Fascin Rigidity and L-plastin Flexibility Cooperate in Cancer Cell Invadopodia and Filopodia

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Invadopodia and filopodia are dynamic, actin-based protrusions contributing to cancer cell migration, invasion, and metas-tasis. The force of actin bundles is essential for their protrusive activity. The bundling protein fascin is known to play a role in both invadopodia and filopodia. As it is more and more acknowledged that functionally related proteins cooperate, it is unlikely that only fascin bundles actin in these protrusions. Another interesting candidate is L-plastin, normally expressed in hematopoietic cells, but considered a common marker of many cancer types. We identified L-plastin as a new component of invadopodia, where it contributes to degradation and invasiveness. By means of specific, high-affinity nanobodies inhibiting bundling of fascin or L-plastin, we further unraveled their cooperative mode of action. We show that the bundlers cannot compensate for each other due to strikingly different bundling characteristics: L-plastin bundles are much thinner and less tightly packed. Composite bundles adopt an intermediate phenotype, with fascin delivering the rigidity and strength for protrusive force and structural stability, whereas L-plastin accounts for the flexibility needed for elongation. Consistent with this, elevated L-plastin expression promotes elongation and reduces protrusion density in cells with relatively lower L-plastin than fascin levels.

Filopodia are finger-like protrusions coordinating movement at the cell front by “sensing” extracellular stimuli (1). Invadopodia on the other hand are formed at the ventral cell side, enabling matrix degradation by localized proteolysis (2). As both actin-based protrusions contribute to migration and invasion, they are associated with cancer metastasis (3, 4), which has also been shown in vivo (5, 6).

The architecture and dynamics of filopodia and invadopodia are controlled by a broad variety of actin-binding proteins (7, 8). Moreover, these regulating proteins show highly similar spatial and temporal distributions in filopodia and invadopodia (9).

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The fascin nanobody FASNb5 (Kd ~ 35 nm) disrupts fascin-mediated actin bundling resulting in impeded filopodium formation, unstable invadopodia, and reduced degradation and invasiveness (14). The L-plastin nanobody LPLNb5 reduces L-plastin bundling by binding the hinge region between the actin binding repeats (Kd ~ 40 nm), resulting in disrupted filopodia formation. Additionally, another L-plastin nanobody (LPLNb9, Kd ~ 80 nm) is available, binding the N-terminal EF-hand structures in a calcium-dependent manner, thereby sequestering and thus inactivating L-plastin (30). By using these unique tools in several biochemical and cell biological approaches, we analyzed how the bundling proteins fascin and L-plastin cooperate in vitro and in cell protrusions such as invadopodia and filopodia.

Experimental Procedures

Antibodies and Reagents—Actin and acti-stain 670 phalloidin were obtained from Cytoskeleton (Denver, CO). Mouse monoclonal anti-actin (C4, 691002) was purchased from MP Biomedicals (Santa Ana, CA), mouse monoclonal anti-L-plastin (LPL4A.1, MA5–19121) from Thermo Scientific (Waltherm, MA), and rabbit polyclonal anti-cortactin (H-222, 3503) from Cell Signaling (Danvers, MA). Mouse monoclonal anti-V5 antibody (R960-25) and Alexa Fluor 594-labeled phalloidin were obtained from Invitrogen (Merelbeke, Belgium). Rabbit polyclonal anti-fish/Tks5 (M-300, sc-30122) and rabbit polyclonal anti-T-plastin (C-15, sc-16655-R) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-fascin (55K2, M356701) was obtained from Dako (Glostrup, Denmark), rabbit monoclonal anti-MMP-9 (ab137869) from Abcam (BurCambridge, United Kingdom), and anti-V5-agarose beads (V5–10) from Sigma. Glutathione-Sepharose was obtained from GE Healthcare (Diegem, Belgium). Alexa Fluor-labeled secondary goat anti-rabbit or anti-mouse IgG antibodies were obtained from Molecular Probes (Eugene, OR).

Nanobody Generation, cDNA Cloning, and Recombinant Protein Productions—Nanobody generation by the VIB Nanobody service facility, further cloning and recombinant production of nanobodies and antigens was described (14, 30).

Transmission Electron Microscopy—Proteins were centrifuged at 12,000 × g to remove potential aggregates. 2 μM monomeric actin was polymerized 15 min in the presence of 2 μM fascin and/or L-plastin in buffer (100 mM KCl, 1 mM MgCl2, 0.2 mM ATP, 1 mM DTT, 0.5 mM EGTA, and 10 mM HEPES, pH 7.0). 4 μM nanobody was preincubated with the bundlers for 30 min. Samples were evaluated with a transmission electron microscope (Jeol 1200 EXII; Jeol Ltd., Akishima, Japan) at an accelerating voltage of 100 kV and ×100,000 view and analyzed as described (14).

