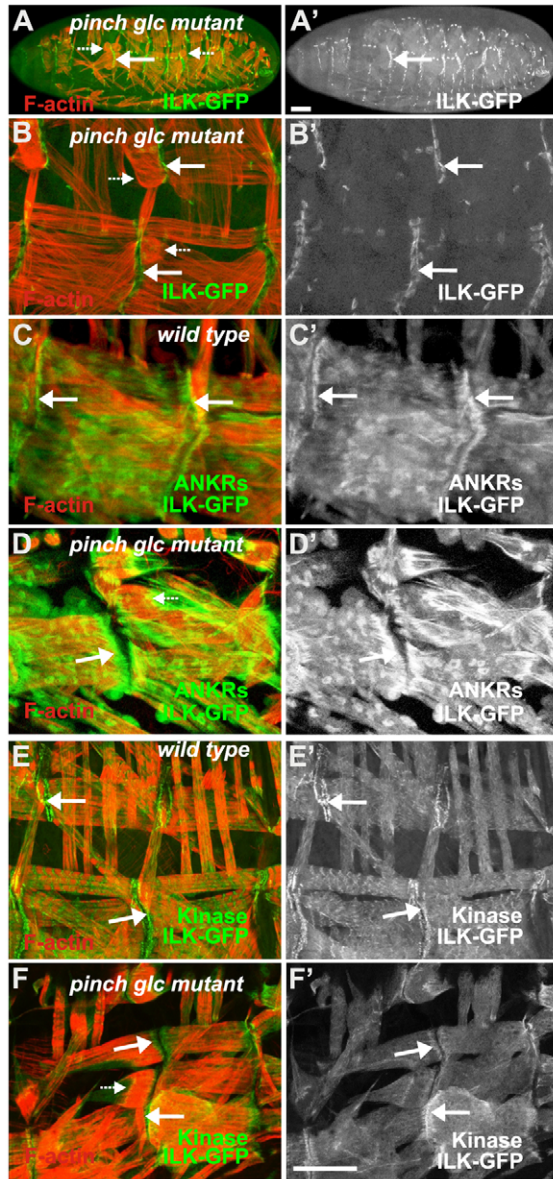


Fig. 6. PINCH is not required for ILK stability or localization in the *Drosophila* embryo. Stage 17 embryos expressing full-length or truncated forms of ILK–GFP (green and white) and stained for F-actin (red). (A,B) Embryos lacking both maternal and zygotic PINCH [*stck*^{T2} glc (germ line clone)] retain ILK–GFP at MASSs; (A,A') Low magnification image of ventro-lateral view showing retraction of sarcomeric actin (dashed arrows) and ILK–GFP at MASSs (arrows). (B,B') High magnification image of lateral longitudinal muscles showing ILK–GFP localization at MASSs (arrows) as actin retracts (dashed arrow). (C–F) The partial recruitment to MASSs (arrows) of overexpressed ILK^{ANKRs}–GFP (C) or ILK^{kinase}–GFP (E) was not affected by removing PINCH (D,F, *stck*^{T2} glc). Scale bars: 50 μ m (A), 25 μ m (B–F).

wings. Overexpression of this mutant form of ILK rescued embryonic lethality, but unexpectedly only 20% of the resulting 1stIL survived to adulthood (Fig. 5F). A few rescued 1stIL had a detached-actin phenotype, but the majority died at later stages (data not shown). The expression level of ILK^{R208D,R210D}–GFP appeared the same as wild-type ILK–GFP (Fig. 3A,B,I,J), and overexpression in wild-type embryos had no effect, so this mutant is not acting as a dominant-negative. Therefore, we suspect that *24B-Gal4* drives

suboptimal expression in certain tissues, so that, in combination with the reduced activity caused by the point mutations, it fails to rescue the lethality of the *ilk* null mutation. Thus, mutation of the phosphoinositide-binding site appears to have the most significant effect in non-muscle tissues, such as the wing. Adding the R208D, R210D mutations did not further impair the partial function of the isolated kinase domain (Fig. 5C,F).

Mutation of the putative paxillin-binding site (T384A or T384D) had no effect on the ability of ILK to rescue activity (Fig. 5F). Similarly, we found no alterations in the functions of ILK in the mutants S176G, T180D and F436A. All the site-directed mutants we engineered and overexpressed with the *24B-Gal4* line rescued the *ilk* mutants in a manner equivalent to that with wild-type ILK–GFP (Fig. 5F).

