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RESEARCH ARTICLE

PI(4,5)P2 regulates myoblast fusion through Arp2/3 regulator localization at the fusion site

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ABSTRACT

Cell-cell fusion is a regulated process that requires merging of the opposing membranes and underlying cytoskeletons. However, the integration between membrane and cytoskeleton signaling during fusion is not known. Using *Drosophila*, we demonstrate that the membrane phosphoinositide PI(4,5)P2 is a crucial regulator of F-actin dynamics during myoblast fusion. PI(4,5)P2 is locally enriched and colocalizes spatially and temporally with the F-actin focus that defines the fusion site. PI(4,5)P2 enrichment depends on receptor engagement but is upstream or parallel to actin remodeling. Regulators of actin branching via Arp2/3 colocalize with PI(4,5)P2 *in vivo* and bind PI(4,5)P2 *in vitro*. Manipulation of PI(4,5)P2 availability leads to impaired fusion, with a reduction in the F-actin focus size and altered focus morphology. Mechanistically, the changes in the actin focus are due to a failure in the enrichment of actin regulators at the fusion site. Moreover, improper localization of these regulators hinders expansion of the fusion interface. Thus, PI(4,5)P2 enrichment at the fusion site encodes spatial and temporal information that regulates fusion progression through the localization of activators of actin polymerization.

KEY WORDS: Arp2/3 regulators, *Drosophila*, Myoblast fusion, PI(4,5)P2, PtdIns(4,5)P_2, Actin regulation

INTRODUCTION

Cell-cell fusion is a conserved and highly regulated process that is important in development, homeostasis and disease. Cell-cell fusion requires signaling between transmembrane receptors, the lipid bilayer and the cytoskeleton. These interactions lead to membrane and cytoskeletal rearrangements necessary to achieve cytoplasmic continuity. Although the role of the cytoskeleton during cell fusion has been investigated (Abmayr and Pavlath, 2012), the nature of the membrane-based signaling during fusion remains unclear.

The body wall musculature of the *Drosophila* embryo is a well-characterized system with which to study cell-cell fusion as it allows for genetic analysis and time-lapse imaging of subcellular processes *in vivo*. Myotube syncytia in *Drosophila* arise from two distinct myoblast populations termed founder cells (FCs) and fusion-competent myoblasts (FCMs). Fusion is initiated via recognition and adhesion through two pairs of Nephrin-like family transmembrane receptors: Dumbfounded (Duf; also known as Kirre) and Roughest (Rst) in the FC/myotube and Sticks and stones (Sns) and Hibris (Hbs) in the FCM. Engagement of these receptors prompts bidirectional signaling, resulting in the invasion of the FCM into the FC/myotube. Invasion is mediated by a podosome-like structure (PLS) within the established fusogenic synapse (Abmayr and Pavlath, 2012; Chen, 2011). The PLS is dependent on the Arp2/3 regulators Scar (also known as Wave) and WASp. Within the FCM, Arp2/3 activity is crucial for the formation and dissolution of the F-actin focus, an accumulation of branched actin that demarcates the fusion site in *Drosophila*. Disruption of either Scar or WASp activity leads to fusion defects with persisting F-actin foci (Kesper et al., 2007; Kim et al., 2007; Richardson et al., 2007). However, the mechanisms by which actin branching is coordinated downstream of receptor engagement are unclear.

In mammalian systems, cell membrane-based signaling pathways, including the redistribution of phosphoinositides (PIs), are required for myotube formation (Bach et al., 2010; Leikina et al., 2013; Nowak et al., 2009). One phosphoinositide that is essential for fusion is phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2; also known as PtdIns(4,5)P_2], an important regulator of actin polymerization (Shewan et al., 2011). Direct actin regulators, including WASp, rely on PI(4,5)P2 for localization and activation in other cellular processes (Miki et al., 1996; Papayannopoulos et al., 2005; Tal et al., 2002). Consistent with these data, global depletion of PI(4,5)P2 through chemical treatment in C2C12 myoblasts causes a fusion block (Bach et al., 2010; Leikina et al., 2013). Since the fusion site has not been identified in mammalian systems, how, where and when PI(4,5)P2 acts in fusion remain open questions.

Here, we demonstrate that PI(4,5)P2 enrichment at the fusion site is crucial for fusion in *Drosophila*. PI(4,5)P2 enrichment shows striking similarities with respect to the spatial and temporal dynamics of the F-actin focus, which is the landmark for the *Drosophila* myoblast fusion site. PI(4,5)P2 signaling activity is downstream of receptor-mediated cell-cell attachment, but either upstream or in parallel to branched actin polymerization. Reduction of PI(4,5)P2 leads to a fusion block, with smaller, misshapen actin foci due to mislocalized actin regulators. In addition, mislocalization of these actin regulators, including RacGTP, contributes to a failure in the expansion of the fusion interface. Thus, PI(4,5)P2 functionally links the membrane to receptor-mediated signaling and to actin cytoskeleton rearrangements, establishing crosstalk between these compartments during cell-cell fusion.

RESULTS

PI(4,5)P2 is enriched at the fusion site

PI(4,5)P2 distribution during embryonic myogenesis was recorded *in vivo*, employing time-lapse imaging using two previously described transgenic reporter constructs: $PH^{plc\gamma}$*::GFP* (Pinal et al., 2006) and $PH^{plc\delta}$*::GFP* (Verstreken et al., 2009). Both constructs showed similar localization and dynamics in our system. Signal from the reporters localized evenly throughout the plasma membranes of individual FCMs and FCs/myotubes, except for an enrichment where an FCM adhered to the FC/myotube. The fluorescence intensity at the aligned membranes increased dramatically, indicating an increase in PI(4,5)P2 at this site (Fig. 1A). This enrichment lasted on average 784±30 s

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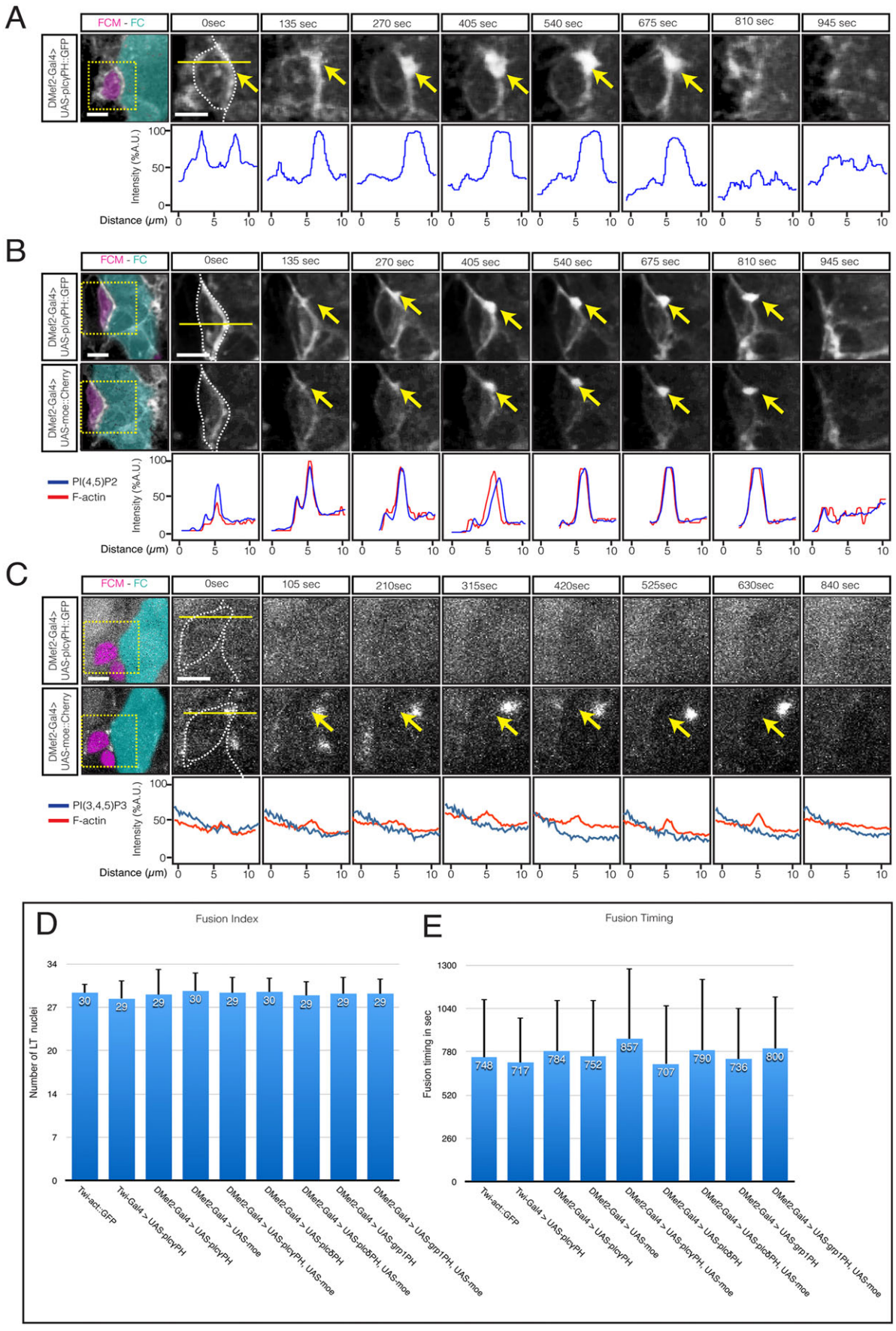