Cell Culture, Transfection, and Transduction—HeLa, HEK293T, MDA-MB-231, PC-3, HT1080, and H1299 cells were maintained at 37 °C in a humidified 10% CO2 incubator; HNSCC61 and THP-1 cells were maintained at 5% CO2. All cells were seeded in serum-free medium onto Matrigel-coated cell culture insert membranes with 3-μm pores (Corning Inc., Corning, NY). 0.05 mM hydrocortisone. Transient expression was achieved with Jetprime (Polyplus Transfection Inc., New York, NY). Stable expression was achieved by transduction, making use of the Lenti-X Tet-On advanced system (Clontech, Mountain View, CA) (14). Expression was induced by addition of 500 ng/ml of doxycycline.

Pulldown Experiments—Cells were lysed as described before (30). In the plastin pulldown, 10 μg of V5-tagged nanobody was incubated with cell lysate and anti-V5-agarose beads. For the GST pulldown experiment, 15 μg of GST or GST-L-plastin were incubated with 10 μg of LPLNb5 or fascin and glutathione-Sepharose beads.

Immunofluorescence and Microscopy—Immunofluorescence was performed as described (14). Cells were imaged at room temperature using a Zeiss Axiovert 200M Apotome epifluorescence microscope equipped with a cooled CCD Axioimager camera (Zeiss ×63 1.4-N.A Oil Plan-Apochromat objective, Carl Zeiss, Oberkochen, Germany) and Axiovision 4.5 software (Zeiss) or an Olympus IX81 Fluoview 1000 confocal laser scanning microscope (Olympus ×60 1.36-NA Oil PlanApo objective, Olympus, Tokyo, Japan) with FluoView FV1000 software (Olympus).

Analysis of Invadopodium Formation and Lifetime—To study invadopodia, cells were seeded onto 0.01% gelatin (Sigma-coated coverslips or on 0.01% Oregon Green 488-conjugated gelatin matrix (Invitrogen) as described (14). Then, (co)transfection was performed, followed by fixation and phalloidin labeling 24 h later. Lifetime analysis was performed on constitutive LifeAct-mCherry expressing cells in gelatin-coated 96-well plates. Invadopodium formation was followed for 15 h at 37 °C and 10% CO2, and pictures were taken every 5 min with an Olympus IX81 microscope (Olympus ×40 0.60-NA LUCPlanFl N objective) equipped with a x8-zytox stage and Cell M software (Olympus).

Chemo-invasion Assay—Chemo-invasion assays were executed as described (9). Briefly, 24 h after (co)transfection, cells were seeded in serum-free medium onto Matrigel-coated cell culture insert membranes with 3-μm pores (Corning Inc., Corning, NY). Attraction medium with 20% fetal bovine serum and 5 ng/ml of doxycycline and degradation was allowed to occur for another 24 h. Cells were scored positive for degradation when black holes were present in the matrix underneath it. The parameter “degraded area per cell area” was determined by dividing the total degraded area by the total cell area per picture. Gelatin zymography was performed as described.

3 The abbreviations used are: FASNb, fascin nanobody; LPLNb, L-plastin nanobody; MMP, matrix metalloprotease; ANOVA, analysis of variance; EGF, enhanced green fluorescent protein.

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before (14). For the Oris invasion assays (Platypus Technologies, Madison, WI), cells were allowed to adhere into 96-well plates coated with 2 mg/ml of collagen type I (BD Biosciences, San Jose, CA) in the presence of cell stoppers to create a cell-free area. Next, cells were covered with an additional collagen layer and stoppers were removed to allow invasion into the cell-free zone, which was monitored at 37 °C and 10% CO2 on an Olympus IX81 microscope (Olympus) equipped with a xyz-robotic stage and Cell M software (Olympus).

Analysis of Filopodium Formation and Lifetime—Cells were seeded onto 50 µg/ml of collagen type I (BD Biosciences)-coated coverslips, (co)transfected with the corresponding constructs, fixed after 24 h, and labeled with phalloidin. For the images used to determine intensity profiles, a pre-extraction step of 1 min was performed with 0.5% Triton to reduce soluble background. Lifetime analysis was performed on constitutive LifeAct-mCherry expressing cells in collagen-coated 35-mm µ-dishes (Ibidi, Planegg/Martinsried, Germany). 24 h later, expression was induced with doxycycline. Time-lapse images were generated every 10 s at 37 °C during 10 min using the Olympus confocal microscope.

Image and Statistical Analysis—Image analysis (protein intensities, intensity profiles, live cell imaging analysis, and quantifications) was performed with ImageJ (National Institutes of Health, Bethesda, MD). Statistical analysis was performed with Sigmaplot (Systat Software Inc., San Jose, CA) using one-way ANOVA or ANOVA on ranks, both with \( p = 0.05 \).