ILK domains are required in the same molecule

We have found that both the ANKRs and the kinase domain are important for ILK function. However, we next tested whether it is necessary for them to be present in the same molecule. Coexpressing the two domains as individual GFP-tagged proteins did not improve the ability of each of them to rescue the embryonic lethality (Fig. 5F). Indeed, unexpectedly, it had the opposite effect, as the rescue activity of ILK lacking the ANKRs was reduced by adding the ANKRs in trans. Thus, the two domains of ILK need to be present in the same molecule, supporting the view that ILK functions as an adaptor, linking at least two other molecules together in *Drosophila* MASSs.

Testing ILK and PINCH function in regulating each other's localization

It has been previously shown that removing PINCH does not affect the localization of ILK to *Drosophila* MASSs (Clark et al., 2003). This contrasts with results from mammalian cells showing that ILK recruitment requires PINCH (Tu et al., 2001). One possible explanation for this difference is that existing mutant alleles in the gene encoding PINCH in *Drosophila*, *steamer duck* (*stck*), produce short proteins containing the ILK-binding LIM1 domain; these could recruit ILK because the alleles cause premature stops only after LIM1. To rule this out, we generated new alleles by excision of a P-element close to the main coding exons (see Materials and Methods). This resulted in a new allele, *stck*^{T2}, that deletes the region encoding LIM domains 1–3 and part of LIM4. The molecular nature of this mutation means it is unlikely that any PINCH protein is produced, but even if protein were produced, it would lack LIM1. We used this allele to generate embryos lacking both maternal and zygotic PINCH, and found that ILK–GFP was still recruited to MASSs, at levels that were not significantly altered from those in embryos with wild-type PINCH (Fig. 6A–B'). This result enables us to conclude definitively that PINCH is not required for ILK stability and localization at MASSs. We then tested whether the localization of ILK^{ANKRs}–GFP at MASSs is mediated by its interaction with PINCH, and found that this mutated ILK form was still recruited in embryos lacking PINCH (Fig. 6C–D'). Similarly, the kinase domain of ILK localized at MASSs in the absence of PINCH (Fig. 6E–F'). These results demonstrate that even when ILK is split into two halves, neither is dependent on PINCH for MAS recruitment. This implies that the essential function of PINCH in muscles is not to stabilize or recruit ILK, in contrast with recent studies in mammalian cells (Stanchi et al., 2009). Thus, even though ILK and PINCH have shared functions in diverse cells, the precise way they function together differs.