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Fig. 1. PI(4,5)P2 is enriched at the fusion site. (A-C) Live imaging stills of stage 14 *Drosophila* embryos. Boxed areas are magnified to highlight the cell-cell interface of VA1 muscle (turquoise) with attached FCM (magenta). (A) Myoblasts expressing *UAS-PH$^{plc\gamma}$::GFP*. A strong enrichment of PI(4,5)P2 signal with a single peak in fluorescence intensity (line scan at yellow bar; a.u., arbitrary units) is found at the contact site (arrow), which resolves with the fusion event (945 s). (B) Myoblasts expressing *UAS-PH$^{plc\gamma}$::GFP* and *UAS-mCherry::moesin*. Enrichments of PI(4,5)P2 and F-actin reporters (arrows) overlap spatially and temporally. (C) Myoblasts expressing *UAS-PH^{Grp1}::GFP* [PI(3,4,5)P3 reporter] and *UAS-mCherry::moesin*. PI(3,4,5)P3 signal is cytoplasmic and undetectable at the membrane. No signal enrichment can be detected at the F-actin focus before, during or after fusion (arrows, line scans; *n*=14 embryos). Frame rate: 45 s for A,B; 15 s for C. Scale bars: 5 µm. (D) Fusion index showing the total number of dsRed-positive nuclei in LT muscles (*n*=15 embryos). (E) Average time of F-actin focus or PI(4,5)P2 accumulation in control and reporter embryos (*n*=14 embryos). The reporter constructs used in these experiments had no detectable effect on fusion dynamics. Error bars indicate s.d.

(Fig. 1E). Its disappearance coincided with fusion, as determined by the disappearance of the dividing membranes (Fig. 1A; supplementary material Movie 1). Fusion was not affected by the expression of either reporter, as assessed by the lateral transverse (LT) muscle fusion index [total LT muscle nuclei per hemisegment: 29±4 versus control 30±2 (Fig. 1D,E, *n*=14 embryos)].

The enrichment of PI(4,5)P2 at FCM/myotube adhesion sites suggested localization at the fusion site. A well-described hallmark of the *Drosophila* myoblast fusion site is the F-actin focus. To determine whether PI(4,5)P2 colocalized both spatially and temporally with actin at the fusion site, we co-expressed either *PH$^{plc\gamma}$::GFP* or *PH$^{plc\delta}$::GFP* with *mCherry::moe^{act}* (Millard and Martin, 2008) to simultaneously monitor PI(4,5)P2 and F-actin. Importantly, expression of the reporter constructs for PI(4,5)P2 and F-actin did not alter myoblast fusion dynamics nor the fusion index (Fig. 1D,E). PI(4,5)P2 accumulation colocalized both spatially and temporally with F-actin at the fusion site (Fig. 1B; supplementary material Fig. S1 and Movie 2). Together, these data established the discrete temporal and spatial enrichment of PI(4,5)P2 signal at the fusion site.

One aspect of PI(4,5)P2 signaling is its conversion into other phosphoinositide species, most prominently PI(3,4,5)P3 [also known as PtdIns(3,4,5)P_3]. PI(3,4,5)P3 itself is a powerful signaling molecule and a known actin regulator (Insall and Weiner, 2001). Our detection of PI(4,5)P2 enrichment at the fusion site raised the question of whether PI(3,4,5)P3 could also be involved in myoblast fusion.

We examined PI(3,4,5)P3 localization during the fusion process. Expression of a PI(3,4,5)P3 reporter in the developing musculature revealed only cytoplasmic localization [pleckstrin homology (PH) domain of Grp1 (also known as Step), *UAS-PH^{grp1}::GFP* (James et al., 1996; Pickering et al., 2013; von Stein et al., 2005)] (Fig. 1C; supplementary material Movie 3). Moreover, no increase in signal was found in the proximity of the F-actin focus before, during or after the fusion event (Fig. 1C). Expression of this reporter construct in conjunction with mCherry::moe^{act} did not alter fusion dynamics or the fusion index (Fig. 1D,E). Together, these data suggested that PI(3,4,5)P3 is not associated with fusion under these conditions.

PI(4,5)P2 is enriched at the fusion interface of both FCs/myotubes and FCMs

Myoblast fusion is an asymmetric process in which single FCMs actively and irreversibly invade and fuse with the growing FC/myotube (Abmayr and Pavlath, 2012; Chen, 2011). On the subcellular level, this asymmetry manifests in the uneven distribution of F-actin. Specifically, the F-actin focus is present in the FCM, and it has been suggested that the focus forms the backbone for an invasive PLS, which extends from the FCM into the FC/myotube. A thin F-actin sheath is found in the FC/myotube at the fusion site (Sens et al., 2010). To determine whether PI(4,5)P2 localized asymmetrically, the PI(4,5)P2 reporter was first expressed in a single FC/myotube using *5053-GAL4* (Ritzenthaler et al., 2000). When expressed only in the FC/myotube, PI(4,5)P2 accumulated, lasting on average 630±294 s (Fig. 2A, *n*=6 embryos; supplementary material Movie 4). The observed enrichment correlated with fusion, as identified by the sudden discernibility of the fusing FCM via cytoplasmic mixing (Fig. 2A, 720 s, red arrow).

To test whether PI(4,5)P2 accumulated at the fusion site in the FCM, the reporter was expressed specifically in FCMs using *sns-GAL4* (Galletta et al., 2004). To prevent reporter appearance in the myotube after a fusion event, the reporter was expressed in FCMs of *myoblast city* (*mbc*) mutant embryos, in which fusion is blocked prior to cytoplasmic mixing (Doberstein et al., 1997; Rushton et al., 1995).

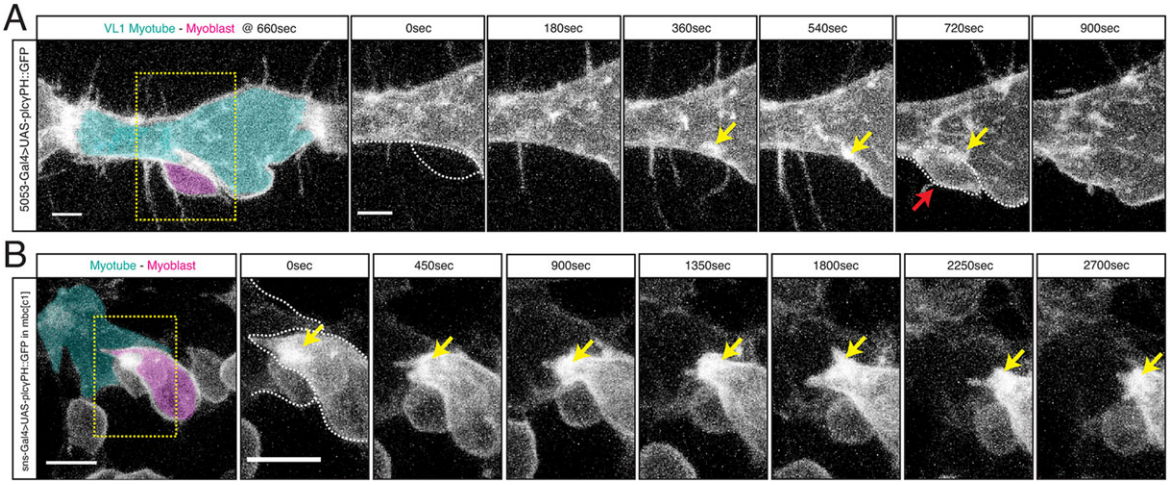

Fig. 2. PI(4,5)P2 is enriched at the fusion interface of FCs/myotubes and FCMs. Time-lapse imaging stills of stage 14 embryos expressing *UAS-PH$^{plc\gamma}$::GFP* under the control of the VL1-specific *5053-Gal4* in wild type (A) or the FCM-specific *sns-GAL4* driver in an *mbc^{c1}* embryo (B). *z*-stack projection stills of VL1 (A) and unidentified FC (B) in turquoise, with an attached FCM in magenta. Boxed area is magnified to highlight the cell-cell interface. (A) Myotube-specific expression revealed PI(4,5)P2 enrichment on the FC/myotube side (yellow arrows) prior to cytoplasmic mixing with FCM (red arrow). Note the numerous filopodial extensions from the myotube. (B) FCM-specific expression in a fusion-defective background shows PI(4,5)P2 accumulation at the fusion site. Frame rate: 45 s. Scale bars: 5 µm.

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FCMs in this mutant established contact with the FC/myotube and formed a stable interface. Furthermore, the F-actin focus formed, grew in size, but did not resolve (Haralalka et al., 2011; Richardson et al., 2007). In this background, the PI(4,5)P2 reporter signal strongly accumulated at the contact site (Fig. 2B; supplementary material Movie 5). Owing to the resulting fusion block, the dynamics of the PI(4,5)P2 accumulation were altered. The overall size of the signal increased, and it persisted for the full length of the time-lapse acquisition (Fig. 2B, 3240 s, n=6). This altered behavior mirrored the changes in F-actin focus dynamics in this mutant background (Haralalka et al., 2011). Together, these results showed that the PI(4,5)P2 enrichment is present at the fusion site both on the myotube and the FCM side. This suggested a role of PI(4,5)P2 in fusion in both cell types.

