Supplemental Materials

Molecular Biology of the Cell

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Online Supplemental Material

Supplemental Figure legends

Figure S1: FMNL2 accumulation at lamellipodium tips requires the FH2-domain plus a 40aa residue, proline-rich, N-terminal region. (A) Left panel: Representative cell expressing EGFP-tagged FH2-domain of FMNL2, as indicated, illustrating the lack of accumulation at the lamellipodium tip. Right panel: Pull-down assay demonstrating interaction of EGFP-tagged FMNL2-FH2 with actin, with the Ponceau-stained 42kDa-band confirmed to constitute actin by Western Blotting. (I: Input; S: Supernatant; P: Pulldown). These data indicate that actin binding alone by EGFP-FMNL2-FH2 is insufficient for lamellipodial tip localization. (B) Left panel: An EGFP-construct encompassing the FH2 domain of FMNL2 and 40 amino acid residues derived from the more N-terminal FH1 domain (as illustrated in the graph on the right panel) is sufficient for accumulation at lamellipodial tips. (C, D) Domain swap experiments revealing that the nature of the FH2 domain is driving the localization specificity of FMNL formins. As FMNL1 is incapable of lamellipodial localization and FH1-FH2 expression constructs derived from FMNL2 and -3 but not FMNL1 accumulate at lamellipodia tips (data not shown), the 40aa FH1-derived, proline-rich stretch derived from FMNL2 employed in (B) was combined with the FH2-domain of FMNL1, and vice versa (see graphical representation of constructs used in D). Note that the FH2-domain of FMNL2 is well capable of targeting to the lamellipodium tip when combined with an FMNL1-derived poly-proline stretch, but not the other way around (top panel), demonstrating that nature of the FH2 domain defines whether or not FMNL formin family members accumulate at lamellipodia tips. (E) Western blot using anti-GFP antibody to confirm that the EGFP-tagged, chimeric FMNL2-L40aa-FMNL1-FH2 protein is expressed at its expected molecular weight, excluding its absence from lamellipodia tip to be simply caused by protein degradation. Monoclonal anti-GFP (101G4B2) was obtained from Synaptic Systems (Göttingen, Germany).

Figure S2: Lamellipodial tip localization of EGFP-tagged constructs is characterized by a sharp and well-pronounced peak of fluorescence. (A) Representative images of the subcellular localization of each construct expressed as EGFP-tagged version in migrating B16-F1 mouse melanoma cells, as well as EGFP alone as control (CTRL), as indicated. (B) Linescans of approximately 10μm in length drawn starting from outside the cell across the lamellipodial width (green lines in A) and deeper into the cytoplasm, to demonstrate differences in accumulation patterns between various constructs. Note the sharp peak in fluorescence for VASP, FMNL2-Full length, as expected, and the FMNL2(8P)-C construct, in all cases characteristic for constructs specifically accumulating at the lamellipodium tip. Although a slight increase in fluorescence throughout the entire lamellipodium was also observed for FMNL1-FH1-FH2 and mDia1-FH1-FH2 constructs, these patterns were by no means comparable to the sharp peak of fluorescence accumulation observed for former constructs. Moreover, although less evident at first glance, linescans of EGFP control expressing cells also revealed an increase of fluorescence throughout the lamellipodium (see bottom right), so the latter accumulations are considered to be distinct from the sharp peak localization of factors like VASP and FMNL2 variants presumed to be targeting to the interface of plasma membrane and polymerizing actin network.

Figure S3: Correlation analyses of expression levels of individual constructs versus lamellipodial protrusion rates in B16-F1 cells. EGFP-fluorescence images of cells overexpressing the five EGFP-tagged constructs as indicated plus EGFP alone as control (CTRL) were acquired prior to performing time-lapse microscopy for determination of
lamellipodial protrusion rates. Panels display correlation analyses between protrusion rates (in μm/min) and expression levels for each individual construct, the latter being determined as average EGFP fluorescence intensity normalized to external background of each individual cell. Correlation coefficients and p-values determined by Spearman Rank Order correlation tests are given in top right of each panel. Correlations between expression levels of individual constructs and their effects on protrusion rates are comparably poor, with lack of statistical significance in all cases except for FMNL1-FH1-FH2.