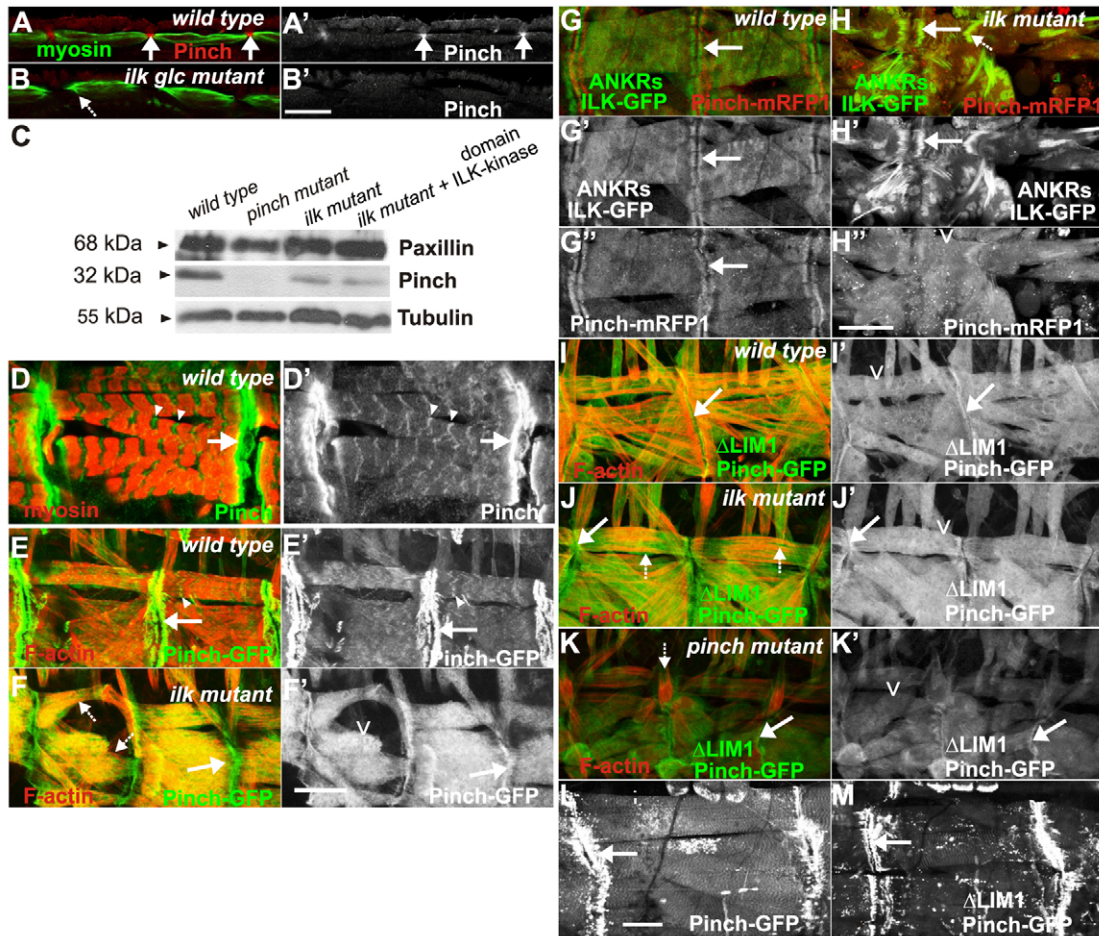


Fig. 7. ILK is crucial for PINCH stability and localization in the *Drosophila* embryo. Most panels show projections of optical sections of muscles, with A, B showing horizontal optical sections. (A, B) Stage 17 embryos labelled for muscle Myosin (green) and PINCH (red and white). (C) Western blot showing levels of paxillin, PINCH and Tubulin in lysates from embryos as labelled. (D) Stage 17 embryos showing PINCH (green and white) recruitment to MASs (arrow) and Z-lines (arrowhead) in wild-type muscles labelled with Myosin (red). (E, F) Lateral muscles in stage 17 embryos, showing PINCH-GFP (green and white) and phalloidin-stained F-actin (red). Overexpressed PINCH-GFP was still well-localized at MASs in wild-type embryos (arrow) (E, E'); in the absence of ILK (F, F') PINCH-GFP was not degraded but was poorly localized. (G, H) Dorsal muscles from live stage 17 embryos expressing ILK^{ANKRs}-GFP (green and white) and PINCH-mRFP1 (red and white). Both ILK^{ANKRs}-GFP and PINCH-mRFP1 were localized at MASs in wild-type embryos (G), and in the absence of endogenous ILK (H) ILK^{ANKRs}-GFP still concentrated at muscle ends (arrow), whereas PINCH-mRFP1 was only faintly localized. (I–K) Lateral muscles from stage 17 embryos expressing PINCH^{ΔLIM1}-GFP (green and white) showing phalloidin-stained F-actin (red). Deletion of the LIM1 domain from PINCH results in a substantial recruitment of PINCH^{ΔLIM1}-GFP to MASs (arrow) (I), which was not affected by removing ILK (J) or endogenous PINCH (K). PINCH lacking the LIM1 domain (M) was recruited to the same extent as full-length PINCH (L) in third-instar larval lateral muscles. Dashed arrows, detached actin; angles, cytoplasmic distribution. Scale bars: 25 μm (A–K), 200 μm (L, M).

We next examined whether ILK has a role in PINCH localization. As discussed above for PINCH, it is possible that the characterized *ilk^l* allele produces enough truncated ILK to bind and stabilize PINCH, because the *ilk^l* has converted W211 into a stop, which is after the PINCH-binding ANKRs (Zervas et al., 2001). We tested this possibility by using a new allele that converts W7 into a stop (*ilk^{5/4}*), removing both the maternal and the zygotic contribution. This resulted in the loss of PINCH at MASs (Fig. 7A–C), and the levels of PINCH protein in the cytoplasm were reduced, even when the kinase domain of ILK was provided (Fig. 7C).

Because ILK is required to maintain PINCH protein levels, this could account for the loss of PINCH localization at MASs in the absence of ILK. We therefore examined whether overexpression of PINCH would restore its localization. In wild-type embryos, overexpressed PINCH-GFP was distributed in a manner similar to that of endogenous PINCH detected by antibody staining (Fig.

7D–E'). In *ilk^{5/4}* zygotic mutant embryos, overexpressed PINCH-GFP was detected, demonstrating that it had evaded the dependence on ILK for stability, but was not strongly localized at MASs (Fig. 7F, F'). This demonstrates that ILK is required for both the stabilization and localization of PINCH.

