

Supplementary Information

**Quantitative investigation of membrane curvature sensing and generation by I-BARs in living cells**

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## Custom script for semi-automated analysis of filopodia

The custom script used for image analysis identifies filopodia, and extracts intensities along their length for the fluorescence channel of the protein and of the membrane. The intensity of the protein channel is used to measure the density of the protein along the filopodia, while the corresponding radius is derived from the intensity of the membrane channel. The employed theoretical method used to extract the two variables of interest is based on the work of Rosholm et al.<sup>1</sup>. We describe below in four steps the custom script.

### 1. Filopodia identification and intensity extraction

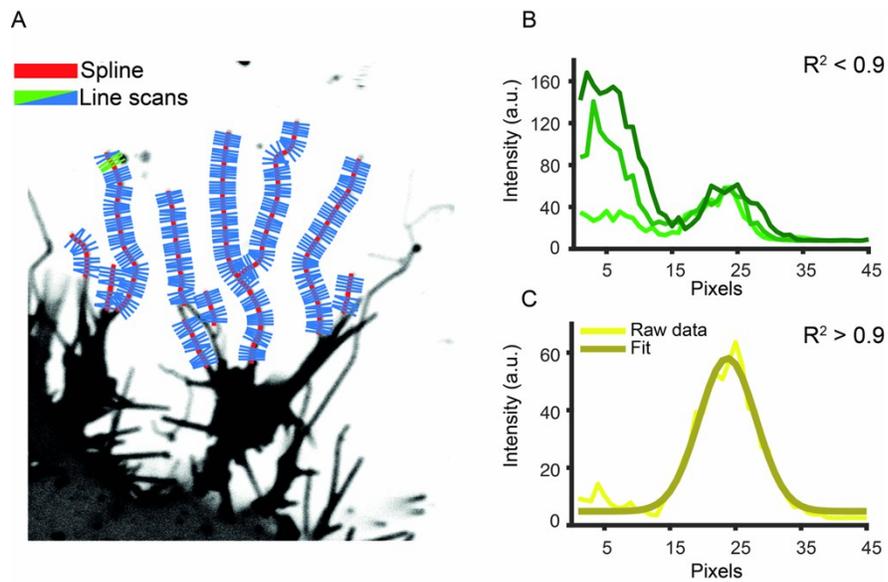
The custom script functions as a plug in of the microscopy image browser of Belevich et al.<sup>2</sup>. The initial stage of the analysis involves filopodia identification using the algorithm described in Nilufar et al. 2013<sup>3</sup>. The algorithm defines a skeleton of 1 pixel for each identified tubular structure and generates a mask. After identification, the custom script removes tubular structures with a length lower than 50 pixels.

Due to the dynamic nature of filopodia, we verify the overlapping of the identified filopodia in the two channels. The skeleton of the filopodia in the protein channel is dilated by 2-pixels, if the produced mask does not overlay with the corresponding filopodia of the membrane channel, we exclude it. Additionally, the user has the option to remove filopodia manually.

The program fits each selected filopodia with a spline function of order 3, and equidistant knots separated by 9 points. The spline function generates a smooth description of a filopodium, which is derivable, and continuous at every point. The continuity condition allows us to take the second order derivative and identify segments perpendicular to the skeleton of filopodia (see Fig. S1). From each segment, we extract the underlying intensity, through a Matlab built in function called `lmpofile`, which extracts the intensity of the pixels crossed along the segment length. An intensity profile of a segment perpendicular to a cylindrical structure with a size below the confocal resolution has a Gaussian profile. Therefore, we extract the integrated intensities by Gaussian fitting with a linear background (Fig. S1 C).

The fit provides additional information as the full width at half maximum (FWHM) and the coefficient of determination  $R^2$ . These parameters are used to exclude noisy data and sample artifacts, using a FWHM  $> 7$  pixels and  $R^2 < 0.9$ . We chose 7 pixels to exclude profiles above the diffraction limit (7 pix  $\sim$  280 nm). A FWHM above the diffraction limit typically indicate the presence of two parallel filopodia or non-perpendicular segments and ensures that the intensity profiles are gaussian. The cut-off for  $R^2$  is empirically based. An example of an artifact identified using  $R^2$  is the presence of dye aggregates neighbouring a filopodium (Fig. S1 B). The user can tune each parameter to adapt the analysis to microscopes with different resolution, and experimental conditions.

After the intensity extraction, the custom script defines a region of interest composed by the average integrated intensity  $I$  of 5 adjacent segments for both channels. One region of interest produces a single data point with error the standard deviation between the integrated intensities of the 5 segments included in each region of interest. This analysis produces intensity values for the protein and the membrane channel, i.e.  $I^{(protein)}_{filo}$  and  $I^{(membrane)}_{filo}$ .