Results

Fascin-induced Actin Bundles Are Rigid, whereas L-plastin Generates Thinner and More Flexible Actin Bundles—Both fascin and L-plastin are well known actin bundling proteins (12, 22), however, they do not interact directly (Fig. 1A). To compare their bundling characteristics, transmission electron microscopy was performed (Fig. 1B). This enabled quantification of the number of filaments per bundle and the interfilament distance (as a measure for filament “packing”) (Fig. 1C). Fascin induces thick bundles composed of 14 filaments, which are closely packed to each other, whereas L-plastin generates thin bundles with only 2–3 loosely packed actin filaments. An intermediate phenotype can be observed upon combination of fascin and L-plastin, consisting of 9 actin filaments (“bundler alone”: Fig. 1, B, left panel, and C, white bars). As shown before (14), disruption of fascin bundling activity by the fascin nanobody FASNb5 leads to thinner (less filaments per bundle) and improperly organized actin bundles, with the edges of the filaments being hard to discern. The L-plastin nanobody LPLNb5 leads to a substantial loss of...
L-plastin-induced bundles (return to actin-only phenotype) (30). Remaining bundles mainly consist of loosened filaments (increased interfilament distance), which are less clearly organized. Also, the combination of both nanobodies mainly affects filament organization (“/H11001 Nanobody”: Fig. 1, B, middle panel, and C, gray bars). We wondered if the bundling proteins could compensate for each other (rescue phenotype) when their activity is inhibited by a respective nanobody. L-plastin addition to FASNb5-disrupted bundles restores the structural filament organization, but not the number of filaments, nor the interfilament distance. Fascin addition to LPLNb5-disrupted bundles does not rescue the original phenotype but goes beyond it with increased packing and filament numbers typical for fascin (“/H11001 Other bundler” (rescue): Fig. 1, B, right panel, and C, black bars). In conclusion, fascin and L-plastin form strikingly different non-interchangeable actin bundles and adopt an intermediate phenotype upon combination.

**L-plastin Is a New Component of Invadopodia, Regulating Matrix Degradation, and Cancer Cell Invasion**—Invadopodia are formed at the ventral cell side and contribute to cancer cell invasiveness by performing proteolytic degradation (2). Fascin has been described previously as a component of cancer cell invadopodia (13, 14). We wondered if the actin bundling protein L-plastin is also enriched in these protrusions. Indeed, we found that L-plastin colocalizes with the invadopodium markers actin (phalloidin) (Fig. 2 A–D), Tks5 (Fig. 2 B), cortactin (Fig. 2 C), and spots of fluorescent gelatin matrix degradation (D) in MDA-MB-231 breast cancer cells. Boxed areas are enlarged in the lower panels and arrowheads depict some invadopodia. Scale bars = 10 μm. E, confocal image sections of MDA-MB-231 cells labeled with phalloidin and stably overexpressing EGFP-tagged fascin and mCherry-labeled L-plastin in a chemo-invasion assay. xy1 represents the cells at the focal plane of the filter. The boxed area is enlarged in the lower panels and arrowheads depict some invadopodia. xy2 shows the cells with invadopodia 2.25 μm lower. A three-dimensional reconstructed xz projection over the transversal cut (dashed rectangle) through 2 invadopodia is shown in the bottom panels. Corresponding intensity profiles of fascin, L-plastin, and actin (phalloidin) were determined in the direction of the arrows in the xy1 and xz plane. Scale bars = 10 μm. F, confocal images showing that EGFP-tagged fascin and mCherry-tagged L-plastin are enriched in cancer cell invadopodia of several cell systems (HeLa cervix cancer, HT1080 fibrosarcoma, HNSCC61 head and neck squamous cancer, and PC-3 prostate cancer) upon transient overexpression. Boxed areas are enlarged in the lower panels. Examples of invadopodia are indicated with arrowheads. Scale bars = 10 μm.

**FIGURE 2.** L-plastin is a new component of cancer cell invadopodia. A–D, epifluorescent images showing that L-plastin colocalizes with phalloidin (A), Tks5 (B), cortactin (C), and spots of fluorescent gelatin matrix degradation (D) in MDA-MB-231 breast cancer cells. Boxed areas are enlarged in the lower panels and arrowheads depict some invadopodia. Scale bars = 10 μm. E, confocal image sections of MDA-MB-231 cells labeled with phalloidin and stably overexpressing EGFP-tagged fascin and mCherry-labeled L-plastin in a chemo-invasion assay. xy1 represents the cells at the focal plane of the filter. The boxed area is enlarged in the lower panels and arrowheads depict some invadopodia. xy2 shows the cells with invadopodia 2.25 μm lower. A three-dimensional reconstructed xz projection over the transversal cut (dashed rectangle) through 2 invadopodia is shown in the bottom panels. Corresponding intensity profiles of fascin, L-plastin, and actin (phalloidin) were determined in the direction of the arrows in the xy1 and xz plane. Scale bars = 10 μm. F, confocal images showing that EGFP-tagged fascin and mCherry-tagged L-plastin are enriched in cancer cell invadopodia of several cell systems (HeLa cervix cancer, HT1080 fibrosarcoma, HNSCC61 head and neck squamous cancer, and PC-3 prostate cancer) upon transient overexpression. Boxed areas are enlarged in the lower panels. Examples of invadopodia are indicated with arrowheads. Scale bars = 10 μm.