We next examined whether the interaction between the ILK ANKRs and the LIM1 domain of PINCH is important for the ILK-dependent recruitment of PINCH. We showed above that the ANKR-containing N-terminus of ILK localized independently of PINCH, so we tested whether this ILK construct could restore PINCH localization in the absence of ILK, and we were surprised to discover that it could not (Fig. 7G–H'). However, PINCH with LIM1 deleted (PINCH^{ΔLIM1}) was poorly localized (Fig. 7I, I'), having a distribution similar to full-length PINCH in the absence of ILK (Fig. 7J, J'). Thus, LIM1 is crucial for PINCH MAS localization, but this does not occur through an interaction with the

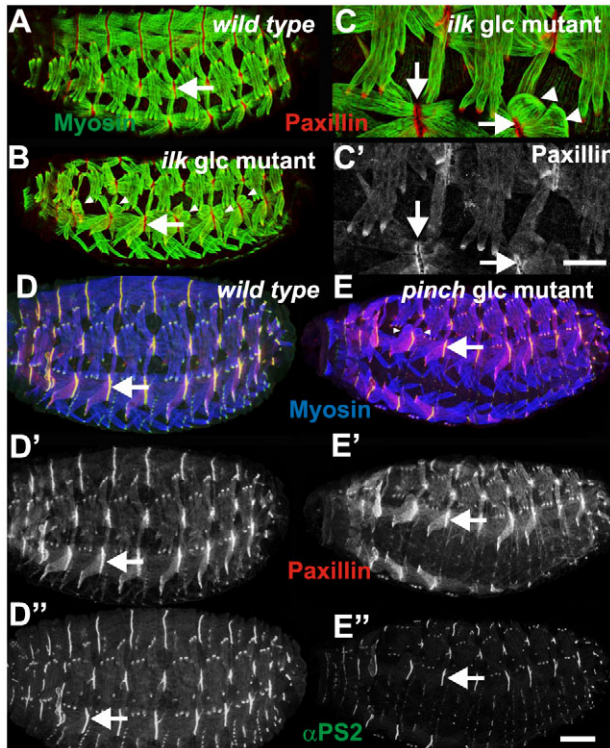


Fig. 8. Paxillin recruitment to MASSes does not require ILK or PINCH. Stage 17 embryos showing paxillin (red and white) and muscle Myosin (green in A–C; blue in D–E), and α PS2 integrin subunit (D,E, green and white). Arrows indicate paxillin localization in wild-type embryos (A,D), and embryos lacking both maternal and zygotic ILK (B,C) or PINCH (E). White arrowheads show detached muscles. Scale bars: 50 μ m (A,B,D,E), 25 μ m (C).

ILK ANKRs. Instead, these results suggest that ILK is indirectly required to recruit PINCH. We were intrigued by the small amount of $\text{PINCH}^{\Delta\text{LIM1}}$ localized to MASSes, suggesting a second recruitment mechanism. This small amount of localization was retained when the zygotic contribution of ILK or PINCH was removed (Fig. 7K,K'). The protein Rsu-1 binds tightly to the LIM5 domain (Kadmas et al., 2004), but mutant animals lacking Rsu-1 still recruited $\text{PINCH}^{\Delta\text{LIM1}}$ (data not shown). Furthermore, we noted that $\text{PINCH}^{\Delta\text{LIM1}}$ was localized to the same extent as full-length ILK in third-instar larvae (Fig. 7L,M), suggesting that the level of the protein that recruits $\text{PINCH}^{\Delta\text{LIM1}}$ increases during development.

Thus, our results show that, in muscles, the LIM1 domain is essential for efficient PINCH localization as MASSes form during embryogenesis. We have also shown that PINCH localization is dependent on ILK, but unexpectedly ILK recruits PINCH indirectly, rather than through the known binding between PINCH LIM1 domain and the ILK ANKRs. This suggests that ILK recruits an unknown factor, which in turn recruits PINCH through the LIM1 domain. We also determined that there is a LIM1-independent pathway for PINCH recruitment, which arises as development progresses.

Paxillin does not require ILK or PINCH for its localization

Finally, we used our new null alleles of *ilk* and *stck* to test whether the interaction between paxillin and ILK plays a role in paxillin localization. We found that embryos lacking both maternal and zygotic ILK or PINCH still recruited paxillin, at levels similar to

that in wild-type embryos (Fig. 8A–E''). This is consistent with the robust paxillin localization at myotendinous junctions in zebrafish embryos lacking ILK (Postel et al., 2008).