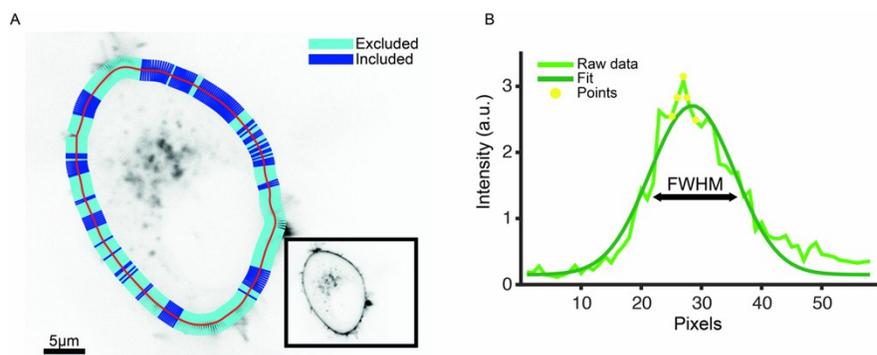
**Fig. S1 Spline fitting of the identified filopodia and extraction of the integrated intensities.** (A) Section of a confocal fluorescent image of a PC12 cell expressing the I-BAR of IRSp53 tagged with eGFP, where the focus is at the surface of the glass slide. The red line shows the spline fit for each identified filopodium. In blue and green the perpendicular segments to the spline function used to extract the underlying intensity. The green segments correspond to the segments in (B). (B) Examples of the intensity profile for three perpendicular segments with  $R^2 < 0.9$ . (C) Intensity profile of a segment passing the exclusion criteria with an  $R^2 > 0.9$  and corresponding Gaussian Fit.

## 2. Plasma membrane identification and intensity extraction

To normalize for heterogeneity in the transfection level and labeling efficiency of the membrane, and combine data from multiple cells, we collect a second set of images taken at the equator of the cell. For the transfection level of the I-BAR domain, we use the average intensity signal of the cytosol. In contrast, for the labeling efficiency of the membrane we use the average intensity of the plasma membrane (PM) at the cell equator as a normalization factor, as previously done by Rosholm et al.<sup>1</sup> Considering that the optical thickness of a confocal microscope ( $\sim 0.8 \mu\text{m}$ ) is much smaller than the thickness of the cells we work with ( $\sim 10\text{-}20 \mu\text{m}$ ), the plasma membrane at the cell equator is approximately perpendicular to the image plane. Membrane patches that deviate substantially from this approximation are excluded during the data analysis.

To extract the normalization factors, the custom script initially identifies an outline of the PM in the membrane channel using a watershed algorithm. The watershed algorithm requires the user to select a point within and a point outside of the PM borders. The program then fits the membrane outline with a spline function, of order 3 and knots distance 50, and produces a smooth description of the PM (Figure S2 A). The program defines perpendicular segments to the cell membrane and extracts the underlying intensities, as described for the identification of filopodia. The extracted intensities are then fitted with a Gaussian function and the FWHM is measured. We use an empirically based upper limit of 15 pixels on the FWHM to exclude segments that overlap with structured membrane folds as tubular protrusions and endomembrane in proximity of the PM. The limit on the FWHM of  $\sim 600\text{nm}$  also excludes membrane patches that deviate more than  $>37^\circ$  from a perpendicular orientation to the image plane. We can calculate an upper limit on the deviation from perpendicularity as the angle adjacent to the major cathetus of a right triangle with catheti the z-resolution ( $\sim 0.8 \mu\text{m}$ ) of the confocal microscope and the upper limit on the FWHM ( $\sim 0.6 \mu\text{m}$ ), i.e.  $\theta = \tan^{-1} 0.6/0.8$ .

For every one of the included segments along the PM the custom script averages the intensity of the five neighboring points to the maximum of each intensity profile (Figure S2 B yellow points) for the membrane and protein channel respectively. The normalization factors are calculated as the average intensity of all the selected points both for the protein and the membrane channel. These normalization factors are then used to combine data points obtained from filopodia of different cells, and we denote them  $I(\text{protein})_{PM}$  for the protein and  $I(\text{membrane})_{PM}$  for the membrane label.



**Fig. S2 Extraction of the integrated intensities of the plasma membrane.** (A) Cell cross-section imaged  $\sim 5 \mu\text{m}$  above the coverslip for the membrane channel. The outline of the PM determined by the watershed algorithm is fitted with a spline function (red line). The intensity profiles extracted from perpendicular cuts are included (blue) or excluded (cyan) based on the FWHM. (B) Example of the intensity profile from one of the included perpendicular segments fitted with a gaussian function. The gaussian fitting allows to estimate the FWHM. The yellow colored points are the 5 points used to estimate the intensity in the protein channel.

