Lamellipodin and the Scar/WAVE complex cooperate to promote cell migration in vivo

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Cell migration is essential for development, but its deregulation causes metastasis. The Scar/WAVE complex is absolutely required for lamellipodia and is a key effector in cell migration, but its regulation in vivo is enigmatic. Lamellipodin (Lpd) controls lamellipodium formation through an unknown mechanism. Here, we report that Lpd directly binds active Rac, which regulates a direct interaction between Lpd and the Scar/WAVE complex via Abi. Consequently, Lpd controls lamellipodium size, cell migration speed, and persistence via Scar/WAVE in vitro. Moreover, Lpd knockout mice display defective pigmentation because fewer migrating neural crest-derived melanoblasts reach their target during development. Consistently, Lpd regulates mesenchymal neural crest cell migration autonomously in Xenopus laevis via the Scar/WAVE complex. Further, Lpd’s Drosophila melanogaster orthologue Pico binds Scar, and both regulate collective epithelial border cell migration. Pico also controls directed cell protrusions of border cell clusters in a Scar-dependent manner. Taken together, Lpd is an essential, evolutionary conserved regulator of the Scar/WAVE complex during cell migration in vivo.

Introduction

Tightly controlled cell migration is essential for the development of multicellular organisms, and deregulation is a hallmark of diseases such as metastatic cancer (Hanahan and Weinberg, 2011). The force for cell migration is largely provided by actin polymerization at the leading edge of cells, the lamellipodium, and is controlled by actin-binding proteins including Ena/VASP and the Arp2/3 complex. These proteins are recruited to the leading edge by regulators such as Scar/WAVE for the Arp2/3 complex or Lpd for Ena/VASP proteins. The Scar/WAVE complex is composed of five proteins (Sra1/Pir121, Nap1, Scar/WAVE1-3, Abi1-3, and HSPC300) and is activated by Rac to interact with the Arp2/3 complex, thereby nucleating branched actin filament networks. In this way, both Scar/WAVE and Arp2/3 complexes regulate cell migration (Suet sugu et al., 2003; Yan et al., 2003; Insall and Machesky, 2009; Campellone and Welch, 2010; Michael et al., 2010; Suraneni et al., 2012; Wu et al., 2012). However, the regulation of the Scar/WAVE complex in migrating cells is not well understood.

Ena/VASP proteins localize to lamellipodia, tips of filopodia, and focal adhesions, and regulate lamellipodial dynamics and cell migration. Ena/VASP regulate actin filament length at the leading edge of cells by temporarily protecting actin filament ends from capping protein and recruiting polymerization-competent G-actin bound to profilin. Scar/WAVE–Arp2/3–mediated actin filament branching and Ena/VASP-regulated actin filament elongation together control speed and stability of lamellipodial protrusions, but it is not known how these mechanisms are coordinated (Bear et al., 2001, 2002; Krause et al., 2003; Pula and Krause, 2008).

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Supplemental material can be found at: http://doi.org/10.1083/jcb.201304051
Lpd and its Drosophila melanogaster orthologue Pico interact with Ena/VASP proteins, and harbor a proline-rich region with putative SH3 domain binding sites, a Ras association (RA) domain, and a pleckstrin homology (PH) domain. Lpd localizes to lamellipodia, and both RA and PH domains cooperate in membrane targeting of Lpd upon growth factor stimulation of fibroblasts. Lpd recruits Ena/VASP proteins to lamellipodia and to dorsal ruffles of fibroblasts, thereby controlling lamellipodia protrusion dynamics, dorsal ruffling of fibroblasts, axon elongation, and branching of primary hippocampal neurons, but its role in mesenchymal and epithelial cell migration is unknown. Surprisingly, knockdown of Lpd decreased F-actin content, resulted in the absence of a dense lamellipodial F-actin meshwork, and impaired lamellipodium formation (Krause et al., 2004; Lyulcheva et al., 2008; Michael et al., 2010). These phenotypes were not observed with loss of Ena/VASP, which suggests that Lpd regulates other effectors of the actin cytoskeleton in addition to Ena/VASP. Interestingly, recent reports suggest that the Lpd orthologue in Caenorhabditis elegans, MIG-10, may directly or indirectly bind to Abi-1, and both genetically interact to regulate axon guidance, synaptic vesicle clustering, and excretory canal outgrowth in C. elegans (Stavoe et al., 2012; Xu and Quinn, 2012; McShea et al., 2013).

Here, we show that Lpd is in complex with Scar/WAVE, mediated by a direct binding of the Abi SH3 domain to three sites in Lpd. In addition, Lpd directly interacts with active Rac, which positively regulates the Lpd–Scar/WAVE interaction. Therefore, Lpd functions as a Rac effector and controls lamellipodia formation via the Scar/WAVE complex. Lpd knock-out (KO) mouse embryonic fibroblasts (MEFs) are impaired in cell migration, whereas Lpd overexpression dramatically increased cell migration speed in a Scar/WAVE-dependent manner. Most Lpd KO mice die shortly after birth, and the few surviving mice are reduced in body weight and display missing pigmentation on their ventral side because fewer migrating neural crest (NC)–derived melanoblasts reach their target during development. In agreement, Lpd and the Scar/WAVE complex cooperate to regulate NC migration in vivo and in vitro in Xenopus laevis. This cooperation is evolutionary conserved because Lpd’s orthologue Pico also binds the Scar/WAVE complex and regulates epithelial collective border cell migration in the fly ovary in a Scar/WAVE-dependent manner. Taken together, we have identified a novel pathway in which Lpd functions as an important evolutionary conserved regulator of the Scar/WAVE complex during cell migration in vivo.

Results
Lpd colocalizes and interacts with the Scar/WAVE complex
We previously reported that Lpd knockdown impairs lamellipodium formation (Krause et al., 2004). In contrast, loss of the Lpd-binding proteins Ena/VASP only altered lamellipodial dynamics (Bear et al., 2002; Krause et al., 2003). Therefore, we hypothesized that the Scar/WAVE complex, a key regulator of lamellipodia, may mediate Lpd’s function in lamellipodium formation.

Coimmunoprecipitation with GFP-Lpd and Myc-tagged Scar/WAVE complex revealed that Lpd interacts with both Scar/WAVE1 (Fig. 1 A) and Scar/WAVE2 complexes (Fig. 1 B). Endogenous Lpd and Scar/WAVE1 also coimmunoprecipitated from lysates of primary cortical neurons (Fig. 1 C), which suggests that Lpd and Scar/WAVE indeed form a protein complex in cells.

The stability of the Scar/WAVE complex is tightly controlled, such that knocking down one protein of the core complex results in proteasomal degradation of the remaining proteins of the complex (Kunda et al., 2003). The expression of the Scar/WAVE complex was unaltered when Lpd protein levels were reduced using siRNA (Fig. 1 D), which suggests that Lpd is not part of the core complex but may associate with it to control its function.

Both Lpd and the Scar/WAVE complex have been reported to localize to lamellipodia (Hahne et al., 2001; Stradal et al., 2001; Krause et al., 2004). Consistently, immunofluorescence analysis revealed that Lpd colocalizes with Scar/WAVE1 (Figs. 1 E and S1), Abi1 (Figs. 1 F and S1), and Sr1 (Figs. 1 G and S1) at the very edge of lamellipodia in B16F1 mouse melanoma cells (Fig. 1, E–G) or CAD mouse neuronal cells (Fig. S1).

The interaction between Lpd and the Scar/WAVE complex is mediated by the Abi SH3 domain and three sites in Lpd
Abi harbors an SH3 domain, which could bind to putative SH3 binding sites in the C terminus of Lpd. To test this, we purified a GST-Abi1-SH3 domain fusion protein and pulled down Lpd from NIH3T3 cell lysates, revealing an interaction of the Abi SH3 domain with Lpd (Fig. 2 A). In Far-Western blot experiments with fragments of Lpd, we observed that only full-length Abi1 but not a truncated form without the SH3 domain (Abi1ΔSH3) directly interacted with two fragments (GST-Lpd-C4 and GST-Lpd-C6) and weakly with a third (GST-Lpd-C5; Fig. 2, B–D). A Far-Western experiment on a peptide array covering the C terminus of Lpd as 12-mer peptides that overlap each other by three amino acids revealed two strong class II SH3 domain–binding sites: one in each fragment Lpd-C4 and Lpd-C6, and one weak class II binding site in fragment Lpd-C5 (Fig. 2, E and F).