Discussion

Establishing a hierarchy of function in the assembly of the integrin–actin link

In cell culture models, focal adhesions are the prototype form of sites of integrin adhesions. Several lines of evidence suggest that the molecular assembly of focal adhesions is a multistep process, where different cytoplasmic proteins enter the adhesion sites in a defined sequential order (Zamir and Geiger, 2001). Here, we provide genetic evidence that supports a hierarchical model for formation of integrin adhesions, using the *Drosophila* embryonic MASSes as a model system. A summary diagram comparing the factors that contribute to ILK recruitment in vertebrate cell focal adhesions and *Drosophila* MASSes is shown in Fig. 9.

ILK recruitment by integrins

First, we re-examined whether integrins are essential for the localization of ILK to MASSes. In a previous study, we found that ILK was recruited independently of integrins (Zervas et al., 2001). Here, we corrected this conclusion, and demonstrated that complete elimination of integrins resulted in loss of ILK recruitment. This result, therefore, reveals that the small amount of maternally provided integrin recruits unexpectedly high levels of ILK. This surprising finding has two important implications: that the recruitment of ILK and maintenance of cell–matrix adhesion have different requirements for the amount of integrin (low levels of integrin are sufficient to recruit ILK, but high levels are needed for functional adhesion); and that the amount of ILK recruited by integrins is not set by the quantity of integrin at MASSes, suggesting that there is an amplification mechanism so that a single integrin can recruit multiple ILK molecules (although our experiments do not reveal the relative number of molecules). A similar, but less extreme, lack of correlation between the levels of integrin and an intracellular integrin-associated protein at MASSes was found previously for talin (Devenport et al., 2007). This apparent amplification could be explained by a multidocking scaffolding protein that is able to recruit, either directly or indirectly, several ILK molecules. Alternatively, ILK could have the ability to employ a variety of additional interactions with different binding partners in the integrin-containing junctions, so that even if integrins or other components are much reduced, multiple ILK molecules are recruited. The mechanism of the amplification is unknown, with a number of the integrin-associated proteins being potential components; the kindlin proteins are particular good candidates, because they bind both integrins and ILK (Karaköse et al., 2010; Rogalski et al., 2000), and Wech has been shown to bind both talin and ILK and to contribute to ILK recruitment (Löer et al., 2008).

Talin is essential for the assembly of the ILK complex

Talin binding to integrins is one of the first molecular events, and is a prerequisite, in the formation of the integrin–cytoskeleton link (Tadokoro et al., 2003). Therefore, we examined whether talin is required to recruit components of the ILK-containing complex at sites of integrin adhesion. As with integrins, it was essential to remove both the maternal and zygotic production of talin to reveal defects in ILK, PINCH and paxillin recruitment. Consequently, these results show an important function for talin in recruiting the ILK-containing complex. A similar role for talin in ILK recruitment

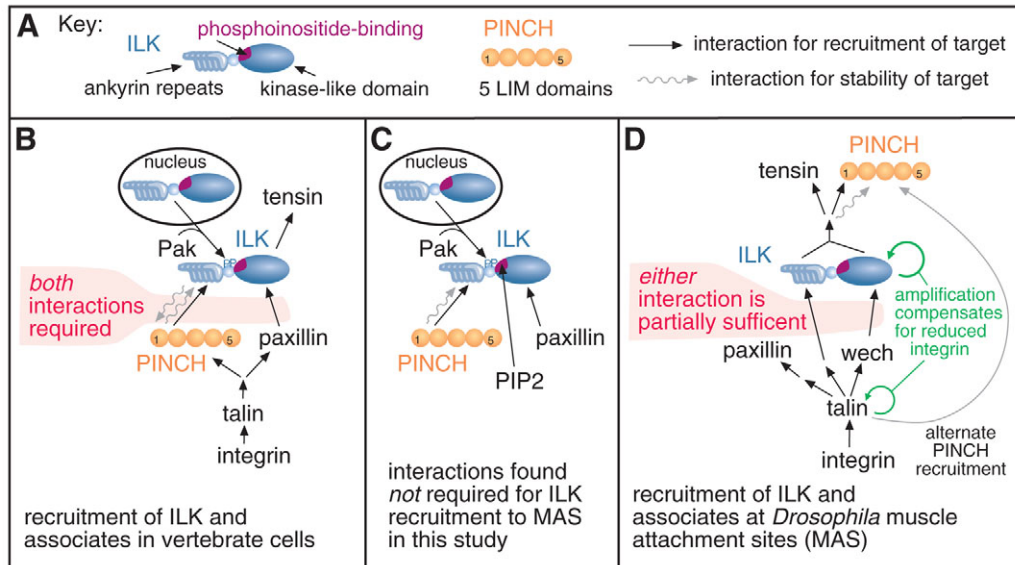


Fig. 9. Comparison of ILK recruitment mechanisms. (A) Key to components in B–D. (B) Factors that contribute to ILK and PINCH recruitment in focal adhesions that we have tested at MASs (see text for references). Note that eliminating the interaction with either PINCH or paxillin results in loss of ILK recruitment. (C) Recruitment and stability interactions that did not contribute to ILK recruitment at MASs. (D) Summary of interactions required for recruitment of ILK and PINCH at MASs. In contrast with focal adhesions, each half of ILK can be recruited on its own, demonstrating that there are parallel recruitment mechanisms, and PINCH functions downstream, rather than upstream, of ILK. The alternate pathway of PINCH recruitment does not require the first LIM domain, and becomes more substantial as larvae develop.