Figure S4: Correlation of construct expression versus fold increase of cytosolic F-actin (as compared to non-transfected controls) in B16-F1 cells. EGFP-fluorescence images of cells overexpressing the five EGFP-tagged constructs as indicated plus EGFP alone as control, acquired in parallel to phalloidin images, with the latter used for determining fold increase of cytosolic F-actin in construct overexpressing cells versus non-transfected cells (as described for data shown in Figure 2). Panels display correlation analyses for increase of cytosolic F-actin versus expression level of each individual construct determined as EGFP intensity for a given cell normalized to background outside the cellular region. Correlation coefficients and p-values determined by Spearman Rank Order correlation test are given in top right of each panel.

Figure S5: Total actin levels measured in B16-F1 cells overexpressing EGFP-tagged constructs. Quantification of actin expression in cells transiently transfected and selected for construct expression using puromycin, as specified in Materials and Methods. On average, at least 80-90% of cells having survived puromycin selection were confirmed to express detectable levels of EGFP-tagged constructs by fluorescence microscopy. Actin staining of total cell extracts of each experimental group was normalized to the expression of GAPDH on the same membrane (see bottom panel of representative Western blot used for quantifications), and displayed as fold-change compared to EGFP alone overexpressing cells used as control (CTRL). Data correspond to five independent experiments.

Figure S6: FMNL2(8P)-C overexpression reduces lamellipodium protrusion in B16-F1 cells irrespective of the fluorescent tag employed. Analysis of lamellipodium protrusion in migrating B16-F1 cells overexpressing EGFP alone (as control) or FMNL2(8P)-C tagged with EGFP or mCherry, as indicated. Data are arithmetic means ± standard errors of means. Both FMNL2(8P)-C variants markedly reduced rates of lamellipodium protrusion as compared to EGFP alone, but to a degree that was highly comparable and not statistically different (n.s.).

Figure S7: Alternative, fluorescent tagging of Lifeact does not affect rates measured for lamellipodium protrusion or actin network polymerization B16-F1 cells overexpressing Lifeact fused to EGFP versus mCherry were explored and analyzed concerning rates of lamellipodium protrusion (A) or actin network polymerization (B), as indicated.

Figure S8: Effects on lamellipodium width upon VASP and formin variant overexpression. Correlation analysis of lamellipodal protrusion rate versus lamellipodial width indicates a clear tendency of correlation between both parameters. A statistically significant correlation between the two parameters was established for all EGFP-tagged constructs except for mDia1-FH1-FH2, which might be due to comparably low values of both parameters observed upon expression of the latter construct. The fact that the correlation between the two parameters was highly significant also in cells expressing mCherry alone as
control (CTRL; bottom right) demonstrated the correlation to be uncoupled from individual construct expression and that the extent of effects on both parameters were comparable for the majority of constructs employed.

**Figure S9:** Differential translocation of PA-GFP-actin from cytosol to lamellipodium in B16-F1 cells overexpressing VASP and formin constructs. (A) Average change of fluorescence intensities of PA-GFP-actin over time in lamellipodial regions following photoactivation in the cell cytoplasm. Data are provided as percentage of intensities in respective photoactivation regions at t0 (left panel). Experimental conditions are color-coded as indicated, and data are arithmetic means ± standard errors of means. (B) Analysis of dispersal of cytosolically photoactivated PA-GFP-actin from the activation spot in cells overexpressing the 6 indicated constructs, as indicated. (C) The table summarizes the average area (± standard deviation) of the photoactivated region employed for each construct (in μm²), as well as the average distance (± standard deviation) between the edge of the photoactivated region and the tip of the lamellipodium for each construct tested (in μm).