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To better understand the function of L-plastin in invadopodia, the bundling inhibitor LPLNb5 as well as LPLNb9, binding L-plastin in a calcium-dependent manner (27, 30), were applied. Although LPLNb5 is a tool to study the role of L-plastin bundling, LPLNb9 rather reflects the role of L-plastin as a whole by locking the protein in the calcium-bound (inactivated) state. The L-plastin nanobodies did not affect invadopodium lifetime (Fig. 3 A). Matrix degradation activity, however, which is typically executed by mature invadopodia and can be...
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Easily studied in PC-3 prostate cancer cells (14, 39), is reduced with both nanobodies in terms of “degraded area” (Fig. 3B). In the case of the inhibitory LPLNb5, this is not due to an effect on matrix metalloprotease 9 (MMP-9) secretion or activity, the main gelatinase in PC-3 cells (14, 39). LPLNb9 on the other hand significantly reduces MMP-9 secretion and activity (Fig. 3C). These effects result in reduced three-dimensional cancer cell invasion in an Oris assay, which is more pronounced for LPLNb9 (Fig. 3D). Altogether, our data show that L-plastin is a new component of cancer cell invadopodia with its actin bundling activity contributing to matrix degradation and invasion, as shown by means of LPLNb5. Independent from its bundling activity, L-plastin further regulates MMP-9 secretion and activity as shown by means of LPLNb9. This additionally contributes to matrix degradation and therefore cancer cell invasiveness.

**Fascin and L-plastin Cooperate in Invadopodium Formation, with L-plastin Stimulating Elongation whereas Counteracting Formation—Invadopodium precursors are defined as phalloidin dots in the perinuclear region of the cell, which do not necessarily protrude. FASNb5, LPLNb5, or a combination of both nanobodies did not affect precursor density in MDA-MB-231 cells (Fig. 4A), suggesting that actin bundling is not involved in the initial stage of invadopodium formation. To effectively study protruding invadopodia, a chemo-invasion assay was performed, allowing elongation of invadopodia through membrane pores (Fig. 4B). This assay revealed that both inhibitory nanobodies reduce density and/or protrusion length of invadopodia (Fig. 4C), suggesting that fascin as well as L-plastin bundling is involved in invadopodia protrusions. Based on their different bundling properties in vitro, we wondered if fascin and L-plastin could compensate for each other in this cell-based system. Remarkably, fascin can rescue invadopodium density but not length, whereas the opposite is true for L-plastin (Fig. 4, A and B, rescues). This is not due to spatial differences as fascin and L-plastin are similarly distributed over the invadopodium length (Fig. 2E).

To further investigate this phenomenon, several cancer cell types were screened for their fascin and L-plastin levels (Fig. 5A). T-plastin, the plasmin isoform particularly present in solid tissues, is abundant in all the cell systems studied. Importantly, the L-plastin nanobodies used do not cross-react with the T-plastin isoform (Fig. 5B). PC-3 prostate cancer and HT1080 fibrosarcoma cells express the highest L-plastin levels (Fig. 5A). However, MDA-MB-231 breast cancer cells also express relatively more L-plastin than fascin, whereas the opposite is true for cell types such as H1299 (non-small cell lung cancer) and HNSCC61 (head and neck squamous cancer) (Fig. 5C). The latter two are of particular interest to study the effect of increased L-plastin expression. Of note, an indication of the level of overexpression in rescue phenotypes, which is rather modest, is presented in Fig. 5D. HNSCC61 cells were chosen for further analysis as they form nicely quantifiable invadopodia (Fig. 2F). Here, L-plastin overexpression increases invadopodium length while reducing density numbers (Fig. 5E). This suggests that L-plastin stimulates invadopodium elongation and counteracts formation when its levels are increased. Fascin overexpression on the other hand reduces invadopodium length.