at myotendinous junctions in mice was reported recently (Conti et al., 2009). Currently there are no data to support a direct association between components of the ILK complex and talin, but Wech binds to talin and ILK suggesting that it could be the key linker between the two proteins (Löer et al., 2008), at least in muscles. Therefore we favour a model whereby talin, in concert with integrins, recruits Wech and additional proteins. These proteins then in turn recruit ILK and paxillin, and, as shown here, ILK indirectly recruits PINCH (Fig. 9).

In muscles, ILK is needed to recruit and stabilize PINCH, but PINCH is not required for ILK recruitment or stability

In vertebrate cells ILK and PINCH have mutually dependent functions. Their association is required to protect each other from a proteasome-dependent degradation (Fukuda et al., 2003) and to facilitate each other's localization to focal adhesions (Zhang et al., 2002). The direct interaction between ILK and PINCH has also been confirmed in *Drosophila* (Clark et al., 2003; Kadrmas et al., 2004), and PINCH-mediated stabilization of ILK in mice has been confirmed genetically (Li et al., 2005). In *Drosophila*, we found that neither ILK protein stability nor its recruitment to MASs was affected in the absence of PINCH. This is in agreement with the persistent subcortical localization of ILK in the *pinch-1*-knockout mouse embryoid bodies (Li et al., 2005), as well as in *unc-97* mutants in *C. elegans* (Norman et al., 2007). By contrast, loss of ILK function reduced PINCH levels in *Drosophila* embryos. The instability of PINCH in the absence of ILK was overcome by overexpressing PINCH but, under these conditions, PINCH still was not localized to MASs. The isolated ILK ANKRs did localize to MASs in the absence of endogenous ILK, but were not able to recruit PINCH. Thus, rather than ILK recruiting PINCH by binding of its ANKRs to the LIM1 domain of PINCH, it appears to do so indirectly. This finding raises two important points. First, the interaction between

the ILK ANKRs and LIM1 of PINCH might not be always maintained at sites of integrin adhesion. Our finding that LIM1 is crucial for recruitment, but apparently not through interaction with ILK, suggests alternative interactions might be required. This is consistent with our observation that high levels of PINCH–GFP can be recruited to MASs, even when only the endogenous levels of ILK are present. Second, recent work in mammalian cells provided strong evidence that the primary function of PINCH was the recruitment of ILK through the ANKRs–LIM1 interaction (Stanchi et al., 2009). Notably, the recruitment of tensin and ILK at focal adhesions in cells lacking PINCH could be rescued by a direct fusion of the ILK kinase domain to integrin. However, our result suggests that, in *Drosophila* muscles, the reverse is true, and a primary function of ILK might be the stabilization and recruitment of PINCH.

Interaction between paxillin and ILK does not contribute to ILK recruitment to MASs

Finally, we examined whether ILK and PINCH influence the subcellular localization and protein stability of paxillin. Previous studies have demonstrated an interaction between the kinase domain of mammalian ILK and paxillin, and further suggested that this interaction controls ILK localization at focal adhesions (Nikolopoulos and Turner, 2001). These studies identified key residues in ILK that contribute to paxillin interaction and ILK recruitment, such that ILK mutations at E359 and T384 impaired recruitment of ILK to focal adhesions. However, the ILK E359K mutation does not impair its ability to rescue the null phenotype or alter its recruitment in flies or mice (Sakai et al., 2003; Zervas et al., 2001). Here, we showed that mutating T384 also failed to impair the biological activity or recruitment of ILK, and neither ILK nor PINCH were required for paxillin recruitment. Thus, in MASs, the stability of paxillin and its subcellular localization does not depend on the ILK–PINCH complex.