To test whether the SH3 domain of Abi mediates the interaction between Lpd and the Scar/WAVE complex, we attempted to coimmunoprecipitate GFP-Lpd and all Myc-tagged components of the Scar/WAVE complex, including either full-length Abi1 or Abi1ΔSH3. This analysis revealed that expression of Abi1ΔSH3 disrupts the interaction between Lpd and the Scar/WAVE complex (Fig. 3, A and B), which suggests that the SH3 domain of Abi is required for this interaction. To explore whether the three Abi SH3 domain binding sites are sufficient for the Lpd interaction with Abi, we mutated them in full-length Lpd (LpdAbiMut). Comparing coimmunoprecipitations between wild-type (WT) Lpd or LpdAbiMut and Abi1 revealed that LpdAbiMut is defective in Abi binding (Fig. 3, C and D). Because Lpd interacts with Ena/VASP proteins (Krause et al., 2004), we...
Figure 1. Lamellipodin interacts with the Scar/WAVE complex. (A and B) Coimmunoprecipitation using Lpd or IgG control antibodies from HEK293 cell lysates expressing GFP-Lpd and the tagged Scar/WAVE complex including FLAG-WAVE1 (A) and Myc-WAVE2 (B). Myc-HSPC300 is not shown. (C) Endogenous Scar/WAVE1 and Lpd coimmunoprecipitate from lysates of primary cortical neurons using Lpd antibodies but not with IgG control. (D) Knockdown of Lpd by siRNA in B16F1 cells does not reduce expression of the Scar/WAVE complex (HSPC300 not shown) or Arp3. Loading control: Tubulin. (E–G) Endogenous Lpd (green) colocalizes with Scar/WAVE1 (E), Abi1 (F), and Sra1 (G; red) at the very edge of lamellipodia in B16F1 mouse melanoma cells. Representative line scan from multiple experimental repeats across the leading edge (location indicated on merged images) shows colocalization of lpd (green) and Scar/WAVE1 (E), Abi1 (F), and Sra1 (G; red). Bar, 25 µm. See also Fig. S1.
Collectively, we have shown that the interaction between Lpd and Abi is direct and mediated by the SH3 domain of Abi and three binding sites in Lpd, and that this interaction is also coimmunoprecipitated Lpd with GFP-Abi and GFP-VASP from HEK cell lysates (Fig. S2 A), which suggests that Lpd forms a complex with both Abi and Ena/VASP proteins.
interaction is direct we pulled down purified maltose binding protein (MBP)-tagged RA-PH domains of Lpd with GTP\(_{\gamma}\)-S- or GDP\(_{\delta}\)-S-loaded purified GST-Rac protein and found that it preferentially directly interacts with active Rac (Fig. 3 G). This prompted us to explore whether active Rac regulates the interaction between Lpd and the Scar/WAVE complex. We expressed GFP-Lpd and GFP-Abi with and without all Myc-tagged components of the Scar/WAVE complex in HEK cells and found that significantly more Abi coimmunoprecipitates with Lpd when dominant-active Rac (DA Rac) was coexpressed compared with coexpression of dominant-negative Rac (DN Rac; H, middle), or empty vector control (H, right) show increased coimmunoprecipitation between Lpd and GFP-Abi only when dominant-active Rac is coexpressed. Western blot: anti-GFP. [I] Comparison of efficiency of coimmunoprecipitation of Lpd with GFP-Abi from blots in H. Quantification of band intensity of chemiluminescence imaged with a charge-coupled device camera. Coimmunoprecipitation is increased by >100% compared with empty vector and 150% compared with DN-Rac. Error bars indicate mean ± SEM, \(n = 3\). One-way ANOVA and Tukey’s test were used. ***, \(P < 0.001\).

**Figure 3.** The interaction between Lpd and the Scar/WAVE complex is mediated by the Abi SH3 domain and positively regulated by active Rac. (A–D) The Abi SH3 domain and three Abi binding sites in Lpd mediate the interaction between Lpd and the Scar/WAVE complex. Immunoprecipitation using Lpd antibodies or IgG control from HEK293 cell lysates. (A) Expressing GFP-Lpd and all Myc-tagged components of the Scar/WAVE complex, including Myc-Abi\(_{1}\)SH3 (A, left), Myc-Abi\(_{1}\)SH3 (A, right), or GFP-Abi and GFP-Lpd (C, left) or GFP-Lpd\(_{Myc}\) (C, right), show coimmunoprecipitation between Lpd and all components of the Scar/WAVE complex only when the Abi SH3 domain is present ([A]; Myc-HSPC300 is not shown) or between Lpd and GFP-Abi only when the Abi binding sites are present (B). Western blot: anti-GFP. (B and D) Comparison of efficiency of coimmunoprecipitation of Lpd with all components of the Scar/WAVE complex (B) or GFP-Lpd or GFP-Lpd\(_{Myc}\) with Abi (D). Quantification of band intensity of chemiluminescence imaged with a charge-coupled device camera. (B) Coimmunoprecipitation is reduced by >90%. Error bars indicate mean ± SEM, \(n = 3\). One-way analysis of variance (ANOVA) and Tukey’s test were used; **, \(P < 0.01\). (D) Coimmunoprecipitation is reduced by >94%. Error bars indicate mean ± SEM, \(n = 3\). An unpaired t test was used; ***, \(P < 0.001\).

(E and F) Lpd and the RA-PH domains of Lpd are in complex with active Rac. Purified GTP\(_{\gamma}\)-S- or GDP\(_{\delta}\)-S-loaded GST-Rac, or GST only as control Sepharose beads were incubated with lysates from HEK cells expressing GFP-Lpd (E) or GFP-Lpd\(_{Myc}\) (F) and bound with GFP-Lpd or GFP-Lpd\(_{Myc}\). Samples were detected in a Western blot against GFP. (G) The RA-PH domains of Lpd directly interact with active Rac. Purified GTP\(_{\gamma}\)-S- or GDP\(_{\delta}\)-S-loaded GST-Rac or GST only as control Sepharose beads were incubated with MBP-Lpd-RAPH or MBP only as control, and direct interaction was detected in a Western blot against MBP. (H and I) The interaction between Lpd and Abi is positively regulated by active Rac. Immunoprecipitation using Lpd-specific antibodies or IgG control from HEK293 cell lysates expressing GFP-Abi, GFP-Lpd, and dominant-active Rac (DA Rac; H, left) or dominant-negative Rac (DN Rac; H, middle), or empty vector control (H, right) show increased coimmunoprecipitation between Lpd and GFP-Abi only when dominant-active Rac is coexpressed.
and Fig. S2, B and C). This suggests that Lpd functions as a novel Rac effector because the interaction between Lpd and the Scar/WAVE complex is positively regulated by active Rac.

**Lpd regulates cell migration via Abi and the Scar/WAVE complex**

The Scar/WAVE complex regulates lamellipodium formation by activating the Arp2/3 complex (Machesky and Insall, 1998; Miki et al., 1998), and both complexes regulate cell migration (Suetsugu et al., 2003; Yan et al., 2003; Suraneni et al., 2012; Wu et al., 2012). We identified Lpd as a protein that regulates lamellipodia formation (Krause et al., 2004), but its role in mesenchymal and epithelial cell migration had not been determined.

To explore the role of Lpd in mesenchymal cell migration, we generated conditional KO MEFs (Lpd WT MEFs) from Lpd KO mice (see Fig. 6) and transduced them with retro-viruses conferring 4-OH-tamoxifen (4-OHT) inducible CreERT2 (Oskarsson et al., 2006). When treated with 4-OHT, these Lpd WT MEFs lose the Lpd gene and Lpd expression (Lpd KO MEFs; Fig. 4 A). Expression levels of Scar/WAVE1, RIAM, Mena, VASP, or EVL did not change in the Lpd KO MEFs compared with Lpd WT MEFs (Figs. 4 B and S2 D). Lpd KO MEFs were impaired in lamellipodium formation (Fig. 4, C and D), which is consistent with earlier observations that Lpd knockdown cells are devoid of lamellipodia (Krause et al., 2004).