**Figure S10:** Dispersion of PA-GFP-actin within the cytosol of B16-F1 cells is reduced upon overexpression of mDia1-FH1-FH2, as compared to VASP. (A) Time-lapse frames of GFP-channel of representative B16-F1 cells co-overexpressing PA-GFP-actin with mCherry-tagged VASP or mDia1-FH1-FH2, as indicated (not shown), and photoactivated at time point 0 (PA) in cytosolic regions, as marked with white circles. In each experiment, fluorescent intensities were recorded over time in three color-coded regions positioned at varying distance from the activation spot, to monitor efficiency and rate of dispersal of activated fluorescence through the cytosol (see below). (B) Results from quantitation of average fluorescence intensities recorded from regions as highlighted in a representative fashion in A. Significant differences were observed for dispersal of PA-GFP-actin in the two experimental conditions (color-coded in red and blue) and in each region; the respective number of cells (n) analyzed for each region is provided in graph for region 1; color-coding of regions is as shown in A.

**Figure S11:** Cytosolic mobility of PA-GFP-actin correlates with incorporation efficiency in the lamellipodium
Average intensities of PA-GFP-actin in the activation spot in the cytosol 15 sec after time of activation plotted against incorporated PA-GFP-actin intensities measured in the lamellipodium at the same time point. Data from both VASP and mDia1-FH1-FH2 overexpressors were combined for analysis. The apparent negative correlation was confirmed to be statistically significant using Spearman Rank Order Correlation analysis, as indicated.

**Figure S12:** Rates of lamellipodium protrusion and actin network polymerisation are differentially affected by overexpression of various actin-binding proteins in NIH-3T3 cells. (A) Average lamellipodial protrusion rate of B16-F1 cells following overexpression of each of the five different constructs, as indicated, and EGFP alone as control (CTRL). (B) Average rates of lamellipodial actin network polymerisation in NIH-3T3 cells following overexpression of EGFP-tagged constructs and EGFP alone as control (CTRL). All data are arithmetic means ± standard errors of means.

**Figure S13:** Effects of VASP or formin construct overexpression on lamellipodial and cytosolic F-actin distributions in NIH-3T3 cells.
(A) Representative images of NIH-3T3 cells transfected with various constructs, as indicated, and stained for F-actin with phalloidin. Note increased F-actin staining in cells ectopically expressing respective, EGFP-tagged construct (red asterisks). (B) Average values of different
F-actin intensity ratios, as indicated by labels in each row, measured for each of the overexpressed constructs and control (EGFP alone). (C) Ratios of average cytosolic F-actin intensities of EGFP-positive over EGFP-negative cells within the same microscopic field of view for each indicated construct. (D) Average ratios of lamellipodial over cytosolic F-actin intensities in EGFP-positive cells expressing each construct. Data in (C) and (D) are arithmetic means ± standard errors of means.
**Supplemental Movie legends**

**Movie S1:** Representative examples of time lapse phase-contrast microscopy of protrusive edges of migrating B16-F1 melanoma cells transfected with EGFP-tagged VASP or formin variants, as indicated. EGFP overexpression served as control.

**Movie S2:** Time-lapse fluorescence microscopy of lamellipodium and proximal lamella of a B16-F1 cell expressing EGFP-tagged Lifeact, illustrating the rearward movement of fluorescence inhomogeneities (black circle) within lamellipodial actin networks (region between black lines at first frame of movie). Movements of these inhomogeneities were exploited to calculate average actin polymerization rates of these lamellipodial actin networks, corresponding to the distance travelled by these fluorescence inhomogeneities plus the distance of protrusion for any given time.

**Movie S3:** B16-F1 cell co-expressing mRuby-Lifeact to visualize the actin cytoskeleton before and after photoactivation and PA-GFP-actin to explore the mobility of cytoplasmic actin. Photoactivation within a specific cytosolic region was followed by rapid incorporation of photoactivated GFP-actin into closely positioned lamellipodia (east side of the cell), and more delayed so and with reduced intensity at the back of the cell (west side). mRuby-Lifeact and PA-GFP-actin are shown in red and green in merge, respectively, as indicated. The area of photoactivation was marked as green polygonal.