Insights on ILK function from mutational analysis

In this study, we examined the functions of the two main domains that constitute ILK (i.e. the ANKRs and the kinase domain), as well as specific binding sites that have been proposed to mediate interactions with phosphoinositides or paxillin and have been implicated in the regulation of the protein. From this work, it became evident that deletion of either the ANKRs or the kinase-like domain eliminates the essential functions of ILK in the developing organism. These two domains are required in the same molecule to execute the essential function of ILK. The proposed phosphoinositide-binding motif is dispensable for embryonic development but is required in the epithelial tissues of the wing, and therefore does contribute to ILK function. This suggests that the highest levels of ILK function are required in the wing. This is consistent with the finding that elimination of tensin only causes a wing-blister phenotype, even though it is also concentrated at MASs (Torgler et al., 2004). By contrast, hypomorphic mutations in the gene encoding the α PS2 integrin subunit were found to cause muscle defects but not cause wing blisters (Bloor and Brown, 1998), indicating that integrin adhesive sites are differentially sensitive to loss of different components of the integrin adhesion complex. The residue F436, which is located at the far end of the C-terminal region, was found to be crucial in vertebrate cells, but its mutation only mildly affected recruitment of the overexpressed protein and was dispensable for ILK function. It is possible that the F436A mutation reduces the binding affinity of ILK with Wech.

We found that both the isolated ANKRs and the kinase domain were recruited to MASs, in contrast with the recruitment to focal adhesions seen in mammalian cells (Zhang et al., 2002). This suggests that recruitment is more robust at MASs, so impaired ILK recruitment can still be visualized. We were surprised to find that the recruitment of the ANKRs of ILK did not involve an interaction with PINCH, as removing PINCH did not impair recruitment. Given that there is only one *Drosophila pinch* gene, in contrast with two in mammals, the ANKRs must be binding to another type of protein. One alternative candidate is *Mlp84B*, a protein that contains five LIM domains and is localized at MASs in an integrin-independent manner; it is known that the related zebrafish protein CSR3 binds ILK (Clark et al., 2007; Postel et al., 2008).

In summary, our results have demonstrated that, at the robust integrin adhesive sites that form in the developing muscles, ILK recruitment is much less sensitive to perturbation compared with recruitment in focal adhesions in cells in culture. Our results show that integrins and talin are essential for the recruitment of ILK, and ILK in turn is essential for the recruitment of PINCH. How much of the function of ILK is mediated by PINCH, and whether it also functions independently through its interaction with parvin, will be the focus of our future research.

Materials and Methods

Drosophila strains and genetics

The *ilk*¹, *Df(3L)Pc-14d*, and the genomic ILK-GFP rescue construct were as described previously (Zervas et al., 2001). The allele *ilk*⁵⁴ was isolated in an ethyl methanesulphonate (EMS) screen carried out in the laboratory of Danny Brower (University of Arizona, Tucson, AZ) and was kindly provided to us. This allele contains a premature stop codon after the sixth amino acid of ILK. The *stck*^{T2} allele was generated by the imprecise excision of the P-element EY06672, inserted within the *stck* locus. The molecular lesion in the *stck*^{T2} allele is a deletion of 1615 bp, removing 697 bp upstream of the ATG and 918 bp downstream, resulting in the complete absence of the region encoding the amino acid residues 1–228 of PINCH protein. The alleles *ilk*⁵⁴ and *stck*^{T2} were recombined with FRT sites in order to make germline clones (Chou and Perrimon, 1992). Additional alleles used were *stck*^{3R-17}, *stck*^{3R-18}, *ifB4*, *ifB4*^{ef}, *mys*^{XG43}, *rhea*^{79A}, and the *Gal4* lines *mef2* and *24B* (see FlyBase for references).

DNA constructs and rescue crosses

The genomic rescue fragment for *ilk* was subcloned in the P-element transformation vector pWhiteRabbit (Zervas et al., 2001) and used as a template for PCR mutagenesis, verified by sequencing. All transgenes were fused at the C-terminus to mGFP6 (Kaltschmidt et al., 2000) or mRFP1 [provided by Roger Tsien (Howard Hughes Medical Institute and Department of Pharmacology, University of California, San Diego, CA)] and were subcloned downstream of the *Gal4*-dependent *UAS* promoter in the pGreenRabbit vector derived from *pUAST* (Brand and Perrimon, 1993) or in the genomic *ilk* rescue construct. The ILK deletion constructs generated had the following modifications: Δ 34–177, which removes ANKRs 2–4 (amino acids 33–128) and the middle part of the protein (amino acids 129–177); Δ 178–448, a deletion of the C-terminus part of the ILK protein (amino acids 177–448), which contains the putative phosphoinositide-binding motif (amino acids 180–212) and the entire kinase domain (amino acids 192–448); R208D, R210D; the Δ 178–448 deletion with R208D, R210D; T384A; T384D; S176G, T180G; S176D, T180D; and F436A. All the engineered transgenic lines selected for rescue experiments were introduced in the same genetic background carrying the *Df(3L)Pc-14d*, *e*¹ third chromosome and crossed with the *ilk*¹ or *ilk*⁵⁴ alleles at 25°C.