Cells use lamellipodia for spreading on extracellular matrix and during cell migration. Lpd KO MEFs spread significantly more slowly (spread area reduced by 38% at 60 min) compared with Lpd WT MEFs (Fig. 4, E–G). We then analyzed random cell migration and found that migration speed and persistence were significantly reduced by 45% and 36%, respectively (Fig. 5, A and B). Likewise, directional migration into a scratch wound was impaired in the Lpd...
proteasomal degradation of the other members of the Scar/WAVE complex (Kunda et al., 2003). Overexpressing GFP-Lpd resulted in increased wound closure in nontargeting shRNA control but not in Nap1 knockdown cell lines (Fig. 5 E), which suggests that Lpd function in cell migration is mediated by the Scar/WAVE complex.

To further assess whether the direct interaction between Lpd and the Scar/WAVE complex is required for Lpd’s role in cell migration, we overexpressed the Lpd mutant in all three Abi binding sites (GFP-LpdAbiMut), which significantly reduced wound closure compared with GFP expression in MDA-MB231 cells, whereas overexpression of GFP-Lpd increased cell migration (Fig. 5 F).

To explore Ena/VASP’s contribution to Lpd’s role in cell migration, we overexpressed an Lpd mutant in all seven Ena/VASP binding sites (GFP-LpdEVMut) and a GFP-Lpd mutant harboring mutations in both Ena/VASP and Abi binding sites (GFP-LpdEV+AbiMut). We observed that the GFP-LpdEVMut increased cell migration into the scratch wound to a similar extent in KO MEFs compared with the Lpd WT MEFs (reduced by 50%; Fig. 5, C and D). In agreement, we found that directional migration into a scratch wound was also highly reduced when Lpd expression was knocked down in the Rat2 fibroblast cell line (Fig. S3, A and B), which indicates that this defect is not cell line dependent. WT Rat2 fibroblasts migrated with a polarized lamellipodium, whereas Lpd knockdown cells migrated by extending filopodia (Video 2), which was also observed in the Lpd KO MEFs (Video 1), similar to Arp2/3 knockdown and KO cells (Saraneni et al., 2012; Wu et al., 2012). We also overexpressed Lpd in MDA-MB231 breast cancer cells and found that this dramatically increased migration in a scratch wound healing experiment (Fig. 5 E).

Because the Scar/WAVE complex regulates lamellipodia formation and cell migration through activation of the Arp2/3 complex (Machesky and Insall, 1998; Suetusgu et al., 2003; Yan et al., 2003), we hypothesized that Lpd may function upstream of the Scar/WAVE complex to control cell migration. To explore this, we stably knocked down Nap1, which leads to proteosomal degradation of the other members of the Scar/WAVE complex (Kunda et al., 2003). Overexpressing GFP-Lpd resulted in increased wound closure in nontargeting shRNA control but not in Nap1 knockdown cell lines (Fig. 5 E), which suggests that Lpd function in cell migration is mediated by the Scar/WAVE complex.

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Figure 6. Lpd functions to regulate melanoblast migration. (A) Conditional Lpd KO mice were generated by flanking exon 4 with loxP sites. Cre-mediated recombination of the loxP sites results in the removal of exon 4, creating a frame shift between exon 3 and 5 and premature termination. (B and C) Conditional Lpd KO mice crossed with β-actin-Cre mice on a mixed genetic background produced mice with a reduced body size (−20.6 ± 3.0% SEM; ****, P ≤ 0.0001, unpaired t test), which also display missing pigmentation on the ventral side (D). (E and F) To visualize melanoblasts, DCT-LacZβ/β;β-actin-Creβ/+;Lpdflox/flox whole-mount embryos at E14.5 were stained for β-galactosidase expression in the melanoblasts. (E) Areas within three 1 mm × 1.5 mm boxes positioned at the middle of the trunk between the fore and hind limbs were quantified in WT and KO animals. Bar, 1.5 mm. (F) Lpd KO mice show a significant reduction in the number of melanoblasts in all three boxes. Bar, 150 μm. Melanoblast numbers were reduced by ~60%, ~40%, and ~30% for boxes 1, 2, and 3, respectively (20 KO or WT embryos from three litters; unpaired t test; *, P < 0.05; error bars indicate SEM). See also Fig. S4.
as WT Lpd. In contrast, the double mutant GFP-Lpd<sup>EV+AbiMo</sup> decreased cell migration compared with GFP expression to a similar extent as the Lpd Abi binding mutant (GFP-Lpd<sup>AbiMo</sup>; Fig. 5 F).

Collectively, this suggests that Lpd’s function in cell migration is not mediated by Ena/VASP proteins but is predominantly facilitated by the Scar/WAVE complex.

**Lpd KO mice have pigmentation defects**

To explore the role of Lpd in cell migration in vivo, we generated conditional Lpd KO mice (Fig. 6 A). We crossed these mice to PGK-Cre (ubiquitous deletion) mice on a pure C57BL/6 genetic background to delete the Lpd gene in all cells. The PGK-Cre::Lpd<sup>flx/flx</sup> KO mice have a reduced body size, show no ingestion of milk, and tend to die shortly after birth. From crosses of PGK-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> × PGK-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> we obtained the expected Mendelian ratio directly after birth (postnatal day 0 [P0]), but at P10 (n = 208) only 10.6% of PGK-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> compared with the expected 25% survived. Many of these PGK-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice died before the age of 6 wk, and those that reached sexual maturity were infertile.

We also used β-actin-Cre (ubiquitous deletion) mice on a mixed genetic background to obtain more viable Lpd KO mice. Western blots of tissue lysates from homozygous β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice showed loss of Lpd protein expression as expected (Fig. S4 A). Some homozygous β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice died within 4 wk after birth (at P14 only 42% and at P28 only 33% of expected mutants survived). The surviving β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice had a reduced body weight (reduced by 20%; Fig. 6, B and C) but were viable, and some were fertile, most likely due to modifier genes differently expressed in the mixed genetic background. Interestingly, the few surviving PGK-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice and the β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> mice displayed missing pigmentation on their ventral side (“white belly spots”; Fig. 6 D), which suggests NC/melanoblast migration defects. We observed that Lpd is expressed in melanoblasts in agreement with a putative role for Lpd in this cell type (Fig. S4 B).

NC cells that are destined to become melanoblasts emigrate at embryonic day 9.5 (E9.5) from the neural tube after undergoing EMT. Dopachrome tautomerase (DCT) is a melanogenic enzyme required for hair and skin pigmentation. DCT is expressed from E10.5 and serves as a marker for the melanoblast lineage migrating dorsolaterally through the dermis around E11.5. At E13.5, these cells migrate from the dermis into the epidermis, where they distribute evenly at E14.5 (Wehrle-Haller et al., 2001; Lin and Fisher, 2007). To visualize melanoblasts, we used a transgenic mouse line (DCT-lacZ mice) that expresses lacZ from the DCT promoter and allows visualization of melanoblast distribution by β-galactosidase staining in whole mount embryos (Mackenzie et al., 1997). We generated DCT-lacZ<sup>Wt/wt</sup>·β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup> embryos to compare the number and distribution of melanoblasts at E14.5 with that of WT (DCT-lacZ<sup>Wt/wt</sup>·β-actin-Cre<sup>Wt/wt</sup>·Lpd<sup>flx/flx</sup>) littermates. We quantified the number of melanoblasts in the trunk region (three 1.5-mm × 1-mm boxes between fore and hind limb; Fig. 6 E) where ventral depigmentation had been observed (Fig. 6, D and E).

We observed that Lpd KO embryos had significantly fewer melanoblasts in all three areas (reduced by: box 1, ~60%; box 2, ~40%; box 3, ~30%) along the dorso-ventral path (Fig. 6 F), which suggests that Lpd functions to regulate NC/melanoblast development. However, this analysis does not allow us to distinguish between a problem in NC formation versus migration. To test this, we examined NC development in Xenopus embryos, a system in which NC migration can be directly assessed in vivo (Theveneau and Mayor, 2012).

**Lpd and the Scar/WAVE complex cooperate to regulate NC migration in vivo**

X. laevis embryos were injected with Lpd mRNA, antisense morpholinos (Lpd MO, Abi MO), and dominant-negative Lpd constructs (Lyulcheva et al., 2008) containing only the N terminus, including the RA-PH domain of Lpd (Lpd N1, Lpd N6), and NC migration was analyzed by examining expression of the NC marker gene Twist (Hopwood et al., 1989). Overexpression of Lpd did not affect NC migration. Strikingly, knockdown of Lpd or Abi expression and expression of Lpd dominant-negative constructs (Lpd N1, Lpd N6) impaired the migration of the NC streams (Fig. 7, A–D), which indicates that Lpd, Abi, and the Scar/WAVE complex regulate NC migration in vivo.