PI(4,5)P2 enrichment is dependent on cell recognition but not on actin regulation

We next examined where PI(4,5)P2 acts in the fusion pathway by analyzing PI(4,5)P2 distribution *in vivo* in the available fusion mutants. Embryos carrying mutations in the cell-cell recognition molecule Sns showed no PI(4,5)P2 accumulations, placing PI(4,5)P2 signaling downstream of recognition (Fig. 3A,B). Mutations in the Duf recycling protein Rols (TANC1) lead to sporadic contact formation and fusion (Menon and Chia, 2001). In this mutant background, we detected a significant reduction in the number of PI(4,5)P2 accumulations, as we previously showed for the F-actin focus (Richardson et al., 2007); however, in myoblasts that established contact in this background, we found PI(4,5)P2 accumulations concomitant with those fusion events (Fig. 3C).

Mutations in genes that regulate Arp2/3 activity – *loner* (also known as *siz*; mammalian homolog Iqsec), *DWip* [*solitary* (*sltr*), *Vrp1*; Wip], *Rac1*, *mbc* (Dock180), *kette* (*Hem*; Nap1), *blown fuse* (*blow*; no identified mammalian homolog), *Scar* and *WASp* – display F-actin focus formation but no resolution (Abmayr and Pavlath, 2012). In all these backgrounds, PI(4,5)P2 accumulations were detected at the contact sites, indicating that PI(4,5)P2 enrichment is upstream or parallel to Arp2/3 activity (Fig. 3D-K). Finally, mutation of *singles bar* (*sing*; no identified mammalian homolog) leads to a fusion block after contact formation, with clear PI(4,5)P2 enrichment at those sites (Fig. 3L). Thus, PI(4,5)P2 enrichment was dependent on cell-cell recognition but not on Arp2/3-mediated F-actin dynamics, indicating a function downstream of receptor recognition and adhesion but upstream of, or in parallel to, Arp2/3 activation.

PI(4,5)P2 is essential for myoblast fusion

The temporal and spatial colocalization with the specific actin structures at the fusion site suggested that PI(4,5)P2 participates in an essential function during fusion. To address this function, we attempted to manipulate intracellular levels or availability of PI(4,5)P2. Four different approaches were taken to interfere with endogenous PI(4,5)P2 function: (1) targeting PI(4,5)P2-generating pathways using mutant allele and RNAi analysis, taking both zygotic and maternal loss-of-function approaches; (2) sequestering PI(4,5)P2 moieties through overexpression of the reporter to outcompete endogenous binding partners; (3) manipulating the activity of the PI 5-kinase Skittles (Sktl) through overexpression of a kinase-dead variant; and (4) overexpressing Synaptojanin and Inp54 (phosphatases) or NorpA (lipase) to deplete PI(4,5)P2 at the plasma membrane.

Embryos mutant in members of the PI(4,5)P2-generating pathways, including the PI 5-kinase *sktl* and the PI 4-kinase *four wheel drive* (*fwd*), were analyzed for fusion defects. These mutations have previously been used to alter PI(4,5)P2 levels in other contexts. *sktl* mutants display a bristle phenotype (Hassan et al., 1998) due to the loss of PI(4,5)P2 production, whereas *fwd* mutants are defective in spermatid formation (Wong et al., 2005) through the disruption of the PI(4,5)P2 precursor PI(4)P (Polevoy et al., 2009). However, embryos mutant for these genes did not display muscle phenotypes, and fusion appeared normal. Furthermore, double mutants in both genes exhibited normal fusion. Finally, RNAi-mediated knockdown of these genes maternally, zygotically or both, as well as in the mutant backgrounds, did not generate a discernible fusion defect (supplementary material Table S1).

Next, we employed sequestering of PI(4,5)P2 by reporter overexpression. High levels of the reporter are shown to sequester PI(4,5)P2 moieties in the inner leaflet of the plasma membrane and therefore block PI(4,5)P2 signaling (Kwik et al., 2003; Raucher et al., 2000; Wei et al., 2008). To titrate PI(4,5)P2 levels during myoblast fusion, we manipulated both the number of transgenes and temperature. Increased expression levels of the reporter resulted in progressive fusion defects, and fusion was completely blocked at 25°C and 29°C with two copies of both transgenes (Fig. 4A). The severity

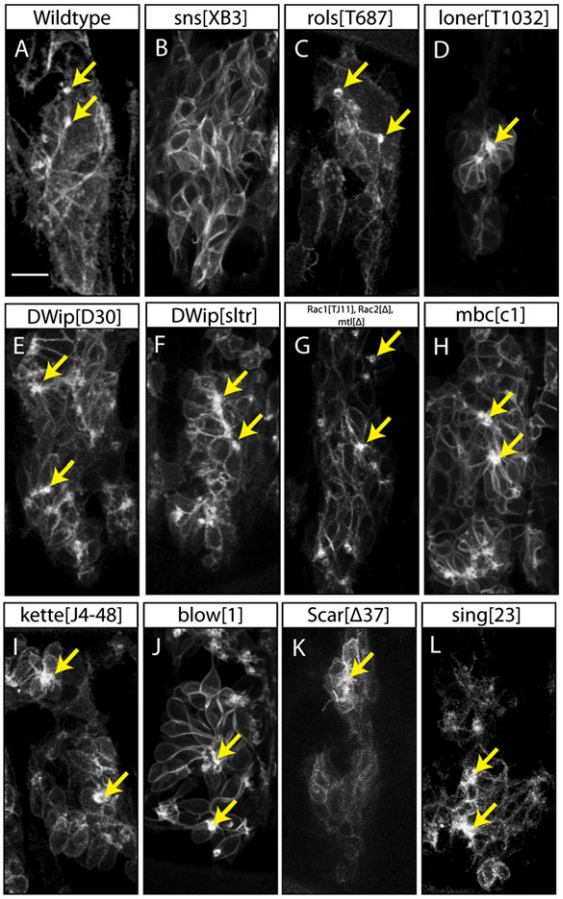

Fig. 3. PI(4,5)P2 enrichment is dependent on cell recognition but not on actin regulators. Time-lapse imaging stills of the PI(4,5)P2 reporter expressed in different fusion mutants. A single ventral section of a stage 14 hemisegment is shown. (A) Wild-type control shows PI(4,5)P2 enrichment at two different fusion sites (arrows). (B) sns^{XB3} homozygous mutant embryo; no PI(4,5)P2 enrichments are detected. (C) $rols^{T687}$ mutants show occasional PI(4,5)P2 enrichments (arrows), consistent with the few fusion events in this background. (D) In $loner^{T1032}$ mutants enrichments are present (arrow), despite a fusion block. In mutants that affect actin focus formation and resolution – (E) $DWip^{D30}$, (F) $DWip^{sltr}$, (G) $Rac1^{j11}$, $Rac2^{\Delta}$, Mtl^{Δ} triple mutant, (H) mbc^{c1}, (I) $kette^{J4-48}$ (J) $blow^{1}$ and (K) $Scar^{\Delta 37}$ – PI(4,5)P2 enrichments are detected (arrows). In $sing^{23}$ (L), PI(4,5)P2 enrichment is unaffected (arrows), despite a block in fusion. Scale bar: 10 µm.

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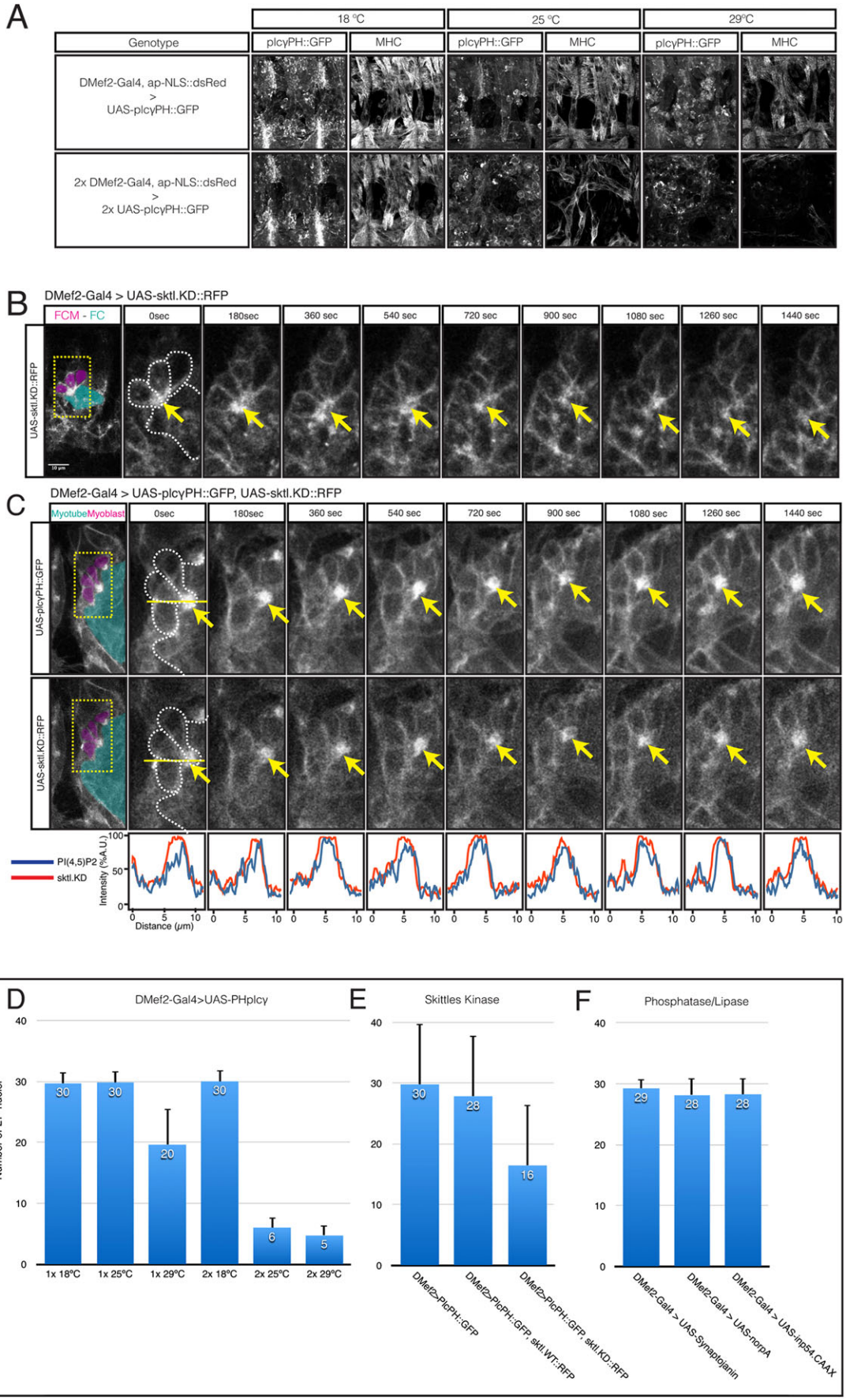