A **Similar Cooperation of Fascin and L-plastin Can Be Found in Filopodia.—**We wondered if the observed collaboration between fascin and L-plastin in invadopodia could be extrapolated toward other actin-based protrusions. Filopodia, for example, show many similarities in composition and formation (9) but are formed at the cell edges to enable environment sensing (1). Moreover, fascin and L-plastin are similarly distributed over the filopodium length in PC-3 prostate cancer cells comparable with the observations in invadopodia (Fig. 6A). Furthermore, fascin and L-plastin are expressed in PC-3 cells at a ratio comparable with the MDA-MB-231 cell system (Fig. 5C). Thus, fascin and L-plastin may also cooperate in filopodium formation. Here, we confirm that both FASNb5 and LPLNb5 significantly reduce filopodium density and length in this cell type (Fig. 6B), as reported before (14, 30). Of note, filopodium formation is not completely abolished. L-plastin can again rescue filopodium length, but not density, which is even more reduced in comparison to the control (Fig. 6B, rescues). Fascin on the other hand cannot rescue length, but density becomes less reduced in comparison to the control. In addition, we studied HNSCC61 cells, which express relatively less L-plastin than fascin (Fig. 5B), but form nicely quantifiable filopodia. L-plastin overexpression here stimulates filopodium elongation, whereas reducing density numbers (Fig. 6C). Fascin overexpression on the other hand has no effect. Thus, not only in invadopodia but also in filopodia, L-plastin stimulates elongation at the expense of density numbers.

**Both Fascin and L-plastin Bundling Activities Contribute to Filopodium Lifetime, whereas Only Fascin Regulates Structural Rigidity—**To better understand the differences in fascin and L-plastin function in filopodia, live cell imaging was performed on LifeAct-mCherry-transduced cells expressing the inhibitory nanobodies or overexpressing the bundlers themselves (Fig. 7A and supplemental Video S1, A–E). Filopodia of control cells are often embedded into the lamellipodial network, whereas this is less common in the several test conditions. When considering changes in filopodium length per unit of time, only fascin turns out to be important (Fig. 7B). Indeed, fascin overexpression leads to static filopodia, whereas FASNb5 stimulates length changes. However, both fascin and L-plastin affect filopodium lifetime similarly when overexpressed or inhibited (Fig. 7C). Of note, FASNb5 expression often coincides with “wavy” and “falling” filopodia (Fig. 7D and supplemental Video S2, A and B), which was also reported by others upon fascin down-regulation or inactivation (12, 40, 41), whereas LPLNb5 does not affect the filopodium phenotype (Fig. 7A). Remarkably, wavy and falling filopodia can also be observed upon L-plastin overexpression (Fig. 7D and supplemental Video S2, C and D). In conclusion, the presence of FASNb5 leads to unstable, curved filopodia, which collapse early. In the presence of LPLNb5 on the other hand, lifetime is reduced without structural issues. In combination with the observed correlation between L-plastin and filopodium elongation, this rather suggests incomplete growth of LPLNb5-expressing filopodia.
FIGURE 3. L-plastin contributes to matrix degradation and cancer cell invasion due to its bundling activity as well as by a bundling-independent role in MMP-9 secretion and activity. A, distribution of invadopodium lifetimes in stable MDA-MB-231 breast cancer cells with doxycycline-induced EGFP-tagged LPLNb5 and LPLNb9 expression as determined in at least 100 invadopodia by live cell imaging with constitutive LifeAct-mCherry expression. B, representative epifluorescent images of red gelatin matrix degradation by PC-3 prostate cancer cells with doxycycline-induced EGFP-tagged nanobody expression. Scale bars = 10 μm. Bar graphs represent the percentage of degrading cells as mean ± S.E. (n = 3, 100 cells per repeat). The boxplots (whiskers from 10 to 90%) show the ratios of degraded area per cell area as determined in at least 12 images obtained over 3 independent experiments (>75 cells per condition). C, representative Western blot analysis of MMP-9 production in crude lysate (left panel); MMP-9 secretion levels in the medium (middle panel) and gelatin zymography of MMP-9 activity in the medium (right panel) upon EGFP-tagged doxycycline-induced LPLNb5 or LPLNb9 expression. Corresponding quantitative densitometry is represented as normalized mean ± S.E. (n = 3). Equal μg amounts of crude lysate or medium were loaded. D, representative epifluorescent stills at the start and end (12 h) of three-dimensional-like invasion upon doxycycline-induced expression of EGFP-tagged LPLNb5 or LPLNb9. Cells were seeded onto, and covered with, a collagen type I layer in the presence of cell stoppers, creating a cell-free zone into which invasion occurs. Cell-free zones at the start and end point are outlined. Boxplots (whiskers from 10 to 90%) represent the invaded area obtained from 15 to 20 measurements over 3 independent experiments. EGFP-only expressing cells were used as a negative control in all experiments. p values were determined with ANOVA on ranks (A and B) or one-way ANOVA (C and D) (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
Discussion