Coexpression of Lpd or Abi mRNA with the Lpd or Abi MOs rescued NC migration, which suggests that the effect of the Lpd and Abi MO is specific (Fig. 7, E and F). No effect on NC formation was observed under any treatment (Fig. S5), which indicates that Lpd and the Scar/WAVE complex specifically function in NC migration.

To test whether Lpd and Abi act within NC cells or in the surrounding tissue to control NC migration, we performed experiments in which we grafted NC tissue from Lpd or Abi MO–injected embryos into nontreated control hosts. Conversely, we grafted control NC tissue into embryos injected with Lpd or Abi MOs. We observed that Lpd- or Abi-deficient NC grafted into normal hosts exhibited migration defects in 60–80% of embryos, whereas when normal NC was grafted into Lpd or Abi deficient hosts, NC migration was normal (Fig. 7, G–I). This experiment indicates that Lpd and Abi function cell-autonomously to regulate NC migration.

Because Lpd functions in cell migration upstream of the Scar/WAVE complex (Fig. 5, E and F) and interacts with the Scar/WAVE complex via the SH3 domain of Abi (Fig. 2 and Fig. 3, A–D), we hypothesized that the Abi<sup>Mo1</sup> construct would block the interaction between Lpd and the Scar/WAVE complex and therefore might have a dominant-negative function. Notably, expression of Abi<sup>Mo1</sup> impaired NC migration (Fig. 7, A–D), which suggests that Lpd and the Scar/WAVE complex cooperate to regulate NC migration.

**Lpd’s function in regulating lamellipodia formation and cell migration is mediated by the Scar/WAVE complex**

As previously described for other cell types (Krause et al., 2004), Lpd was localized to the very edge of lamellipodia in Xenopus NC cells (Fig. 8 A and Video 3). To further analyze the function of Lpd and the Scar/WAVE complex in lamellipodium...
Figure 7. Lpd regulates NC migration via Abi. (A) In situ hybridization for Twist (Hopwood et al., 1989; migratory NC marker) in control embryos or embryos injected with Lpd mRNA, Lpd, or Abi MOs (Lpd MO or Abi MO) or the dominant-negatives Lpd N1, Lpd N6, and Abi1^{SH3}. Black arrowheads, normally migrating NC streams. Red arrowheads, streams with impaired migration. Blue asterisks, the eye. (B) Summary of phenotypes. Lpd overexpression has no effect on overall NC cell migration. Lpd MO, Abi MO, and the dominant-negatives Lpd N1, Lpd N6, and Abi1^{SH3} all impair NC cell migration. (C) Percentages of embryos with normal migration along the dorso-ventral axis (% of control): n_{ctl} = 26, n_{LpdRNA} = 41, n_{LpdMO} = 37, n_{LpdN1} = 26, n_{LpdN6} = 49, n_{AbiMO} = 16, and n_{Abi1^{SH3}} = 24. (D) Mean distance of migration along the dorso-ventral axis as a percentage compared with control embryos: n_{ctl} = 12, n_{LpdRNA} = 14, n_{LpdMO} = 12, n_{LpdN1} = 14, n_{LpdN6} = 14, n_{AbiMO} = 12, n_{Abi1^{SH3}} = 12. One-way ANOVA and Dunnett’s test were used. ***, P < 0.001. (E) In situ hybridization for Twist in Lpd or Abi (Lpd MO or Abi MO), Lpd mRNA and Lpd MO, or Abi mRNA and Abi MO or control MO injected embryos. (F) Mean distance of migration along the dorso-ventral axis compared with control MO embryos: n_{ctl MO} = 13, n_{LpdMO} = 14, n_{LpdMO+LpdRNA} = 26, n_{LpdMO+AbiRNA} = 20, n_{AbiMO} = 19, n_{AbiMO+AbiRNA} = 26. One-way ANOVA and Dunnett’s test were used. **, P < 0.01; ns, nonsignificant. (G–I) Lpd and Abi function cell-autonomously in NC migration. (G) Schematic diagram of graft experiment in H. (H) NC from MO-treated embryos or WT embryos was grafted into WT or MO-treated embryos, and NC migration was analyzed. (I) Quantification of NC migration phenotypes from H. Numbers (embryos with inhibition of NC migration/total): n_{ctl>ctl} = 0/4 (0%), n_{LpdMO>ctl} = 4/6 (66%), n_{ctl>LpdMO} = ¼ (25%), n_{AbiMO>ctl} = 4/5 (80%), n_{ctl>AbiMO} = 0/5 (0%). Bars, 150 µm.
Protrusion and cell migration, the behavior of Xenopus NC explants was analyzed in vitro using time-lapse video microscopy. Overexpression of Lpd or Lpd dominant-negative (Lpd N6) led to a significant increase or decrease in the size of the lamellipodia at the edge of explants, respectively (Fig. 8, B–E; and Video 4). Importantly, the increase in lamellipodia size induced by Lpd could be reversed by coexpression of dominant-negative Abi (Abi1-SH3; Fig. 8, B and E; and Video 4), which indicates that Lpd’s function to regulate lamellipodia size is mediated by the Scar/WAVE complex.

Because Lpd and the Scar/WAVE complex regulate NC migration in vivo, we explored their cooperation in more detail using cells from dissociated explants. Lpd overexpression increased persistence, whereas knockdown reduced migration speeds and persistence of NC cells (Fig. 8, F–L; and Video 5). To test whether the Scar/WAVE complex is required for Lpd’s function in migration of individual NC cells, we overexpressed Lpd and knocked down Abi with a MO. The increase in persistence upon Lpd overexpression was dependent on the presence of Abi (Fig. 8 L and Video 6), which suggests that in NC cells the Scar/WAVE complex functions downstream of Lpd during cell migration.

Collectively, these data suggest that in both mammalian cells and Xenopus NC cells, Lpd’s function in regulating cell migration is mediated by the Scar/WAVE complex.

Lpd and the Scar/WAVE complex regulate epithelial collective cell migration in vivo

So far we have shown that Lpd controls cell migration in mesenchymal cells, such as fibroblasts, melanoblasts, and NC cells; however, epithelial cells are also migratory during collective cell migration. To examine whether Lpd’s function to regulate the Scar/WAVE complex is a general feature of different modes of cell migration and to test whether it is evolutionarily conserved in invertebrates, we turned to a model for collective cell migration in the Drosophila ovary. In this system, a group of epithelial-derived somatic follicle cells are recruited into a migratory cluster of border cells that delaminates from the epithelium and migrates between neighboring nurse cells to the oocyte. First, we examined whether the fly orthologue of Lpd, Pico, forms a complex with Scar/WAVE. GST-Pico but not GST pulled down all five proteins of the tagged Scar/WAVE complex expressed in HEK293 cells (Fig. 9 A), indicating that this interaction is evolutionary conserved. To test the involvement of pico and Scar in border cell migration, we specifically

Lpd overexpression leads to enlarged protrusions. This effect is abolished by co-injection with Abi-ΔSH3.. (C–E) Area of cell protrusions expressed as a proportion of normal protrusions (graph in D: nctl = 58, nLpd800pg = 33, nAbi1-SH3 = 24, nLpd+Abi1-SH3 = 17 [one-way ANOVA and Dunnett’s test; ***, P < 0.001]; graph in E: nctl = 154, nLpd400pg = 148, nLpd800pg = 157, nLpdN6 = 121 [one-way ANOVA and Tukey’s test; ***, P < 0.001]). (F–J) Tracks of NC cells cultured on fibronectin (a subset of the analyzed tracks are shown). (K and L) Velocity and persistence from tracks performed on all conditions (nctl = 543, nLpdMO = 240, nLpd mRNA = 297, nAbi MO = 180, nLpd+Abi MO = 290). One-way ANOVA and Dunnett’s test were used. *, P < 0.05; ***, P < 0.001. Error bars indicate SEM. See also Fig. S5 and Videos 3–6.
knocked down their expression in the border cell cluster by RNAi. Both pico and Scar RNAi resulted in reduced border cell migration at stage 9 (Fig. 9 B). pico overexpression also impaired border cell migration at stage 9 and 10 (Fig. 9, B and C), which suggests that Lpd/Pico and the Scar/WAVE complex regulate collective cell migration.