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Fig. 4. PI(4,5)P2 is essential for myoblast fusion. (A) Sequestration of PI(4,5)P2 leads to myoblast fusion block. Levels of *UAS-PH$^{plc\gamma}$::GFP* reporter were increased through temperature and copy number [1× (top) and 2× (bottom) *DMef2-Gal4>UAS-PH$^{plc\gamma}$::GFP*]. Myoblasts were double labeled for *PH$^{plc\gamma}$::GFP* and Myosin heavy chain (MHC). Single-copy expression appears as wild type at 18°C and 25°C with no fusion defects. At 29°C, single copy leads to a mild fusion defect. Increasing copy number produces a highly penetrant fusion block, with a complete block at 25°C and 29°C. (B) Time-lapse imaging stills of stage 14 embryos expressing two copies of *UAS-sktl.KD::RFP*. Arrows indicate contact sites of FCMs (magenta) with the FC/myotube (turquoise) and an enrichment of Sktl.KD::RFP. Fusion is stalled at stable contact formation and does not resolve over the total acquisition time (65 min). (C) Time-lapse sequence stills of stage 14 embryos expressing single copies of *UAS-PH$^{plc\gamma}$::GFP* and *UAS-sktl.KD::RFP*. Arrows indicate contact sites of FCMs (magenta) with the FC/myotube (turquoise). Signals for Sktl.KD::RFP and *PH$^{plc\gamma}$::GFP* are enriched at the contact site. Fusion is stalled at stable contact formation and does not resolve over the total acquisition time (45 min). Frame rate: 15 s in A,B. Scale bar: 10 µm. (D-F) Fusion indices based on nuclei counts in LT muscles expressing *apterous-NLS::dsRed*. (D) *PH$^{plc\gamma}$::GFP* overexpression. (E) sktl.WT and sktl.KD overexpression. (F) PI(4,5)P2 depletion through phosphatase (Synaptojanin, Inp54.CAAX)/lipase (NorpA) overexpression. No reduction in fusion index was observed. Error bars indicate s.d.

of the fusion defects under these conditions was assessed via the fusion index, which showed a progressive decrease in fusion (Fig. 4D). FC/FCM differentiation was unaffected under these conditions, as demonstrated by the expression of muscle Myosin heavy chain (MHC) (Fig. 4A) and the muscle identity gene *apterous* (Fig. 4D) (Bourgouin et al., 1992). Hence, sequestration of PI(4,5)P2 at the plasma membrane through reporter overexpression blocked myoblast fusion.

We next asked whether the fusion arrest observed in the sequestration experiment was due to masking of PI(4,5)P2 for endogenous binding partners or if the defect was caused by lessening the amount of PI(4,5)P2 in the composition of the plasma membrane at the fusion site. A kinase-dead version of Sktl had been shown to lower PI(4,5)P2 production during photoreceptor biogenesis (Raghu et al., 2009) and during mouse phagocytosis (Fairn et al., 2009). We first examined the expression of wild-type Sktl::RFP in the mesoderm together with PH$^{plc\gamma}$::GFP, which combined did not affect fusion (Fig. 4E, n=14; supplementary material Fig. S2). The prospective fusion site showed clear enrichment of GFP and RFP, indicating the recruitment of Sktl::RFP to the contact site. Expression of one copy of the kinase-dead version of Sktl (*UAS-Sktl.KD::RFP*) (Raghu et al., 2009) in the developing musculature did not affect myoblast fusion (data not shown). However, higher levels of expression, resulting from two copies of the transgene, led to a fusion arrest (Fig. 4B,E, n=14; supplementary material Movie 6). Expression of single copies of both *sktl.KD::RFP* and *PH$^{plc\gamma}$::GFP* (Fig. 4C,E, n=14; supplementary material Movie 7) similarly blocked fusion. In both instances, neither Sktl::RFP nor PH$^{plc\gamma}$::GFP was released significantly from the membrane, suggesting that PI(4,5)P2 is still present and merely masked under these conditions. This finding is in agreement with observations in mouse RAW phagocytes (Fairn et al., 2009). Further, under these conditions several myoblasts were found attached to each myotube. These contact sites showed enrichment of Sktl.KD::RFP and the PH$^{plc\gamma}$::GFP reporter (Fig. 4B,C) over the entire length of time-lapse acquisition (Fig. 4C, t=2700 s, n=14), reinforcing a role for PI(4,5)P2 in fusion.

We next aimed to lower the levels of PI(4,5)P2 through depletion rather than affecting its generation or through sequestration. Overexpression in the mesoderm of the *Drosophila* inositol polyphosphate 5-phosphatase Synaptojanin, which has been shown to alter PI(4,5)P2 levels during synaptic development (Dickman et al., 2006), did not affect fusion (Fig. 4F). Similarly, expression of the *Drosophila* phosphatidylinositol phospholipase C NorpA, which hydrolyzes PI(4,5)P2 into DAG and IP3, did not alter the fusion process (Fig. 4F). We also introduced the gene encoding yeast polyphosphatase *INP54* fused to a membrane-targeting CAAX domain (Inp54.CAAX) into the *Drosophila* genome (Várnai et al., 2006; Bach et al., 2010). Expression of this transgene in the mesoderm/muscle again failed to affect fusion (Fig. 4F).

To summarize, loss-of-function mutations in PI(4,5)P2-generating pathways did not result in discernible muscle phenotypes. Likewise, overexpression of phosphatases or lipases did not affect fusion. We were also unable to perturb PI(3,4,5)P3 signaling by similar approaches (supplementary material Fig. S3). However, two methods that affect PI(4,5)P2 availability blocked fusion: (1) competition of endogenous binding partners of PI(4,5)P2 through overexpression of PH$^{plc\gamma}$ and Sktl.KD; and (2) overexpression of Sktl.KD at high levels to affect PI(4,5)P2 production and mask resident PI(4,5)P2. Together, these data supported a crucial role for PI(4,5)P2 during myoblast fusion.

PI(4,5)P2 sequestering affects actin focus formation and fusion site expansion

We concentrated our analysis on the PI(4,5)P2 sequestration-derived fusion block (*2xDMef2-Gal4; 2xUAS-PH$^{plc\gamma}$::GFP*). We first examined whether an F-actin focus forms under these conditions. Previous reports described either no F-actin focus, as found in embryos carrying mutations in the recognition and adhesion receptors such as Duf or Sns, or normal sized to enlarged foci, as found in embryos carrying mutations in Arp2/3 regulatory pathways (Richardson et al., 2007). Under sequestering conditions, the majority of FCMs showed a clear accumulation of PI(4,5)P2 and F-actin (Fig. 5A). Specifically, over 75% of these PI(4,5)P2 accumulations were also a site of a distinct F-actin accumulation reminiscent of the F-actin focus (Fig. 5A). These foci were considerably smaller than normal actin foci (1.12±0.34 µm^2 versus control 2.01±0.52 µm^2, n=25). In addition, these foci did not resemble the PLS (Sens et al., 2010), as they were flat in morphology and appeared non-protrusive. Such smaller foci have not been described previously in a fusion mutant. In addition, most FCMs in this fixed preparation were not attached to a myotube. Since the F-actin focus only forms in FCMs attached to a myotube (Richardson et al., 2007), these data suggested that these FCMs detached after the initial focus formation. Nevertheless, the reduced actin focus size in both attached and detached FCMs reinforced that PI(4,5)P2 regulates F-actin.