Fascin and L-plastin Collectively Bundle Actin in Invadopodia and Filopodia—Our results reveal that fascin and L-plastin act together as bundling proteins in actin-based protrusions such as invadopodia and filopodia, which show many similarities in composition and formation (9). Others reported before a composite bundle phenotype important in filopodia composed of fascin and the actin bundling formin Daam1, which is recruited and stabilized onto actin by fascin (41). Furthermore, most in vivo structures based on actin bundles, such as Drosophila bristles or hair cell stereocilia, are assembled by two or more actin cross-linking proteins (42, 43). It further makes sense that the diversity of invadopodia and filopodia structures, behaviors, and functions that are found in a wide range of cell types (1, 2) does not arise from one sole bundling protein. Combining the bundle characteristics of fascin and L-plastin may be a strategy of the cell to modulate protrusion possibilities, adaptation to new environmental conditions, and cell behavior dependent on the needs of the moment.

Because both fascin and L-plastin affect protrusion density, length, and lifetime while colocalizing in filopodia and invadopodia and do not bind directly with each other (Figs. 1A, 2E, and 6A), these proteins may act simultaneously and cooperatively. A possible mechanism is that L-plastin loosely gathers and tethers the actin filaments, which can then be tightened by fascin. Such a mechanism has been reported before during Drosophila oogenesis and bristle formation where the singed protein (a functional homologue of fascin) is collaborating with forked or quail (a villin-like protein), respectively (43, 44). In these cases, forked or quail loosely packs actin filaments, which are subsequently tensed by fascin. Depletion of forked led to thin bundles with fewer filaments, whereas singed (fascin) depletion affected filament “tightening” (43). This is in line with our in vitro transmission electron microscopy data on actin bundles with fascin and L-plastin in the presence of a nanobody (Fig. 1, B, right panel, and C, black bars). Composite bundles with LPLNb5 contain 20% fewer filaments per bundle and are 25% more tightened compared with the composite bundles with FASNb5. Indeed, if LPLNb5 affects L-plastin-mediated tethering of actin filaments, smaller bundles with fewer incorporated filaments are expected, whereas the tightening still occurs efficiently by fascin. The presence of FASNb5 on the other hand mainly causes loosening of the filaments.

FIGURE 4. Fascin can restore density, whereas L-plastin rescues protrusion length in cancer cell invadopodia. A, representative confocal images of MDA-MB-231 breast cancer cell invadopodia upon transient EGFP- or mCherry-tagged expression of one or both (combi) inhibitory nanobodies. Invadopodium precursors are visualized with phalloidin. Scale bars = 10 μm. Boxplots (whiskers from 10 to 90%) represent invadopodium precursor density per 100 μm² cell surface as determined in at least 89 cells obtained over 3 independent experiments. B, representative confocal images of invadopodia in chemo-invasion assays with MDA-MB-231 breast cancer cells upon transient EGFP- or mCherry-tagged expression of one or both (combi) inhibitory nanobodies. Rescue was executed by overexpression of the other bundling protein. Cells co-transfected with empty pEGFP-N1 and pmCherry-N1 vector were used as a negative control. Scale bars = 10 μm. Invadopodia are visualized with phalloidin and their length can be studied in yz sections (right panels), taken along the vertical solid lines. Cell bodies are present at the left side of the dashed line, representing the focal plane of the membrane, whereas the protruding invadopodia (arrowheads) can be observed at the right side. Scale bars = 5 μm. C, boxplots (whiskers from 10 to 90%) of invadopodium length and density as determined in at least 40 cells obtained over 3 independent experiments for the conditions depicted in B. All p values were determined with ANOVA on ranks (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
**FIGURE 5.** L-plastin overexpression in HNSCC61 cells, which barely contain endogenous L-plastin, induces filopodium elongation and counteracts formation.

A, representative Western blot experiment showing actin (ACT), fascin (FAS), L-plastin (LPL), and T-plastin (TPL) levels in 50 μg of several human cancer cell line lysates (HeLa cervix cancer, MDA-MB-231 breast cancer, PC-3 prostate cancer, HT1080 fibrosarcoma, H1299 non-small cell lung cancer, and HNSCC61 head and neck squamous cancer) and a non-cancer cell line (HEK293T embryonic kidney). The lower L-plastin panel indicates the presence in other cell lines upon prolonged exposure.

B, Western blot of a representative pulldown experiment with V5-tagged LPLNb5 or LPLNb9 on HEK293T or THP-1 cell lysates. HEK293T cells mainly contain T-plastin, which is not bound by the nanobodies. THP-1 monocytes on the other hand are rich in L-plastin, which is pulled down by both nanobodies. Negative control reactions (−) were performed in the absence of a nanobody.

C, representative Western blot experiment allowing comparison of fascin (FAS) and L-plastin (LPL) levels in 75 μg of cell lysate with recombinant fascin or L-plastin as internal standards. Arrowheads indicate the estimated amount of protein corresponding to the level in the lysate resulting in the LPL/FAS ratios depicted at the right.