To explore whether pico function in this process is mediated by the Scar/WAVE complex, we tested if pico and Scar genetically interact. The effect of pico overexpression on border cell migration was ameliorated by Scar RNAi, which suggests that pico function is indeed mediated by the Scar/WAVE complex during collective cell migration (Fig. 9 C). To further test this, we quantified the migration speed by live-cell imaging and observed that both pico RNAi and pico overexpression reduced the rate of border cell cluster movement per frame in the first half of migration (Fig. 10 A). Consistent with our earlier observations, Scar RNAi rescued the migration defect induced by pico overexpression.

Border cell migration is characterized by polarized cell behavior in the first half of migration, followed by dynamic collective behavior, in which the cluster travels in shuffling and tumbling movements (Bianco et al., 2007). Unlike WT border cells, which normally show little or no tumbling in the first half of migration, we observed a high frequency of premature tumbling in pico-overexpressing clusters, which was dependent on Scar (Fig. 10 B), similar to what has been observed when overexpressing a dominant-negative PDGF receptor (PVRDN) in border cells (Poukkula et al., 2011). To explore the underlying causes of migration defects further, we quantified the number and direction of actin-based cellular protrusions from the border cell clusters. Overexpression of PVRDN resulted in a reduction in the total number of protrusions and a higher proportion of protrusions at the rear of the cluster when compared with WT, as reported previously (Poukkula et al., 2011). In many respects, the effect of pico and Scar RNAi on both the number and directionality of protrusions was similar to, but weaker than, PVRDN. pico overexpression too showed similar effects to PVRDN, with the notable exception that the absolute number of protrusions did not change, but many more rear facing protrusions appeared (Fig. 10, C and D). Importantly, Scar knockdown ameliorated the pico overexpression phenotype and restored the WT distribution of protrusions at the back of the cluster (Fig. 10 D), further demonstrating that pico function is mediated by the Scar/WAVE complex.

Discussion

Here we reveal that Lpd colocalizes with the Scar/WAVE complex at the very edge of lamellipodia and directly interacts with this complex by binding to the Abi-SH3 domain. Active Rac directly binds Lpd, thereby regulating the interaction between Lpd and the Scar/WAVE complex. We therefore postulate that Lpd acts as a platform to link active Rac and the Scar/WAVE complex at the leading edge of cells to regulate Scar/WAVE-Arp2/3 activity and thereby lamellipodium formation and cell migration.
Knockdown of Lpd expression (Krause et al., 2004) or KO of Lpd (Fig. 4) highly impaired lamellipodium formation, phenocopying the effect of Scar/WAVE complex knockdown on lamellipodium formation (Machinesky and Insall, 1998; Innocenti et al., 2004; Steffen et al., 2004). Conversely, we observed that overexpression of Lpd increased lamellipodia size in Xenopus NC cells, and this was dependent on the interaction with Abi, linking it to the Scar/WAVE complex. Overexpression of Pico, the Lpd fly orthologue, aberrantly increased the number and frequency of cellular protrusions at the rear of border cell clusters in a Scar-dependent manner, which suggests that the regulation of Scar/WAVE by Lpd is evolutionary conserved. Collectively, these data suggest that Lpd functions to generate lamellipodia via the Scar/WAVE complex.

We found that Lpd or Pico knockdown or Lpd KO impaired cell migration in vitro and in vivo in Drosophila, Xenopus, and mice. Lpd KO or knockdown cells were unable to migrate via lamellipodia but instead migrated very slowly by extending filopodia. The same residual migration mode had been observed for Arp2/3 knockdown cells (Saraneni et al., 2012; Wu et al., 2012). Arp2/3 is activated by the Scar/WAVE complex to regulate cell migration (Insall and Machesky, 2012; Wu et al., 2012). Arp2/3 is activated by the Scar/WAVE complex and frequency of cellular protrusions at the rear of border cell clusters. Consequently, we found that Lpd or Abi knockdown impaired NC migration in vivo.

Consistently, we found that Lpd and Abi-Scar/WAVE are in both Lpd and Abi knockdown impaired NC migration in vivo.

Figure 10. pico regulates Drosophila border cell migration via SCAR. (A–D) Analysis of time-lapse images of LifeAct-GFP–labeled border cells, using c306-GAL4 to drive the indicated genotypes (graphs indicate mean ± SEM; WT, n = 9; PVR RNAi, n = 12; pico RNAi, n = 7; SCAR RNAi, n = 8; SCAR RNAi, pico RNAi, n = 7). (A) Graph summarizing migration rate/frame calculated using a custom macro (Poukkula et al., 2011). (B) Graph showing percentage frames from the first half of migration with tumbling border cell clusters (see Materials and methods). (B, right) GFP–labeled clusters display a polarized or tumbling phenotype. Bar, 15 µm. (A and B) Tests used were one-way ANOVA and Tukey’s test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Graph showing number of cellular extensions per frame, irrespective of their direction. One-way ANOVA and Dunnett’s test were used. *, P < 0.05; **, P < 0.01. (D) Graph summarizing percentage extensions/frame at front, back, or sides of the cluster. (D, right) Image of a border cell cluster before and after image segmentation. The body of the cluster is shown in blue, cellular extensions at the front in green, the back in red, and the sides in white. White lines indicate quadrants, representing front, back, and sides for quantification. Bar, 5 µm.

Lpd not only interacts with the Scar/WAVE complex but also directly binds to Ena/VASP proteins (Krause et al., 2004; Michael et al., 2010). Ena/VASP proteins regulate actin filament length by temporarily preventing capping of barbed ends and by recruiting profilin-actin to the growing end of actin filaments (Bear et al., 2002; Krause et al., 2003). In contrast, the Scar/WAVE–Arp2/3 complexes increase branching of actin filaments. Lamellipodia with a highly branched actin network protrude more slowly but are more persistent, whereas lamellipodia with longer, less branched actin filaments protrude faster but are less stable and quickly turn into ruffles (Bear et al., 2002; Krause et al., 2003). We observed that Lpd overexpression increases cell migration in a Scar/WAVE- and not Ena/VASP-dependent manner (Fig. 5F). This is consistent with a predominant function of Scar/WAVE downstream of Lpd to regulate a highly branched actin network supporting persistent lamellipodia protrusion and cell migration. Other actin-dependent cell protrusions such as axon extension or dorsal ruffles of fibroblasts require Lpd-Ena/VASP–mediated F-actin structures (Michael et al., 2010).

Collective cell migration describes a group of cells that moves together and affect each other (Rorth, 2012), and various types of collective cell migration exists during development and cancer invasion (Friedl and Gilmour, 2009; Friedl et al., 2012). Xenopus NC cells migrate as loose streams, whereas Drosophila border cells migrate as a cluster of cells with close cell–cell contacts (Rorth, 2012; Theveneau and Mayor, 2012). We found that Rac regulates Lpd and Scar/WAVE interaction and that both are required for Xenopus NC migration, which is consistent with our previous work in which Rac activity mediates this type of migration (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Theveneau et al., 2010). Similarly, NC–derived melanoblast migration in the mouse depends on Rac–Scar/WAVE–Arp2/3 (Li et al., 2011), and we found that Lpd functions in this process as well.
Drosophila border cell clusters migrate through the fly egg chamber in two phases: an early part characterized by large and persistent front extensions, which are regulated predominantly by PVR (the fly PDGF receptor); and a late part characterized by dynamic collective “tumbling” behavior (Bianco et al., 2007; Poukkula et al., 2011). Surprisingly, Pico overexpression resulted in the appearance of a higher proportion of rear facing extensions, a phenotype previously observed with dominant-negative PVR (Prasad and Montell, 2007), causing premature tumbling of the border cell cluster. This suggests that Pico function is normally tightly controlled to stabilize specific extensions and functions also in guidance of collective cell migration. Because Lpd-Scar/WAVE control single cell migration as well as collective cell migration, this suggests that they function as general regulators of cell migration.

Collectively, we have identified a novel pathway in which Lpd functions as an essential, evolutionary conserved regulator of the Scar/WAVE complex during cell migration in vivo.