Focus formation relies on recognition and adhesion between the FC/myotube and the FCM to create a fusogenic synapse competent to form an F-actin focus. We examined whether localization of the recognition and adhesion receptors Duf and Sns was affected when PI(4,5)P2 was sequestered. The FC/myotube-specific receptor Duf was correctly localized at the membrane, in close proximity to the PI(4,5)P2 enrichment when an FCM was attached (Fig. 5B). No enrichment for Duf could be detected on free FC/myotube membranes or on detached FCMs that contained an F-actin focus (Fig. 5B, bottom panel). Additionally, Sns localized correctly to the fusion site in attached FCMs (Fig. 5C, top panel), colocalizing with increased PI(4,5)P2 signal and decreased F-actin (Fig. 5C). In detached cells, Sns was detected in close proximity to the PI(4,5)P2 enrichment and F-actin focus. However, the Sns signal appeared to be in puncta (Fig. 5C, bottom panel) that did not resemble the ring structure described previously (Kesper et al., 2007). Consistent with

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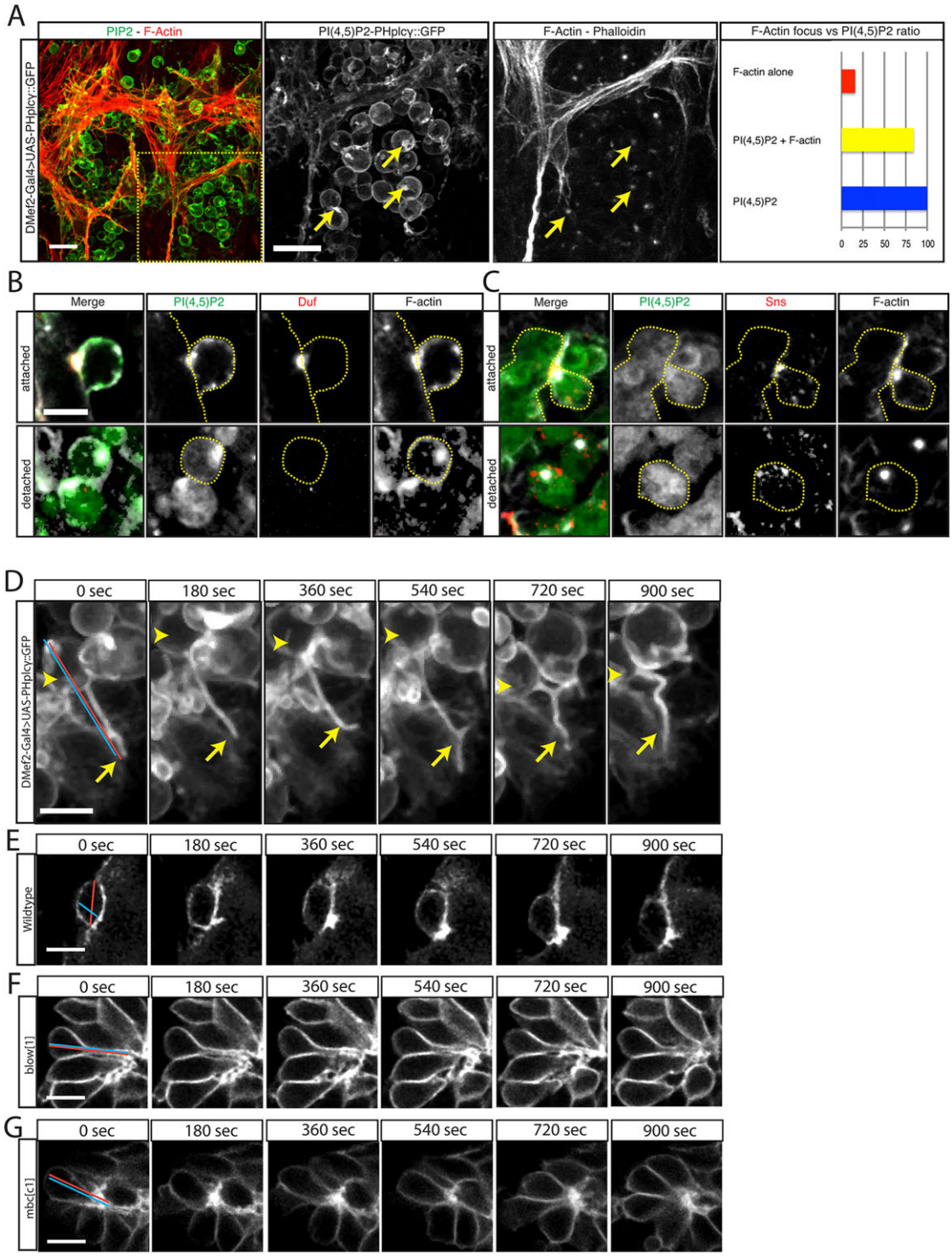

Fig. 5. Effect of PI(4,5)P2 sequestering on FC/myotube-FCM attachment. (A) Fixed preparations of sequestration-induced fusion block in stage 15 embryos. FCMs display distinct PI(4,5)P2 aggregations (arrows) that colocalize with remnant F-actin foci (Phalloidin, arrows) in 75% of all observed enrichments [bar chart: F-actin focus without PI(4,5)P2 accumulation, red; focus with PI(4,5)P2 accumulation, yellow; all observed PI(4,5)P2 accumulations, blue]. (B,C) Receptor localization is largely unaffected by PI(4,5)P2 sequestration. FC/myotube receptor Duf (B) is present at the fusion site at the attached FCM (top), but absent in unattached FCM (bottom). FCM receptor Sns (C) is present at the fusion site in attached myoblasts, yet displays uneven distribution around the F-actin focus remnant in detached FCMs (bottom). (D) Time-lapse sequence stills of stage 14 embryos with sequester-induced fusion block. A long thin protrusion (arrow) links the FCM (arrowhead indicates cell body) with the FC/myotube. Note that no PI(4,5)P2 accumulation is visible at this contact site. Cells do not fuse but also do not lose connection. (D-G) Live imaging stills of FCMs attached to a fusion partner. Aspect ratios of the FCMs were measured: red line, longest possible axis in the FCM; blue line, axis perpendicular to fusion interface. (D) In PI(4,5)P sequestration embryos, the FCM is almost spherical with a long protrusion; the longest axis and the axis perpendicular to the fusion interface are similarly long. (E) Control embryo in which the FCM is aligned to the myotube, with the FCM longest axis almost parallel to the cell-cell interface. The axis perpendicular to the fusion site is the shortest axis. In (F) *blow*^{1} and (G) *mbc*^{c1} mutant embryos, FCMs are attached to their fusion partner but unable to fuse; the longest axis and the perpendicular fusion interface axis are similar, indicating a lack of wild-type cell body alignment and fusion interface expansion. Frame rate: 45 s. Scale bars: 10 µm in A; 5 µm in B-G.

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our analysis of PI(4,5)P2 expression in pathway mutants, these data indicated that PI(4,5)P2 sequestration did not inhibit initial cell-cell contact nor initiation of the fusion process.

The detachment of FCMs observed in the fixed preparations motivated us to investigate FCM behavior using live imaging. Time-lapse imaging of the PI(4,5)P2-sequestered myoblasts revealed no fusion events (n=6 movies examined; supplementary material Movie 8). However, unlike the fixed preparations, the majority of FCMs were in contact with an FC/myotube. These FCMs displayed an unusual spherical morphology, with a single long, thin filopod connecting the FCMs to a FC/myotube (Fig. 5D, arrows, arrowhead for cell body). These FCMs did not show any directed movement towards or away from the myotube. Since recognition and adhesion occurred normally in the sequestered conditions and smaller actin foci formed, we interpreted the observed FCM behavior as FCMs successfully making initial contact with the FC/myotube through filopodia. These FCMs, however, were unable to continue with the fusion process, failing to align their cell bodies with the cell body of the FC/myotube fusion partner and to expand the fusion interface (compare Fig. 5D with 5E). Altogether, masking of PI(4,5)P2 did not affect initial recognition and adhesion of FCMs to FCs, but blocked progression of fusion, owing to a smaller F-actin focus and a defect in fusion interface expansion. Importantly, the observed detachment phenotype in fixed preparations is not apparent in live imaging experiments, indicating that it is a fixation artifact.

As further support, we compared FCM morphology and behaviors between control, PI(4,5)P2 sequestration and Arp2/3 pathway mutants. Time-lapse imaging revealed distinct cellular behaviors of the FCMs in these backgrounds. In control embryos, FCMs were in close contact with their fusion partners and their cell bodies were aligned with the fusion interface. In this situation, the shortest axis of the FCM was perpendicular to the fusion interface, while the longest axis was parallel to it (Fig. 5E). By contrast, embryos mutant for regulators of either the Scar or WASp pathway, specifically *mbc* and *blow*, respectively, displayed an elongated FCM morphology (Fig. 5F,G). In both cases, the longest axis of the cell was perpendicular to the fusion interface, a morphology previously described as teardrop-shaped (Doberstein et al., 1997; Abmayr and Pavlath, 2012). Sequestration of PI(4,5)P2 led to cells that were more spherical and that were connected to the FC and the fusion interface only through a single, thin extension (Fig. 5D). These data suggested that PI(4,5)P2 not only controls F-actin focus generation but also enables the enlargement of the fusogenic interface through changes in FCM morphology.