**Movie S4:**
B16-F1 cells co-expressing mRuby-Lifeact (not shown) and PA-GFP-actin to explore the mobility of cytoplasmic actin. Again, photoactivated actin incorporates into lamellipodia, but can also readily be seen to diffuse towards the cell center (south direction in this movie). Colored regions define areas of fluorescence recording over time before and after photoactivation, with data shown in Figure 5C. Note the gradual increase of fluorescence with time, likely corresponding to PA-GFP-actin monomers diffusing towards the cell center after local activation.

**Movie S5:** Representative examples of B16-F1 cells overexpressing mCherry-tagged VASP or mDia1-FH1-FH2 (not shown) and PA-GFP-actin, to compare the mobility of actin in these two experimental scenarios. In these experiments, both the efficiency of incorporation of photoactivated actin into the lamellipodium tip and the loss of fluorescence in the activation region were analyzed (see Figure 6). Both respective activation region and analyzed lamellipodial region are highlighted as turquoise circles and yellow polygonals, respectively. Note that pre-activation regions show autocontrasted, weak actin signals (of non-activated PA-GFP-actin) throughout the cell, which disappear due to the magnitude of photoactivated fluorescence at the time point of activation. These pre-activation frames are displayed to confirm the presence of lamellipodia facing south in both experimental scenarios, yet incorporation of PA-GFP-actin is strongly reduced and delayed in case of mDia1-FH1-FH2 overexpression (see also analysis in Figure 6).
Supplemental Figure S1

A. EGFP-FMNL2-FH2

B. EGFP-FMNL2-L40aa-FH2

C. EGFP-FMNL2-L40aa-FMNL1-FH2

D. FMNL2-L40aa-FMNL1-FH2

E. FMNL2-L40aa-FMNL1-FH2
Supplemental Figure S2
Supplemental Figure S3

VASP

- Spearman Rank Order Correlation: -0.313
- Not statistically significant

FMNL2-Full length

- Spearman Rank Order Correlation: 0.813
- Not statistically significant

FMNL1-FH1-FH2

- Spearman Rank Order Correlation: -0.554
- P = 0.0001

FMNL2(8P)-C

- Spearman Rank Order Correlation: -0.134
- Not statistically significant

mDia1-FH1-FH2

- Spearman Rank Order Correlation: -0.236
- Not statistically significant

CTRL(EGFP)

- Spearman Rank Order Correlation: -0.437
- Not statistically significant
Supplemental Figure S4
Supplemental Figure S5

![Supplemental Figure S5](image-url)
Supplemental Figure S6
Supplemental Figure S7

A

Lamellipodial protrusion rate [μm/min]

- Lifeact-mCherry (n=46)
- Lifeact-EGFP (n=43)

B

Actin polymerization rate [μm/min]

- Lifeact-mCherry (n=12)
- Lifeact-EGFP (n=19)
Supplemental Figure S8
Supplemental Figure S9

A

Supplemental Figure S9 A

B

Supplemental Figure S9 B

C

|                      | Size PA-region [μm sq] | Distance PA-region to lamellipodia [μm] |
|----------------------|------------------------|----------------------------------------|
| CTRL (mCherry)       | 106.47 +/- 11.69       | 6.86 +/- 1.11                          |
| VASP                 | 105.4 +/- 17.61        | 6.41 +/- 1.36                          |
| FMNL2-Full length    | 97.36 +/- 4.52         | 6.17 +/- 1.01                          |
| FMNL1-FH1-FH2        | 101 +/- 14             | 6.81 +/- 1.23                          |
| FMNL2(8P)-C          | 99 +/- 8.87            | 6.2 +/- 0.94                           |
| mDia1-FH1-FH2        | 102.4 +/- 8            | 6.46 +/- 0.87                          |
Supplemental Figure S10

A

B

Region 1

Region 2

Region 3
Supplemental Figure S11