D, representative Western blot experiment allowing comparison of fascin overexpression (EGFPFAS) and endogenous fascin levels (FAS) in PC-3 cells transfected with LPLNb5mCherry and EGFPFAS (FAS rescue) with recombinant fascin as the internal standard. Arrowheads indicate the estimated amount of fascin corresponding to the level in the lysate (arrowheads pointing down for EGFPFAS and pointing up for endogenous FAS) and the corresponding values are indicated at the bottom. The increase in fascin level due to overexpression is estimated to be 25%.

E, representative confocal images of invadopodia in chemo-invasion assays with HNSCC61 head and neck squamous cancer cells upon transient overexpression of EGFP-tagged fascin or L-plastin. EGFP-only expressing cells were used as a negative control. Scale bars = 10 μm. Invadopodia are visualized with phalloidin and their length can be studied in yz sections (right panels), taken along the vertical solid lines. Cell bodies are present at the left side of the dashed line, representing the focal plane of the membrane, whereas the protruding invadopodia (arrowheads) can be observed at the right side. Scale bars = 5 μm. Boxplots (whiskers from 10 to 90%) represent the invadopodia density and their length as determined in at least 55 cells obtained over 3 independent experiments. p values were determined with ANOVA on ranks (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
Fascin Guarantees Protrusion and Structural Rigidity, whereas L-plastin Mediates Elongation and Counteracts Formation of Invadopodia and Filopodia—The rigidity of fascin provides the mechanical force needed for protrusion and structural stability, as also observed by others (12, 13, 40), and is therefore mainly linked to protrusion density and maintenance. More strikingly however, we found that the actin bundling protein L-plastin stimulates invadopodia and filopodia extension. Protrusion elongation has been mainly linked to proteins of the Ena/Vasp and formin family, which polymerize actin filaments upon de novo formation or protect Arp2/3-generated barbed ends from capping protein, thereby promoting filament elongation (45). However, not all Ena/Vasp and formin proteins perform this equally well, and others have suggested before that a bundling function of these proteins might be involved in elongation of protrusions (46–48). For the case of L-plastin, it is

**FIGURE 6.** Also in filopodia, L-plastin promotes elongation, whereas counteracting formation. A, confocal images showing that EGFP-tagged fascin and mCherry-tagged L-plastin are both present in phalloidin-stained filopodia upon doxycycline-induced expression in PC-3 prostate cancer cells. Scale bar = 10 μm. The boxed area is enlarged in the lower panels. The arrow depicts the line along which the intensity profile was determined for the neighboring filopodium. B, representative confocal images of PC-3 prostate cancer cell filopodia upon transient EGFP- or mCherry-tagged expression of one or both (combi) inhibitory nanobodies. Rescue was executed by overexpression of the other bundling protein. Cells co-transfected with empty pEGFP-N1 and pmCherry-N1 vector were used as a negative control. Filopodia are visualized with phalloidin and the boxed areas are enlarged at the bottom of each panel. Arrowheads indicate some remaining filopodia. Scale bars = 10 μm. Boxplots (whiskers from 10 to 90%) represent filopodium density (per μm of cell perimeter) and length as determined in at least 75 cells obtained over 3 independent experiments. **C**, representative epifluorescent images of phalloidin-labeled filopodia in HNSCC61 cells upon transient overexpression of EGFP-tagged fascin or L-plastin. EGFP-only expressing cells were used as a negative control. Boxed areas are enlarged in the lower panels. Scale bars = 10 μm. Boxplots (whiskers from 10 to 90%) represent filopodium density (per μm of cell perimeter) and length as determined in at least 50 cells obtained over 3 independent experiments. All p values were determined with ANOVA on ranks (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
unlikely that it directly elongates filaments, but its gathering/tethering function upon filament bundling may provide guidance for the polymerized actin filaments, thereby facilitating fascin bundling activity and resulting in further lengthening.

Another explanation for the contribution of L-plastin in protrusion elongation may result from the fact that plastin forms a complex with the intermediate filament protein vimentin in filopodia, retraction fibers, and podosomes upon cell adhesion and spreading. Therefore, plastin is thought to direct the assembly of the vimentin cytoskeleton at cell adhesion sites, thereby integrating the actin and vimentin cytoskeletons (49). Furthermore, vimentin is only present in elongated invadopodia and siRNA significantly reduces invadopodium length (9). Thus, L-plastin may indirectly promote elongation by stimulating the vimentin cytoskeleton at protrusion sites.

Remarkably, fascin and L-plastin also counteract each others functions. A similar combined mode of action has been shown before in filopodia with Ena/Vasp and mDia expression (50). Ena/Vasp can act as a bundling protein in vitro and maintains filopodium stability. The F-actin polymerase mDia on the other hand promotes filopodium elongation, which is in its turn tempered by Ena/Vasp (51).