PI(4,5)P2 regulates RacGTP localization through Mbc

PI(4,5)P2 regulates the localization and activity of actin regulators by binding to the pleckstrin homology (PH) and calcium-binding (C2) domains of these factors (Lemmon, 2008). Several proteins implicated in myoblast fusion, including Mbc, Loner, Blow and WASp, have domains that could bind to phosphoinositides (PIPs), suggesting that one way in which PI(4,5)P2 sequestration blocks fusion is by affecting the localization of these proteins. Thus, we first tested for *in vitro* lipid binding using the following putative PIP-binding domains: two versions of the Mbc C2 domain (short, 444-633; long, 441-678) and the PH domain of Loner (991-1089), Blow (207-305) and WASp (35-104) (Fig. 6A).

Each construct showed binding to phosphoinositides but not to other lipids (Fig. 6A). BlowPH displayed strong interaction with all phosphoinositides except PI(3,4)P2. LonerPH interacted strongly with the singly phosphorylated inositols and all PIP2 species, but weakly with PI(3,4,5)P3. Mbc-short bound to singly phosphorylated PIPs, PI(4,5)P2 and PI(3,5)P2. Mbc-long bound the same phosphoinositides as Mbc-short, yet it bound more weakly to PI(3)P and PI(4)P. Both versions of Mbc bound only weakly, if at all, to PI(3,4,5)P3. WASpPH, like LonerPH, bound all inositides apart from PI(3,4,5)P3, as previously described (Sakurai et al., 2011). In summary, all examined proteins can bind PI(4,5)P2 in this assay and thus could be regulated through PI(4,5)P2 binding in this context.

We next addressed whether phospholipid binding is necessary for the proper localization of these proteins during *Drosophila* myoblast fusion *in vivo*. We compared endogenous protein localization in control embryos (1× *DMef2-Gal4>UAS-PH^{plcγ}::GFP*) with PI(4,5)P2-sequestering embryos (2× *DMef2-Gal4>UAS-PH^{plcγ}::GFP*) (n≥25 embryos/protein). In control embryos, Blow was distributed along the FCM plasma membrane with a slight accumulation at the F-actin focus. Blow was not detected in the FC, as previously described (Jin et al., 2011) (Fig. 6B). In sequestering embryos, Blow was absent from the fusion site and localized in puncta on the plasma membrane of attached FCMs (Fig. 6B). In control embryos, Loner did not colocalize with the F-actin focus (Richardson et al., 2007) nor with the PI(4,5)P2 accumulation at the fusion site, but instead localized in cytoplasmic puncta in both FCs/myotubes and FCMs. This Loner distribution was similar in the PI(4,5)P2-sequestered embryos (Fig. 6C), despite the *in vitro* lipid binding data that showed that Loner could bind PI(4,5)P2 (Fig. 6A). In control embryos, Mbc accumulated at the base of the F-actin focus specifically within the FCM (Fig. 6D). In sequestered embryos, Mbc did not colocalize with F-actin and PI(4,5)P2 reporter at the fusion site (Fig. 6D). Moreover, Mbc was no longer present at the membrane but concentrated in the cytoplasm.

Rac localization is dependent on GEF-phosphoinositide interactions, and Mbc is the primary RacGEF in myoblast fusion (Haralalka et al., 2011). Based on the aberrant localization of Mbc in the PI(4,5)P2-sequestered FCMs, we hypothesized that Rac signaling could also be affected. We find that RacGTP is concentrated at the fusion site in control FCMs (Haralalka et al., 2011) (Fig. 6E), in close proximity to the F-actin focus and PI(4,5)P2 reporter accumulation. By contrast, when Mbc is mislocalized due to PI(4,5)P2 masking, activated Rac is absent from the fusion site and concentrates on the distal side of the cell with respect to the fusion interface (Fig. 6E, bottom panel). We verified that this shift in RacGTP localization is dependent on correct Mbc localization through analysis of *mbc^{c1}* mutant embryos. In these embryos, RacGTP was absent from the fusion site and also concentrated at the distal side of the cell (Fig. 6F). These data indicated that PI(4,5)P2 is necessary for the correct localization of RacGTP through regulation of Mbc at the fusion site.

PI(4,5)P2 localizes the Arp2/3 fusion machinery at the fusion site

The observed mislocalization of activated Rac and its upstream regulator Mbc indicated that downstream targets of the Mbc-Rac pathway might also be affected. Known downstream targets of Rac in myoblast fusion include the actin branching complex Arp2/3. We asked whether the reduced size of the F-actin focus was caused by a change in Arp2/3-mediated actin nucleation. The activator of Arp2/3, Scar, which binds to phosphoinositides through its basic domain (Stephan et al., 2011), was present at the cell membrane and concentrated at the F-actin focus in the control, but its expression was diffuse in the PI(4,5)P2-sequestered embryos (Fig. 7A). We also tested WASp, the second Arp2/3 activator; it was strongly localized to the fusion site in the FCM, where it overlapped with the F-actin focus in control embryos (Fig. 7B). In PI(4,5)P2-sequestered embryos, WASp was unevenly distributed throughout the cell (Fig. 7B).

We further hypothesized that the mislocalization of both Scar and WASp could be caused by the lack of direct binding to PI(4,5)P2, as

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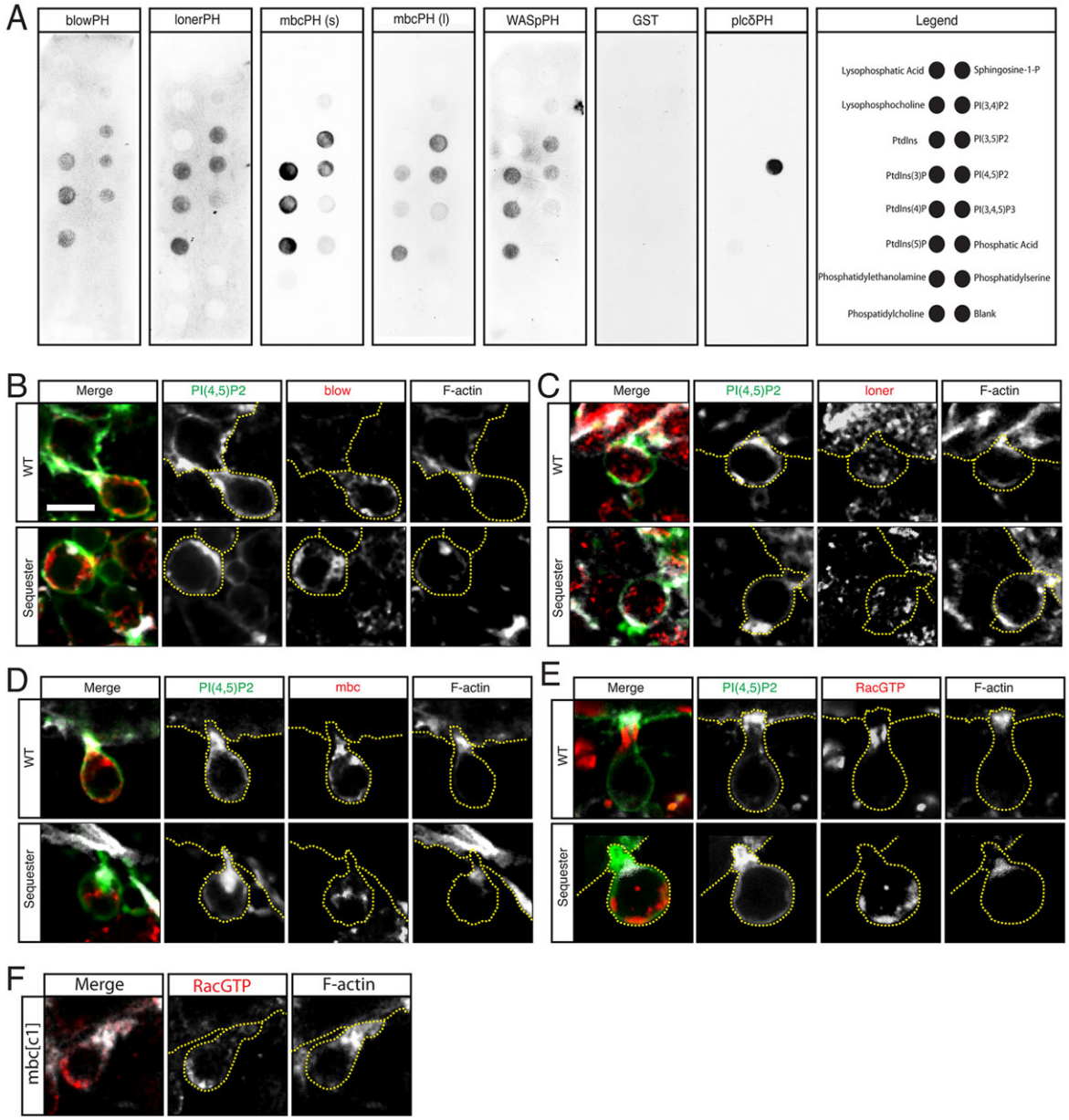