Antibodies, microscopy of muscles and image analysis

Whole-mount labelling of embryos up to stage 16 was performed as described previously (Zervas et al., 2001), whereas stage 17 embryos were heat-fixed (Miller et al., 1989), and was followed by standard antibody labelling. Primary antibodies used were against: α PS2 (PS2HC2) (rat monoclonal; 1:10) (Bogaert et al., 1987); MHC (myosin heavy chain) (mouse monoclonal; 1:60) (Kiehart et al., 1990); PINCH (rabbit polyclonal; 1:500) (Clark et al., 2003); and paxillin (rabbit polyclonal 1:500) (Chen et al., 2005). The secondary antibodies were conjugated to Alexa-Fluor-488, -568 and -633 (1:500, Molecular Probes). F-actin was probed using either Rhodamine or Alexa-Fluor-635-labelled Phalloidin (R-415 and A34054; Molecular Probes). Images were obtained with a Radiance2000 microscope (Bio-Rad Laboratories), using an oil 60 \times /1.4 NA objective, or a Leica SP5 confocal microscope, using the 20 \times /0.7 NA objective or an oil 63 \times /1.4 NA objective. Two types of confocal optical sections of muscle attachment sites are shown. In some cases, single horizontal optical sections were obtained, which allows discrimination between signals coming from muscles and tendon cells. This view was used to demonstrate the loss of the examined molecule from the muscle ends (Fig. 1, Fig. 2 and Fig. 7A,B). When the examined molecule was localized to some degree, a surface view was used to assess recruitment over a larger number of MASs. This surface view is a stack of images over a depth of 4–5 μ m, starting 2–3 μ m below the surface of the epidermis, and focused on either the lateral longitudinal (Fig. 5, Fig. 6B–D, Fig. 7D–J and Fig. 8C) or dorsal oblique muscles (Fig. 3, Fig. 4 and Fig. 7G,H). Leica SP5 software was used for quantification. The confocal laser power and gain was set appropriately to avoid pixel intensity saturation. Selected areas at MASs or nuclei, or the entire muscle area, were outlined, and the total intensity was measured and plotted using Excel. Images were assembled in Photoshop 7 and labelled in Corel Draw 12.

Protein lysates were prepared from selected stage 17 embryos with the appropriate genotype and were analysed by western blotting using antibodies against PINCH, paxillin and tubulin (Sigma) as a loading control. Blots were then probed with horseradish peroxidase (HRP)-conjugated anti-(rabbit Ig) or anti-(mouse Ig) secondary antibodies (Jackson Laboratories; 1:10,000) and were developed using enhanced chemiluminescence (Amersham).

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/8/1316/DC1>

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Putative ILK phosphorylation sites

PAK phosphorylation consensus **K/RxxS/T**

	176	180	227	245
<i>D. melanogaster</i>	QGL - <u>KTR</u> S <u>RDAT</u>		<u>RQCT</u>	<u>RIFS</u>
<i>C. elegans</i>	KGT - <u>KSRT</u> R <u>DAT</u>		Q <u>SRT</u>	RIFA
<i>M. musculus</i>	<u>KGTT</u> RTR P <u>RNGT</u>		<u>RDWS</u>	<u>RIFS</u>
<i>H. sapiens</i>	<u>KGTT</u> RTR P <u>RNGT</u>		<u>RDWS</u>	<u>RIFS</u>
	173	181	228	246

PKB phosphorylation consensus **RxR xxTL**

	180
<i>D. melanogaster</i>	<u>RSRDATL</u>
<i>C. elegans</i>	<u>RTRDATL</u>
<i>M. musculus</i>	<u>RPRNGTL</u>
<i>H. sapiens</i>	<u>RPRNGTL</u>
	181

ILK-GFP engineered constructs

	Genomic	#lines	UAS	#lines
Wild type	yes	3	yes	3
ANKRs (deletion 178-448)	yes	3	yes	2
Kinase (deletion 34-177)	yes	4	yes	3
R208D,R210D	yes	2	yes	4
Kinase, R208D,R210D	yes	3	yes	2
T384A	yes	2	yes	1
T384D	yes	1	yes	2
S176G,T180G	no	-	yes	2
S176D,T180D	no	-	yes	4
F436A	no	-	yes	3