Materials and methods

Molecular biology, plasmids, and reagents

The following materials were used. GFP-Lpd (Krause et al., 2004), Lpd-N1 (aa 1–592) and Lpd-N6 (aa 242–592) was amplified from human Lpd (AY494951), cloned into pKpnI-EcoRI of pENTR3C (Invitrogen), and transcribed into pDEST-EGFP which was generated by subcloning the destination cassette [Invitrogen] into pEGFP-N1 [Takara Bio Inc.]. Picolynuclease et al., 2008) in pDEST27 [Invitrogen]. Sar1 [CIFYP1; HsCD00042136], PIR121 [Harvard Institute of Proteomics]. pDONR221-WAVE-2 (Deutsches Ressourcenzentrum für Genomforschung). hsAbi1d (BC024254; Geneservice) pDONR221 (Harvard Institute of Proteomics). pDONR221-WAVE-2 (Deutsches Ressourcenzentrum für Genomforschung). hsAbi1d (BC024254; Geneservice) in pGEX-KIF (CIFYP1; HsCD00042136), PIR121 [Harvard Institute of Proteomics]. pDONR221-WAVE-2 (Deutsches Ressourcenzentrum für Genomforschung). hsAbi1d (BC024254; Geneservice) full-length and Abi1-dA3SH (aa 1–417) cloned into pENTR11 and transferred to pRXXX-McDST, pV16-gateway (MBP). Abi1d SH3 (aa 410–472) in pGEX-4P1. pCDNA3-2xFLAGhsScar/WAVE was a gift from J. Scott (University of Washington, Seattle, WA). Napi-shRNA-1 (5’-GTCACATCCTCCACGAC-3’) and Napi-shRNA-2 (5’-GCAGTCTGCAGCCITGCTG-3’) in pLL3.7 (puro). Xenopus laevis Lpd (5’-CTTCTACCTGGAAATATCGTC-3’) and Abi1 (5’-AACATCTGATGCTGACCTCCTC-3’) MOs (Gene Tools LLC).

Antibodies

The following antibodies were used: Lpd pab 3917 (Krause et al., 2004), RIAK6613 [Iafonte et al., 2004], VASP pab 2010 (Bear et al., 2000; a gift from F. Gerber, Massachusetts Institute of Technology, Cambridge, MA), ECL mAb 84H1 [Janier et al., 1999], Mena mAb A5317FD9 [Lebrand et al., 2004], Hsc70 mAb (Santa Cruz Biotechnology, Inc.), GST pull down (GE Healthcare), Myc mAb 9E10, FLAG mAb M2 [Sigma-Aldrich], GFP mAb (Roche), Scar1 (BD), Sar1 mAb 30A4 (Synaptic Systems), Napi pab 5151 (peptide HAVYKQSVTSSA; Eurogentec), Abi mAbs (Abcam), Arp3 mAb (BD), Tubulin, α-actin mAbs [Sigma-Aldrich], MBP mAbs [New England Biolabs, Inc.], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb [EMD Millipore]. Secondary antibodies were used: HRP-goat anti-rabbit, anti-goat, mouse, and rabbit anti-goat (Dako).

Cell culture

MEFs isolated from E13 embryos from conditional Lpd KO mice were immortalized using the 3T3 method [Todaro and Green, 1963] and transduced with retroviruses conferring 4-OHT inducible CreERT2 [Oskarsson et al., 2008] in pDEST27 [Invitrogen]. The following antibodies were used: Lpd pab 3917 (Krause et al., 2004), RIAK6613 [Iafonte et al., 2004], VASP pab 2010 (Bear et al., 2000; a gift from F. Gerber, Massachusetts Institute of Technology, Cambridge, MA), ECL mAb 84H1 [Janier et al., 1999], Mena mAb A5317FD9 [Lebrand et al., 2004], Hsc70 mAb (Santa Cruz Biotechnology, Inc.), GST pull down (GE Healthcare), Myc mAb 9E10, FLAG mAb M2 [Sigma-Aldrich], GFP mAb (Roche), Scar1 (BD), Sar1 mAb 30A4 (Synaptic Systems), Napi pab 5151 (peptide HAVYKQSVTSSA; Eurogentec), Abi mAbs (Abcam), Arp3 mAb (BD), Tubulin, α-actin mAbs [Sigma-Aldrich], MBP mAbs [New England Biolabs, Inc.], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb [EMD Millipore]. Secondary antibodies were used: HRP-goat anti-rabbit, anti-goat, mouse, and rabbit anti-goat (Dako).

Immunofluorescence analysis and imaging

For immunofluorescence analysis, cells were plated on nitric acid–washed coverslips (Hecht Assistant) and fixed with 4% paraformaldehyde-PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, and 0.12 M sucrose). Goat anti-rabbit or anti–mouse Alexa Fluor 488 or 568 (Invitrogen) secondary antibodies were used, and cells were mounted (ProLong Gold, Invitrogen). A microscope (IX B1 [Olympus], with filter wheels [Sutter], an ASI-X-Y stage, CascadeII 512B camera [Photometrics], and 4× UVaPlanFL, 10× UVaPlanFL, 60× Plan-Apochromat NA 1.45, or 100× UVaPlan-Apochromat S NA 1.4 objective lenses) was used, and line scans were analyzed with MetaMorph software.

Immunoprecipitation, GST pull-downs, and Western blotting

Cells were harvested in lysis buffer (50 mM Tris HCL, 200 mM NaCl, 1% NP-40, 2 mM MgCl2, 10% glycerol, pH 7.4, 1 mM Na3VO4, 10 mM NaF, and protease inhibitors [complete mini without EDTA]; Roche). Lysates were incubated on ice for 30 min and centrifuged at 17,000 g at 4°C for 15 min. Protein concentration was then determined (Briefer BCA protein assay kit; Thermo Fisher Scientific), lysates were precleared with protein A beads (Thermo Fisher Scientific) and incubated with glutathione beads for GST pull-downs or with antibody or control IgG, followed by 1% BSA blocked protein A beads. Beads were washed with lysis buffer, separated on SDS-PAGE gels, transferred onto Immobilon-P membranes (EMD Millipore), blocked in 5% BSA, and probed with the indicated antibodies, followed by HRP-conjugated antibody (Roche) or with 1:2500 dilution of rat anti-glutathione beads (Amersham Biosciences). Blots were developed with the ECL kit (Thermo Fisher Scientific) and x-ray film, or the Immun-Star WesternC ECL kit (Bio-Rad Laboratories) using the Bio-Rad Imager and ImageJ software.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb [EMDJ Millipore]. Secondary antibodies were used: HRP-goat anti-rabbit, anti-goat, mouse, and rabbit anti-goat (Dako).

Core culture

MEFs isolated from E13 embryos from conditional Lpd KO mice were immortalized using the 3T3 method [Todaro and Green, 1963] and transduced with retroviruses conferring 4-OHT inducible CreERT2 [Oskarsson et al., 2006; a gift from A. Trump, Deutsches Krebsforschungszentrum, Heidelberg, Germany]. The melanoblast stem cell line mel-a was grown using conditions described in Sviderskaya et al. [1995; provided by E. Sviderskaya, Wellcome Trust Functional Genomics Cell Bank at St. George’s, University of London, London, England, UK]. B16.F1, Hek293FT, Nhh/I3, MDAMB231, or Lpd WT and KO MEFs were grown in DMEM (high glucose 4.5 mM/liter). CAD cells were grown in DMEM-Hepes/F12Ham’s (1:1), 10% FCS, or 12.5% FCS for MEFs, or 10% calf serum for MEFs isolated from E13 embryos from conditional Lpd KO mice were imma...
arrays (Cancer Research UK) services were described as previously (Niebuhr et al., 1997) with purified MBP-Abi full-length or MBP-Abi-ΔSH3, and MBP was detected with anti-MBP antibodies (New England Biolabs, Inc.).

**Generation of Lpd conditional KO mice**

All experiments were performed according to UK Home Office regulations. Lpd conditional KO mice were generated using C57BL/6 E5 cells (Taconic-Artemis GmbH) by flanking exon 4 with loxP sites (Fig. S 5 A), and the selection cassette was removed by crossing to Flp mice and crossed to PGK-Cre (Lallemand et al., 1998; general deleter) C57BL6 (provided by A. Behrens, London Research Institute, Cancer Research UK, London, England, UK), β-actin-Cre (Iwandaski and Martin, 1997), or DC-TacZCreβ (Mackenzie et al., 1997; provided by I. Jackson, Medical Research Council, Edinburgh, Scotland, UK) mice on a mixed genetic background.