### 3. Quantification of the MiM I-BAR, and IRSp53 I-BAR Normalized Density

The normalized density is derived from the intensity values extracted from the filopodia and the PM for the protein channel using the described custom script. The normalized density is calculated using the equation:

$$\text{Norm.density} = \frac{I(\text{protein})_{\text{filo}}/I(\text{protein})_{\text{PM}}}{I(\text{membrane})_{\text{filo}}/I(\text{membrane})_{\text{PM}}} \quad (\text{S1})$$

Eq. 1 is characterized by two normalization components. The normalization of the protein signal by the surface area, and the normalization that takes into account heterogeneity in membrane labeling and expression level. The first normalization takes into account that the absolute protein signal needs to be divided by the corresponding surface area to calculate densities. Since the membrane signal does not sort as a function of curvature<sup>1</sup>, the amount of signal is proportional to the underlying membrane area, and can be used to extract densities as:

$$\text{Density} = \frac{I(\text{protein})_{\text{filo}}}{I(\text{membrane})_{\text{filo}}} \quad (\text{S2})$$

The second component of Eq. 1 normalizes each variable of Eq. 2 to compare data from cells with heterogeneous expression level, and membrane label efficiency. Each component in Eq. 2 is, therefore, divided by the corresponding normalization factor:

$$I(\text{protein}) = \frac{I(\text{protein})_{\text{filo}}}{I(\text{protein})_{\text{PM}}}, I(\text{membrane}) = \frac{I(\text{membrane})_{\text{filo}}}{I(\text{membrane})_{\text{PM}}} \quad (\text{S3})$$

#### 4. Estimating the conversion factor by pulling tethers from live cells

The quantification of the radius of filopodia was done by following the same procedure of Rosholm et al.<sup>1</sup>. Here we outline the employed methodology.

To quantify the radius of diffraction limited filopodia we label the plasma membrane with a lipidated dye DiD, which is not sensitive to membrane curvature<sup>1</sup>. Since, filopodia have a tubular membrane geometry, the integrated intensities across the length of a filopodia are theoretically predicted to scale linearly with the radius ( $r$ ):

$$I \propto N_{\text{fluorophores}} \propto A_{\text{surface}} \propto r \quad (S4)$$

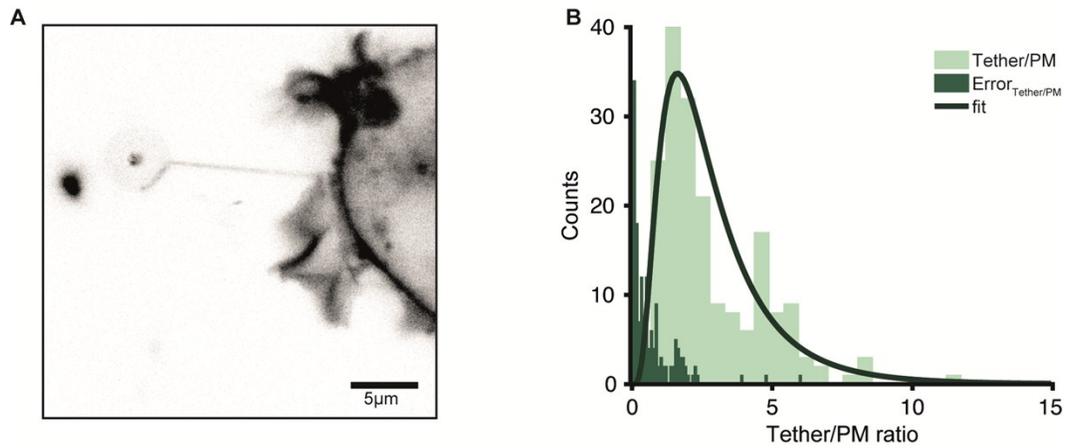
To convert arbitrary units of the intensity measurements into absolute radius in nanometers, Eq.S3 requires the calculation of a conversion factor (C):