Fig. 6. PI(4,5)P2 acts on RacGTP localization through Mbc. (A) Relative phospholipid binding of known fusion facilitators. Equal concentrations of PH::GST fusion proteins were tested on phospholipid strips. s, MbcC2-short; l, MbcC2-long; GST is a negative control; plcδPH is PI(4,5)P2 positive control. Note that binding is predominantly to single phosphoinositide and PIP2 species. (B-F) Single confocal slice of a stage 15 FCM attached to FC showing localization of endogenous (B) Blow, (C) Loner, (D) Mbc and (E) RacGTP in wild-type (WT) and PI(4,5)P2-sequestering (Sequester) embryos with respect to PI(4,5)P2 signal and the F-actin focus. (B) Blow is localized at the FCM cortex, and slightly enriched at the fusion site in WT; it is diffuse and less concentrated in PI(4,5)P2-sequestering conditions. (C) Loner is similarly present throughout the FCM in puncta in WT and PI(4,5)P2-sequestering conditions. (D) In WT, Mbc is localized at the FCM cortex and the F-actin focus, but is depleted from the cortex and fusion site when PI(4,5)P2 is masked. (E) RacGTP localizes specifically to the fusion interface in WT, but displays a reversed, distal localization in the sequestering background. (F) Loss of Mbc prevents activation of RacGDP to RacGTP at the fusion site. mbc^{c1} mutant embryos show the same reversal of RacGTP localization, distally from the F-actin focus at the fusion site. *n*=25 cells/genotype. Scale bar: 5 µm.

well as by the mislocalization of their upstream regulators Mbc and Blow. Analysis of mbc^{c1} and $blow^{1}$ embryos revealed that Scar is correctly localized at the fusion site in these mutant backgrounds, albeit at lower levels in the mbc^{c1} mutant (Fig. 7C). WASp localization was severely affected in the $blow^{1}$ mutant background; its levels were also significantly reduced. In mbc^{c1} embryos, WASp localization and distribution appeared to be unaffected (Fig. 7D). If our hypothesis was correct that the smaller F-actin focus phenotype in the PI(4,5)P2-sequestered embryos was due to diminished Scar and WASp activity, then a mutant background that abolishes activation of both proteins should result in the absence of an F-actin focus. We analyzed double mutants of the Scar and WASp upstream regulators $kette^{J4\text{-}48}$ and $DWip^{D30}$ or $sltr^{1946}$ for F-actin formation. F-actin formation was indeed abolished in these double-mutant embryos, whereas FCM/FC recognition and attachment appeared correct (Fig. 7E,F). The FCMs in these double mutants displayed the characteristic teardrop shape, suggesting a failure in fusion interface expansion. Together, these data indicated that masking of PI(4,5)P2 leads to mislocalization of Scar and WASp, which, in turn, fails to generate the protrusive F-actin focus and fusion

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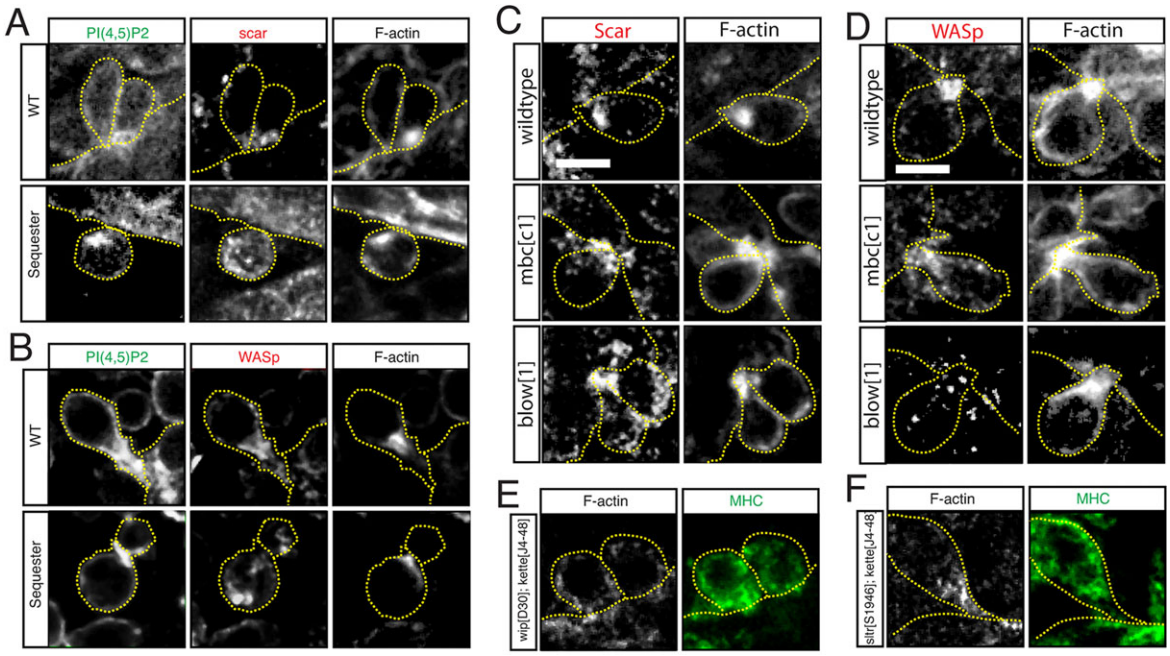

Fig. 7. PI(4,5)P2 acts as a localization signal for the Arp2/3 fusion machinery. Single confocal slice of stage 15 FCMs adhered to FC/myotube. (A,B) Localization of endogenous Scar (A) and WASp (B) in wild-type (WT) and PI(4,5)P2-sequestering (Sequester) embryos with respect to PI(4,5)P2 signal and the F-actin focus. (A) Scar localizes to the fusion interface in WT, whereas it is cytoplasmic in sequestration conditions. (B) WASp localizes to the cortex and to the fusion site in WT; it shows a cytoplasmic localization in the sequestering background. (C,D) Localization of endogenous Scar (C) and WASp (D) in WT, *mbc*^{c1} and *blow*^{1} mutant embryos. Note the absence of WASp signal in the *blow*^{1} embryos. (E,F) Double-mutant analysis for *DWip* and *kette*, which abolishes WASp and Scar activity and shows no F-actin focus at the fusion sites. Two different null alleles for *DWip* were used – (E) *DWip*^{D30} and (F) *sltr*^{1946} – with *kette*$^{J4-48}$. *n*=25 cells/genotype. Scale bars: 5 µm.

interface expansion. The remnant F-actin focus that is observed is most likely due to residual activity of WASp and Scar in the sequestration background.

DISCUSSION

We find that a specific enrichment of PI(4,5)P2 at the interface between a fusing FCM and FC demarcates the fusion site. This PI(4,5)P2 enrichment shows both spatial and temporal overlap with actin at the fusion site and is present on the membranes of both fusion partners. In this work we focused on the role of PI(4,5)P2 and its relationship with an FCM-specific actin structure, the F-actin focus. The F-actin focus, which forms the core of the invasive podosome, is necessary for fusion and has been suggested to provide an invasive force through Arp2/3-mediated actin dynamics (Sens et al., 2010). The relationship that we have uncovered between PI(4,5)P2 and localization of the Arp2/3 regulatory hierarchy at the fusion site significantly extends our knowledge by placing PI(4,5)P2 as an integral part of the invasive fusion machinery and fusion progression in *Drosophila*.

Disruption of Arp2/3-activating pathways blocks cell-cell fusion (Abmayr and Pavlath, 2012). The two Arp2/3-activating proteins important for myoblast fusion are Scar and WASp. These are regulated by the Mbc-Rac-Kette and Blow-DWip pathways, respectively. Mutation in either one of these pathways leads to a fusion block, which is characterized by adherent cells with enlarged actin foci (Kesper et al., 2007; Kim et al., 2007; Richardson et al., 2007). Our phospholipid overlay assays suggest that pathway members that bear a putative lipid-binding domain can interact with phosphoinositides. These phospholipid overlay data, although unsuitable to detect specific binding preferences, provide a rough guide to the binding specificity and suggest that Mbc, Blow, Loner and WASp have the potential to bind PI(4,5)P2. It is interesting to note that Mbc bound only weakly, if at all, to PI(3,4,5)P3 in this assay; this is in contrast to published data in which Mbc binds strongly to PI(3,4,5)P3 (Balagopalan et al., 2006; Côté et al., 2005; Kobayashi et al., 2001). However, examination of the localization of these proteins in FCMs with PI(4,5)P2 sequestration was revealing: we find mislocalization of Scar and its regulator Mbc. Similarly, WASp and its regulator Blow are mislocalized upon PI(4,5)P2 sequestration. Both layers of Arp2/3 regulation require PI(4,5)P2 activity for proper localization. As a result of mislocalization of these regulators, the F-actin focus, which is dependent on Arp2/3 activity, is smaller than normal. Consistent with this result, we show that embryos double mutant for genes in these pathways, i.e. *DWip* (WASp) and *kette* (Scar), are defective in focus formation. This also agrees with data from adult *Drosophila* reporting that Arp2/3 knockdown results in an absence of focus formation (Mukherjee et al., 2011). Nevertheless, small foci are found in our PI(4,5)2 sequestration myoblasts. We reason that this could be due to incomplete masking of PI(4,5)P2 in our assay that allows for baseline Arp2/3 activity at the fusion site.