**Whole-mount β-galactosidase staining of embryos**

DC-TacZCreβ/β-actin-Creβ/+;LpdΔlox/Δlox and DC-TacZCreβ/β-actin-Creβ/+;C57 2lox/labeled lacI1 mice were used at E14, with time of gestation calculated using the day of detection of a vaginal plug as E1. Embryos were dissected in ice-cold PBS and fixed in 0.25% glutaraldehyde at 4°C for 40 min on a rolling platform. Fixed embryos were washed in ice-cold PBS for 15 min at 4°C on a rolling platform before incubating in permeabilization buffer (2 mM MgCl2, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS, without Ca2+ or Mg2+) for 30 min and two further washes for 15 min at room temperature before staining with β-galactosidase substrate solution (2 mM MgCl2, 0.02% NP-40, 0.01% sodium deoxycholate, 5 mM KF, 5 mM KF, and 0.4 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase) for 24–48 h in darkness, at 4°C on a rolling platform. Embryos not fully stained after this time were transferred to 37°C or left at 4°C in stain solution until staining was completed. Embryos were postfixed in 4% PFA for 2 h at 4°C and transferred into PBS.

**Melanoblast quantification**

Both left and right side trunk regions were imaged using a microscope (SMZ1500; Nikon) and camera (Eki Aqua; Almaging). Melanoblast numbers from whole-mount embryos were quantified using MetaMorph Integrated Morphometric Analysis software within three 1 mm x 1.5 mm boxes positioned at the middle of the trunk between the fore and hind limbs. The three boxes were realigned next to each other with the most distal box (from the somites) placed closest to the umbilical cord. Melanoblast numbers were quantified from the boxes by running an automated object count from the MetaMorph Integrated Morphometric Analysis software using automated thresholding of the images. Melanoblasts were identified as objects by screening for objects that are within 10–88 pixels to filter nonspecific staining of veins and other small artifacts.

**Xenopus methods**

**NC culture.** Vitelline membranes were peeled off from embryos at stage 15 using tweezers, and the embryos were left to recover. Around stage 18, the superficial pigmented layer was removed and NC cells were taken out using a hair mounted on a glass pipette. The NC explants were then cut into small pieces and transferred into a fibronectin-coated dish and cultured in modified Danilchik’s medium at room temperature.

**In situ hybridization.** Embryos were fixed in MEMFA for 1 h at room temperature and washed several times in methanol. Embryos were then rehydrated by a series of methanol solutions of decreasing concentration, washed in PBS plus 0.1% Tween 20, and bleached in 5% H2O2 solution for a few minutes. A brief postfixation in 3.7% formaldehyde was performed, then embryos were washed in PBS and passed into a formamide-based hybridization buffer. Embryos were incubated overnight at 67°C in hybridization buffer containing a digoxigenin-labeled antisense probe (1 µg/ml). Probes are washed using formamide-based solutions, and PBS with 0.1% Tween 20. Embryos were passed into a blocking solution (TBS, 10% serum) and incubated overnight with an alkaline-phosphatase-coupled anti-digoxigenin antibody (1/3,000). Antibody was washed in TBS solution for one day and one night. Staining is performed in alkaline-phosphatase buffer containing NBT and BCIP (3 µl/ml). The mRNA probes used were Slug (Mayor et al., 1995) and Twist (Hopwood et al., 1989).

**Time-lapse analysis.** For cell tracking, NC cells were dissociated in low-calcium/magnesium Danilchik’s medium and were cultured as single cells on fibronectin-coated glass coverslips monitored using a 10x objective lens on an inverted microscope (Axiovert; Carl Zeiss) equipped with a camera (Hamamatsu Photonics). Pictures were acquired every 3 min. Cell tracking was performed using ImageJ Manual Tracking plugin and Imaris spot tracker software.

**Cell protrusion analysis.** NC cells were plated on plastic Petri dishes and monitored using a 63x water-immersion lens on an upright microscope (Leica). Protrusions were defined as the region devoid of vitelline platelets.

**Drosophila methods**

**Analysis of fixed Drosophila egg chambers.** Ovaries of 3-d-old females fed on fresh yeast that were dissected in PBS and fixed with 4% paraformaldehyde and stained with Alexa Fluor 633 Phalloidin (Invitrogen). At stage 9, the distance between the center of the border cell cluster and the anterior columnar follicle cells (“border cell position”) was measured in ImageJ (http://rsb.info.nih.gov/ij/). For stage 10 egg chambers, the percentage of the total migration was calculated using the distance from the anterior edge of the egg chamber to the center of the cluster as a percentage of the total migration distance (to the anterior columnar follicle cells). Transgenic lines for RNAi knockdown of pico and SCAR were as described previously (Jenchore and Bennett, 2013). UAS-SCAR RNAi (VDRC 21908) was provided by Vienna Drosophila RNAi Center. Genotypes of fixed samples were as follows:

- slbo-GAL4, UAS-GFP, his2A-RFP/+.
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (line 9).
- UAS-pico RNAi (line 4)/+.
- slbo-GAL4, UAS-GFP, his2A-RFP/+; UAS-pico/+
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (VDRC 21908)
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (VDRC 21908)
- UAS-pico/+

**Live imaging of Drosophila egg chambers.** Egg chambers from fattened ovaries (see "Drosophila methods") were dissected and cultured at 25°C in imaging media (Schneider’s medium, 15% FBS, 1 µg/ml insulin, and 1 µg/ml streptomycin and penicillin) as described by Prasad et al. (2007), with minor modifications. In brief, ovaries were removed from the abdomens of fattened flies and separated into individual fresh droplets of media before dissection of single ovarioles. Egg chambers were placed between two cover slips in the center of a gas-permeable Luxom dish (Sigma-Aldrich). A third cover slip was placed on top to prevent movement while imaging and surrounded by halocarbon oil 27 (Sigma-Aldrich). Confocal stacks were taken at multiple positions using a confocal microscope (LSM 710; Carl Zeiss) equipped with a 488-nm argon ion laser, fitted to an inverted microscope with a 20x 0.75 NA Fluor objective. Z-summer z sections (~3–10 images/stack) were taken at each position at 2-min intervals. If necessary, imaging was paused briefly during the time lapse to adjust the focus. The time lapses were cropped at 50% (early stage) for analysis. Maximum intensity projections were generated, and subsequent analysis was performed using a custom macro for ImageJ to analyze border cell migration behavior and protrusion dynamics, as described in Poukkula et al. (2011; provided by P. Rorth [National University of Singapore, Singapore] for custom macro tool). In brief, time-lapse movies were split into the relevant stages of migration, and processed into binary images after individual thresholding of each movie. Images of border cell clusters were segmented into cluster cell body and cellular protrusions. Protrusions were then categorized by position with respect to the leading cell: front, 315–45°; back, 135–225°; side, 45–135° or 225–315°. In addition to logging the protrusion behavior, the macro automatically tracked the movement of the cluster in xy, enabling in-formation such as migration speed and directed movement to be calculated. Rate per frame was calculated based on the mean distance of the total migration was calculated using the distance from the anterior edge of the egg chamber to the center of the cluster as a percentage of the total migration distance (to the anterior columnar follicle cells). Transgenic lines for RNAi knockdown of pico and SCAR were as described previously (Jenchore and Bennett, 2013). UAS-SCAR RNAi (VDRC 21908) was provided by Vienna Drosophila RNAi Center. Genotypes of fixed samples were as follows:

- slbo-GAL4, UAS-GFP, his2A-RFP/+.
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (line 9).
- UAS-pico RNAi (line 4)/+.
- slbo-GAL4, UAS-GFP, his2A-RFP/+; UAS-pico/+
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (VDRC 21908)
- slbo-GAL4, UAS-GFP, his2A-RFP/UAS-SCAR RNAi (VDRC 21908)
- UAS-pico/+

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism software (see figure legends).


Hahne, P., A. Sechi, S. Benesch, and J.V. Small. 2001. Scar/WAVE is localized to the leading edge of Xenopus NC cells. Video 4 shows that Lpd overexpression increases lamellipodia size. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201304051/DC1.

We thank Pernille Rorth for custom macros; Brian Stamor, Denise Montell, Vienna Drosophila RNAi Center, and Bloomington Drosophila Stock Center for Drosophila strains; Frank Gertler, John Scott, and Andreas Trumpf for sharing plasmids and antibodies; and Ian Jackson and Axel Behrens for sharing mice. We thank Elena Svidraskaya for the melba melanoblast stem cell line. We also thank Ansgar Li (Beatson Institute, Glasgow, Scotland, UK) for help with the β-galactosidase protocol.