$$r = IC \quad (S5)$$

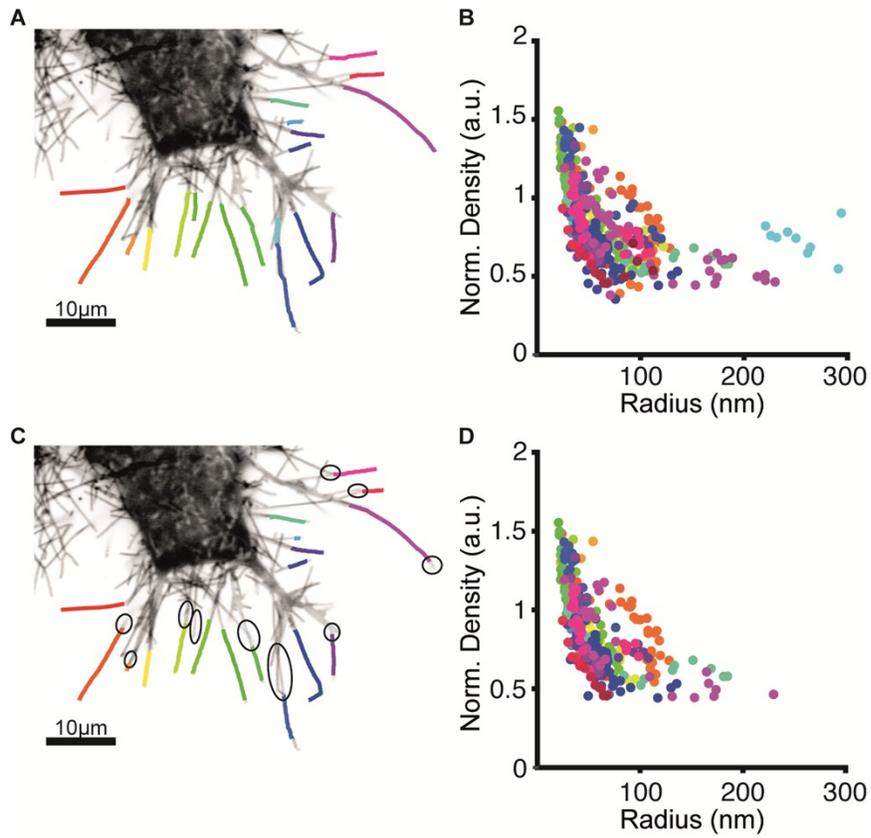
To determine the conversion factor, Rosholm et al.<sup>1</sup> pulled tethers (<15  $\mu\text{m}$ ) of living HEK293 cells labelled with DiO. DiO has the same lipid tails as DiD and has been shown to sort to the same degree as DiD<sup>1</sup>. Rosholm et al.<sup>1</sup> used optical trapped streptavidin-coated beads and imaged the tethers using confocal fluorescence microscopy. Short membrane tethers (<15  $\mu\text{m}$ ) are rapidly buffered by the membrane reservoir and therefore equilibrate to approximately the same radius<sup>4</sup>. Pontes et al.<sup>4</sup> previously characterized by scanning electron microscopy that short tethers (<15  $\mu\text{m}$ ) pulled from NIH3T3 cells have a mean radius of  $r = 49 \pm 4$  nm. Using the custom script, we reanalyzed the images of the tethers collected by Rosholm et al.<sup>1</sup> and we calculated the ratio between the integrated intensities of the tether and the PM using eq. S3,  $I(\text{membrane})$ . We analyzed tethers pulled from 8 distinct cells. The custom script provides multiple intensity values and the corresponding errors for each tether resulting in the histograms shown in Fig S3. We fitted the histograms of the propagated intensities and errors with a lognormal distribution. The fits provide the location and scale parameters  $\mu$  and  $\sigma$ . The two parameters are then used to calculate the arithmetic mean of  $I(\text{membrane})$  and the corresponding errors as:

$$\text{mean} = e^{\mu + 0.5\sigma^2} \quad (S6)$$

This resulted in a mean of  $I(\text{membrane}) = 2.98 \pm 0.77$  that corresponds to a conversion factor of  $C = 49/2.98 = 16.4 \pm 4.5$  nm.



**Fig. S3 Fitting tether ratios to obtain the conversion factor.** (A) Image of a tether pulled from a HEK293 cell using optical trapped streptavidin-coated beads. (B) Histogram of  $I(\text{membrane})$  (light green) and the propagated errors (dark green) of the extracted segments from 8 individual cells. The black continuous line indicates lognormal fit of the ratio between the integrated intensities of the tether and the PM.



**Fig. S4 Data points selection based on the FWHM.** (A) Confocal fluorescence image of a PC12 cell expressing the I-BAR domain of IRSp53 tagged with eGFP, where the focus is at the surface of the glass slide. Each color line represents an identified filopodia. (B) Single cell measurements of normalized I-BAR density plotted against the filopodia radius. In (A) and (B) all data points are included with no filter on the FWHM. (C) and (D) show the same graphs as in (A) and (B) excluding the data points with a FWHM above 280 nm . Examples of excluded parts of filopodia are shown with black circles on (C).

## Supporting References

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- 4 B. Pontes, N. B. Viana, L. T. Salgado, M. Farina, V. M. Neto and H. M. Nussenzveig, *Biophys. J.*, 2011, **101**, 43–52.