The observed change in Rac activity localization in the PI(4,5)P2-sequestering embryos is an intriguing finding. The general importance of Rac activity in myoblast fusion has been described previously (Chen and Olson, 2001; Hakeda-Suzuki et al., 2002; Haralalka et al., 2011). Our finding that RacGTP is mislocalized away from the fusion site on the distal side of the cell in both PI(4,5)P2-sequestering embryos and in the *mbc* mutant embryos suggests a signaling cascade from PI(4,5)P2 via Mbc to Rac activation. It is interesting to note that RacGTP immunoreactivity is not abolished, but is merely relocalized, under these conditions, resulting in deleterious effects on fusion progression. These data agree with the finding that *mbc*^{c1} mutant embryos cannot be rescued by constitutively active Rac1 overexpression, since correct localization of RacGTP is not ensured (Haralalka et al., 2011).

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Live imaging data revealed that masking of PI(4,5)P2 inhibits fusion and leads to an unusual FCM morphology and behavior, specifically a spherical FCM with a single long, thin protrusion connecting to the FC/myotube. This phenotype is reminiscent of published phenotypes in which either the Mbc-Rac-Kette-Scar or the Blow-DWip-WASp pathway is disrupted, resulting in characteristic teardrop-shaped FCMs (Abmayr and Pavlath, 2012). The FCM teardrop morphology (Chen and Olson, 2001; Hakeda-Suzuki et al., 2002; Haralalka et al., 2011; Mukherjee et al., 2011; Richardson et al., 2007) observed in these mutants via time-lapse analysis suggests an additional function of Arp2/3 in the expansion of the fusion interface between the fusion partners through changes in FCM morphology. We note that the use of live imaging is key in this regard, as the movement of the FCMs could be monitored, and the integrity of the filopodial processes, particularly in the sequestering background, could be maintained.

We propose a model in which receptor-mediated recognition and adhesion triggers a signaling cascade that leads to a build up of PI(4,5)P2 at the fusion site through an as yet unidentified kinase(s). PI(4,5)P2 then facilitates correct localization of Mbc, which in turn concentrates Rac activity and Blow at the fusion site. PI(4,5)P2 and these upstream regulators recruit Scar and WASp, which then activate Arp2/3. The extensive actin cytoskeletal rearrangements that are mediated via Scar and WASp activation of Arp2/3 are crucial for the formation of an invasive F-actin-based podosome structure as well as for expansion of the fusogenic synapse, without which fusion cannot progress.

Myoblast fusion is an inherently asymmetric process, with single myoblasts invading and fusing to larger multinucleated myotubes. In *Drosophila*, this asymmetry extends to the subcellular level: the F-actin focus is an FCM-specific fusion site structure, whereas a thin actin sheath is characteristic of the FC/myotube fusion site. We demonstrate that PI(4,5)P2 enrichment is not asymmetric, suggesting that PI(4,5)P2 signaling functions on both sides of the fusion interface. These data agree with the requirement for Arp2/3 activity in both fusion partners (Abmayr and Pavlath, 2012; Chen, 2011). It will be interesting to address the function of PI(4,5)P2 on the myotube side in the future.

Published *in vitro* work on mammalian cell lines indicates a global requirement for PI(4,5)P2 in myoblast fusion. Specifically, depletion of PI(4,5)P2 through chemical treatment prevents fusion (Bach et al., 2010; Leikina et al., 2013). We corroborate this finding through sequestration of PI(4,5)P2 moieties in the developing *Drosophila* musculature. Furthermore, our finding of a fusion block due to overexpression of a kinase-dead form of PI(4)-5-kinase further validates the *in vitro* data on a PI(4,5)P2 requirement in myoblast fusion (Bach et al., 2010; Leikina et al., 2013). Significantly, we show not only the temporal and spatial localization of a PI(4,5)P2 enrichment but also the accumulation of the PI(4)-5-kinase Sktl at the fusion site, suggesting specific recruitment of Sktl and reinforcing a crucial activity of PI(4,5)P2 during fusion. We were unable to demonstrate endogenous Sktl localization to the fusion site because no antibody is currently available. Furthermore, loss-of-function approaches for PI(4)-5-kinase failed to replicate the fusion defect observed in mammalian tissue culture (Bach et al., 2010). We hypothesize that the failure to replicate this phenotype in our system is partly due to maternal loading, and partly to a high degree of redundancy with the PI(4)-5-kinases Fab1 (CG6355) and PIP5K59B. It was outside the scope of this study to generate double- and triple-mutant embryos to identify the degree of functional redundancy in this system. Taken together, our findings suggest that the previously published fusion phenotypes upon PI(4,5)P2 depletion are not due to a global effect of the treatment but to the actual inhibition of PI(4,5)P2 signaling at the fusion site.

The importance of changes in general lipid composition of the plasma membrane for fusion events have been reported for other systems. Phosphoinositide dynamics have been demonstrated in viral-cell (Barrero-Villar et al., 2009) and macrophage (Oikawa et al., 2008, 2012) fusion, whereas only the role of phosphoserine has been noted for sperm-egg (Petcoff et al., 2008; Yu et al., 2012) and trophoblast (van den Eijnde et al., 2001) fusion. It is tempting to hypothesize that most of these processes also involve PI(4,5)P2 mechanisms, similar to those described here.

We have demonstrated that PI(4,5)P2 is enriched at the fusion site and that it controls fusion through regulation of Arp2/3 activity. This is accomplished by providing localization cues, which help to enrich proteins of the Scar and WASp pathways at the fusion site, with the net result of building the protrusive actin focus and expanding the fusion interface. Our work provides novel insight into the functional role of the plasma membrane during the fusion process. We establish PI(4,5)P2 as a key signaling component essential for the crosstalk between the plasma membrane and the actin cytoskeleton in fusion.

MATERIALS AND METHODS

Fly strains

The stocks used are listed in supplementary material Table S2.

Immunohistochemistry

Whole-mount embryo staining was performed as in Richardson et al. (2007). Embryo flat preps were performed as in Lee et al. (2009), with the exception that no detergents were added. For Blow and Loner stainings, 0.01% Saponin was added. Antibodies were: Blow (1:100; E. Chen, Johns Hopkins, USA), Duf (1:200; K.-F. Fischbach, Freiburg, Germany), Loner (1:100; E. Chen), WASp (1:500; E. Schejter, Weizmann Institute, Israel), Mbc (1:100; S. Abmayr, Stowers Institute, USA), Rac1GTP (1:250; Neweast Bioscience 26903), Sns (1:250; Bour et al., 2000). Alexa Fluor 488-, 555- and 647-conjugated fluorescent secondary antibodies were used (1:200; Invitrogen). Fluorescent images were acquired on a Leica SP5 laser scanning confocal microscope with a 63×1.4 NA HCX PL Apochromat oil objective and LAS AF 2.2 software. Maximum intensity projections of confocal *z*-stacks were rendered using Volocity Visualization software (Improvision) and further processed in ImageJ (NIH).

PI(4,5)P2 sequestration assay

PI(4,5)P2 sequestration was achieved using multiple copies of *DMef2-GAL4* and *UAS-PH$^{plc\gamma}$::GFP* to increase reporter expression. Further changes in expression levels were achieved using temperature shifts (18°C, 25°C and 29°C).

Fusion index

The number of nuclei in the four lateral transverse (LT) muscles were counted in live, stage 17 embryos expressing *ap^{ME}-NLS::dsRED* and averaged (Richardson et al., 2007); n=14-16 embryos.

Phospholipid binding assay

To generate GST fusion proteins, the PH domains of Mbc (short), Loner, Blow and WASp were cloned into pENTR/D-TOPO vector (Invitrogen) and recombined into the pDEST15 vector (Invitrogen). The Mbc-long PH domain was cloned into pGEX-6p-1 vector (GE Healthcare Life Sciences). All constructs were sequenced to confirm structure. Protein expression was induced by 0.5 mM IPTG and incubated at 37°C for 3 h. Cell pellets were collected and treated with lysis B buffer [50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 µg/ml Leupeptin (Sigma), 1 µg/ml Pepstatin (Sigma)] followed by sonication, 10% Triton X-100 treatment and centrifugation. Proteins were purified with Glutathione Sepharose 4B (Amersham Pharmacia Biotech) and concentrated through

centrifugal filter units (Millipore). The concentration of GST::PH protein was determined by protein assay (Bio-Rad) and western blot. 0.5 µg/ml purified protein was applied to each membrane (Echelon Biosciences) and incubated for 1 h at room temperature. Bound proteins were detected by HRP-conjugated GST antibody (1:1000; Santa Cruz) and visualized using the HyGLO HRP detection kit (Denville Scientific).

Time-lapse imaging

Embryos were collected, dechorionated and mounted on Teflon membrane in Halocarbon oil 700 (Halocarbon Products, Series 700, 9002-83-9). Images were acquired every 15, 45 or 60 s as indicated, single z=0.5 µm, 18-20 mm total, on an upright Leica SP5 laser scanning confocal microscope with a 63×1.4 NA HCX PL Apochromat oil objective and LAS AF 2.2 software. Maximum intensity projections of confocal z-stacks were rendered using Volocity Visualization software.

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Competing interests

The authors declare no competing financial interests.

Author contributions

I.B. and M.B. developed the project and designed the experiments. I.B. and S.D. performed and analyzed the experiments. I.B., S.D. and M.B. contributed to the interpretation of the data and the writing of the manuscript.

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Supplementary material

Supplementary material available online at
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