L. Dodgson was supported by a Medical Research Council Capacity Building studentship. D. Soong is supported by a British Heart Foundation grant (RE/08/003). M. Kotini is supported by a PhD scholarship from the Latsis Public Benefit Foundation. E. Theveneau was supported by a Wellcome Trust Value in People award. M. Michael was supported by a Medical Research Council studentship. C. Navarro was supported by a European Research Council (ERC) starting grant (230105) and the Huma-NE platform (between 2008 and 2011). R. Mayor is supported by grants from Medical Research Council UK (077429/Z/05/Z; 082907/Z/07/Z). G00319X/1; BB/F011431/1; BB/J000590/1) and the Wellcome Trust UK (084659/Z/08/Z). D. Bennett is supported by grants from Cancer Research UK (C20691/A11834; C20691/A16678), North West Cancer Research (CRB47), and the Wellcome Trust (084659/Z/08/2). R. Mayor is supported by grants from Medical Research Council UK (VR/000655/1), Biotechnology and the Biological Sciences Research Council, and the Wellcome Trust. M. Krause is supported by grants from the Biotechnology and Biological Sciences Research Council (BB/G00319X/1; BB/F011431/1; BB/J000590/1) and the Wellcome Trust (077429/Z/05/2, 082907/Z/07/2).

Author contributions: A.-L. Law, C. Bodo, C. Navarro, and M. Krause performed the biochemistry. A. Vehlow, A.-L. Law, U. Perera, and C. Navarro performed the cell biology, and A.-L. Law, C. Bodo, and M. Krause performed the mouse experiments. C. Navarro quantified cell migration and spreading. G.A. Dunn conceived and wrote the Mathematica notebook for analysis of cell migration. L. Dodgson and E. Taylor performed experiments on fixed Drosophila samples; L. Dodgson performed all Drosophila live imaging experiments and analyzed the data. D. Bennett conceived, designed, and analyzed the Drosophila data. M. Kotini and E. Theveneau performed all the Xenopus experiments; R. Mayor designed and analyzed the Xenopus data. M. Krause conceived, designed, and analyzed the mammalian experiments and wrote the manuscript. All authors contributed to the writing of the manuscript.

Submitted: 8 April 2013
Accepted: 21 October 2013

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Figure S1. Lamellipodin colocalizes with the Scar/WAVE complex at the leading edge of cells. Endogenous Lpd (green) colocalizes with Scar/WAVE1, Abi1, and Sra1 (red) at the very edge of lamellipodia in CAD mouse neuronal cells. Bar, 25 µm.
Figure S2. Lpd is in complex with both Abi and VASP, the interaction between Lpd and the Scar/WAVE complex is positively regulated by active Rac, and expression of RIAM and Eno/VASP proteins is not changed in Lpd KO MEFs. (A) Lpd forms a complex with both Abi and VASP. Immunoprecipitation using Lpd-specific antibodies or IgG control from HEK293 cell lysates expressing GFP-Lpd and GFP-Abi1 (left), GFP-Lpd and GFP-VASP (middle), or GFP-Lpd, GFP-Abi1, and GFP-VASP (right) show coimmunoprecipitation between Lpd and both GFP-Abi and GFP-VASP. Western blot: anti-GFP. (B) The interaction between Lpd and the Scar/WAVE complex is positively regulated by active Rac. Immunoprecipitation using Lpd-specific antibodies or IgG control from HEK293 cell lysates expressing GFP-Lpd, Myc-PIR121, Myc-Nap1, Myc-Scar/WAVE1, Myc-HSPC300, GFP-Abi1, and dominant-active Rac (DA Rac; left), dominant-negative Rac (DN Rac; middle), or empty vector control (right) show increased coimmunoprecipitation between Lpd, GFP-Abi1, and Myc-PIR121, Myc-Nap1, and Myc-Scar/WAVE1 only when dominant-active Rac is coexpressed. Western blot: anti-GFP (B) and anti-Myc reprobe (C). Myc-HSPC300 is not shown. (D) Western blot analysis of whole cell lysates of Lpd WT and Lpd KO MEFs using specific anti-RIAM, -Mena, -VASP, and -EVL antibodies. β-Actin and GAPDH antibodies serve as loading controls.
Figure S3. **Lpd regulates cell migration in Rat2 fibroblasts.** (A) A confluent layer of WT, scrambled control shRNA−, or Lpd-specific shRNA−expressing Rat2 fibroblasts was scratched, and the area of the scratch was measured at 0 and 8 h. (B) Area closure is shown as the percentage of wound closure. Values of three independent experiments are shown as box and whisker plots. ***, P ≤ 0.01; one-way ANOVA. Bar, 100 µm.
Figure S4. **Lpd is not expressed in Lpd KO mice but is expressed in melanoblasts.** (A) Lpd is not expressed in Lpd KO mice. Western blots of brain tissue lysates from WT β-actin-Cre<sup>+/+;</sup>Lpd<sup>flox/+</sup>, heterozygous β-actin-Cre<sup>tg/+;</sup>Lpd<sup>flox/+</sup>, and homozygous β-actin-Cre<sup>tg/+;</sup>Lpd<sup>flox/flox</sup> mice showed loss of Lpd protein expression as expected only in the homozygous mice. Western blot: anti-Lpd. (B) Lpd is expressed in melanoblasts. Western blots of lysates from the melanoblast stem cell line mElb-a (Sviderskaya et al., 1995) show expression of Lpd. Western blot: anti-Lpd.

Figure S5. **Lpd does not affect NC induction.** (A) In situ hybridization for Slug (NC induction marker) in control embryos or those injected with Lpd mRNA, Lpd MO (LpdMO), or dominant-negative Lpd N1. No condition affects NC induction.

Video 1. **Lamellipodin KO MEFs are impaired in cell migration.** This movie shows WT control MEFs (Lpd WT MEFs; top) and Lpd KO MEFs (Lpd KO MEFs; bottom). Confluent monolayers were scratch-wounded and imaged every 5 min for 24 h by time-lapse phase contrast wide-field microscopy (IX81 [Olympus] with MetaMorph software [Molecular Devices]). Bar, 200 μm.
Video 2. **Lamellipodin regulates cell migration.** This movie shows scrambled control shRNA (top) and Lpd-specific shRNA expressing Rat2 fibroblasts. Confluent monolayers were scratch-wounded and imaged every 5 min for 8 h by time-lapse phase contrast wide-field microscopy (IX81 [Olympus] with MetaMorph software [Molecular Devices]). Note that the Lpd knockdown Rat2 cells show slow residual cell migration by extending filopodia. Bar, 100 µm.

Video 3. **Localization of Lpd-GFP in Xenopus NC cells.** This movie shows the localization of Lpd-GFP in Xenopus NC cells coinjected with Life-Act-mCherry. Note that Lpd localizes at the tip of cell protrusions (lamellipodia and filopodia). Projection of a z stack acquired with a 100× objective lens on a Elmer spinning disk confocal [PerkinElmer] inverted IX81 microscope Olympus controlled by Volocity. Cells were imaged every 30 s for 5 min. The same sequence is looped three times.

Video 4. **Lpd regulates Xenopus NC cell lamellipodia via Scar/WAVE.** Examples of representative cell protrusions in control Xenopus NC cells (top left), NC cells overexpressing Lpd (top right), NC cells expressing Lpd dominant-negative N6 (bottom left), and NC cells coinjected with Lpd mRNA and Abi dominant-negative Abi-Δ-SH3 (bottom right). Note that Lpd overexpression promotes the formation of large protrusions. This effect is abolished by the coinjection of Abi dominant-negative. Cells were imaged every minute for 40 min using a 63× water-immersion objective lens on an upright microscope (Leica) equipped with a camera (Hamamatsu Photonics).

Video 5. **Lpd regulates cell migration in Xenopus NC cells.** This movie shows representative examples of control Xenopus NC cells (green tracks), NC cells overexpressing Lpd (red), and NC cells injected with Lpd MO (blue). Cells were imaged every 3 min for 3 h using a 10× objective on an inverted microscope (Axiovert; Carl Zeiss) equipped with a camera (Hamamatsu Photonics).

Video 6. **Lpd regulates Xenopus NC cell migration via Abi.** This movie shows representative examples of control Xenopus NC cells (left), NC overexpressing Lpd (top middle), NC cells injected with Abi MO (middle and right bottom), or cells coinjected with Lpd mRNA and Abi MO (right column, top and middle). Cells were imaged every 3.5 min for 3 h using a 10× objective lens on an inverted microscope (Axiovert; Zeiss) equipped with a camera (Hamamatsu Photonics).

Reference