Fascin and L-plastin co-existence is not exclusive for some cancer cell types, as they are reported to be both up-regulated in colorectal, breast, ovarian, pancreatic, prostate, and fibrosarcoma cancer cells among others (17, 26). Although, other actin bundling proteins may provide similar functions in other cell types. In HNSCC61 cells, with relatively lower L-plastin than fascin levels, nice filopodia and invadopodia can be observed (Figs. 5E and 6C), which are most likely not only composed of fascin. Another comparable bundling protein equilibrium will therefore exist in this cell type, again pointing to the flexibility of cells in modulating actin-based protrusions.

FIGURE 7. Both fascin and L-plastin regulate filopodium lifetime, but only fascin is important for structural rigidity. A, filopodium dynamics was studied in PC-3 prostate cancer cells constitutively expressing Lifeact-mCherry and inducibly expressing the EGFP-tagged inhibitory nanobodies or the bundlers themselves. Representative confocal stills of Lifeact-mCherry-labeled filopodia (arrowheads) during their lifetime are shown. EGFP-only expressing cells were used as a negative control. Scale bar = 5 μm. Corresponding Videos are available (supplemental Video S1, A–E). B, boxplots (whiskers from 10 to 90%) of filopodium length changes per unit of time as determined over the lifetime of 20 filopodia obtained over 3 independent experiments. C, boxplots (whiskers from 10 to 90%) of filopodium lifetime, as determined in at least 30 cells obtained over 3 independent experiments (>300 filopodia). D, representative Lifeact-mCherry-labeled filopodia with wavy appearance or with a tendency to fall down (falling, arrowheads) as observed in cells expressing FASNb5 or L-plastin. Scale bar = 5 μm. Corresponding Videos are available (supplemental Video S2, A–D). All p values were determined with ANOVA on ranks (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
It is also possible that a similar collaboration between fascin and L-plastin exists in other actin-based protrusions such as podosomes. However, the effective mechanism will differ due to distinctions in ultrastructure (2). Bundling proteins are enriched around the core and into a cap structure on top of the podosome, although they are present near the tip of invadopodia (2, 31). Of interest, however, we observed that podosome lifetime increases upon fascin bundling inhibition with FASNb5, suggesting a role of fascin in disassembly (31). By contrast, podosome lifetime decreases upon L-plastin bundling inhibition with LPLNb5 (27). This points to an attenuating role of both bundlers when simultaneously present, which may again find its origin in their different bundle characteristics.

**L-plastin Is a New Component of Cancer Cell Invadopodia—**
L-plastin was first reported as a component of specific adhesion structures in neutrophils (52) and osteoclasts (53), which would now be classified as podosomes (2). This was further confirmed by proteomic analysis of macrophage podosome fractions (54). Therefore, further research mainly focused on the role of L-plastin in podosomes and immune cell function (27–29). The ubiquitous T-plastin on the other hand has been reported to be a component of cancer cell invadopodia, although siRNA only affected invadopodium length, not their number or matrix degradation capacity (9). We show also that L-plastin is a constituent of cancer cell invadopodia (Fig. 2). Several similarities with the role of fascin in invadopodia can be observed. In both cases, bundling inhibition reduces matrix degradation activity and cancer cell invasiveness (Fig. 3). Moreover, both fascin and L-plastin seem to play a bundling independent role in the regulation of MMP-9 secretion and activity, which further contributes to invasiveness (14).

In conclusion, our results show that fascin and L-plastin adopt an intermediate phenotype upon coexistence (Fig. 8A) until an optimal balance of fascin-mediated rigidity and L-plastin-supplied flexibility is achieved. The rigidity is needed for protrusive force and internal structural stability, whereas the flexibility mediates elongation in both invadopodia and filopodia (Fig. 8B). Disturbance of one of the bundling partners...
causes distinct defects (structural instability versus incomplete growth), ultimately resulting in reduced density, length, and lifetime (Fig. 8C). In protrusions expressing relatively less L-plastin than fascin such as in HNSCC61 cells, L-plastin performs its elongation activity upon addition and thereby impairs fascin-induced protrusion formation (Fig. 8D). Similar collaborations between other bundling proteins are likely and future research in this area should lead to a better understanding of the structural build-up and protein interplay in actin-based protrusions.

Author Contributions—I. V. A. designed, performed, and analyzed most of the experiments and wrote the paper. C. B. performed Western blotting and its analysis and executed immunofluorescence studies. M. D. conducted and analyzed the experiments shown in Fig. 3. L. P. and M. C. designed and conducted the transmission electron microscopy experiments shown in Fig. 1. J. G. conceived and coordinated the study and wrote the paper with I. V. A. All authors reviewed the results and approved the final version of the manuscript.

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