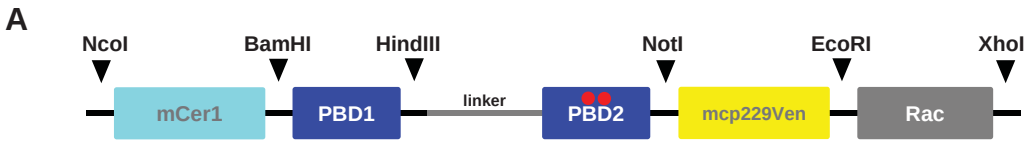


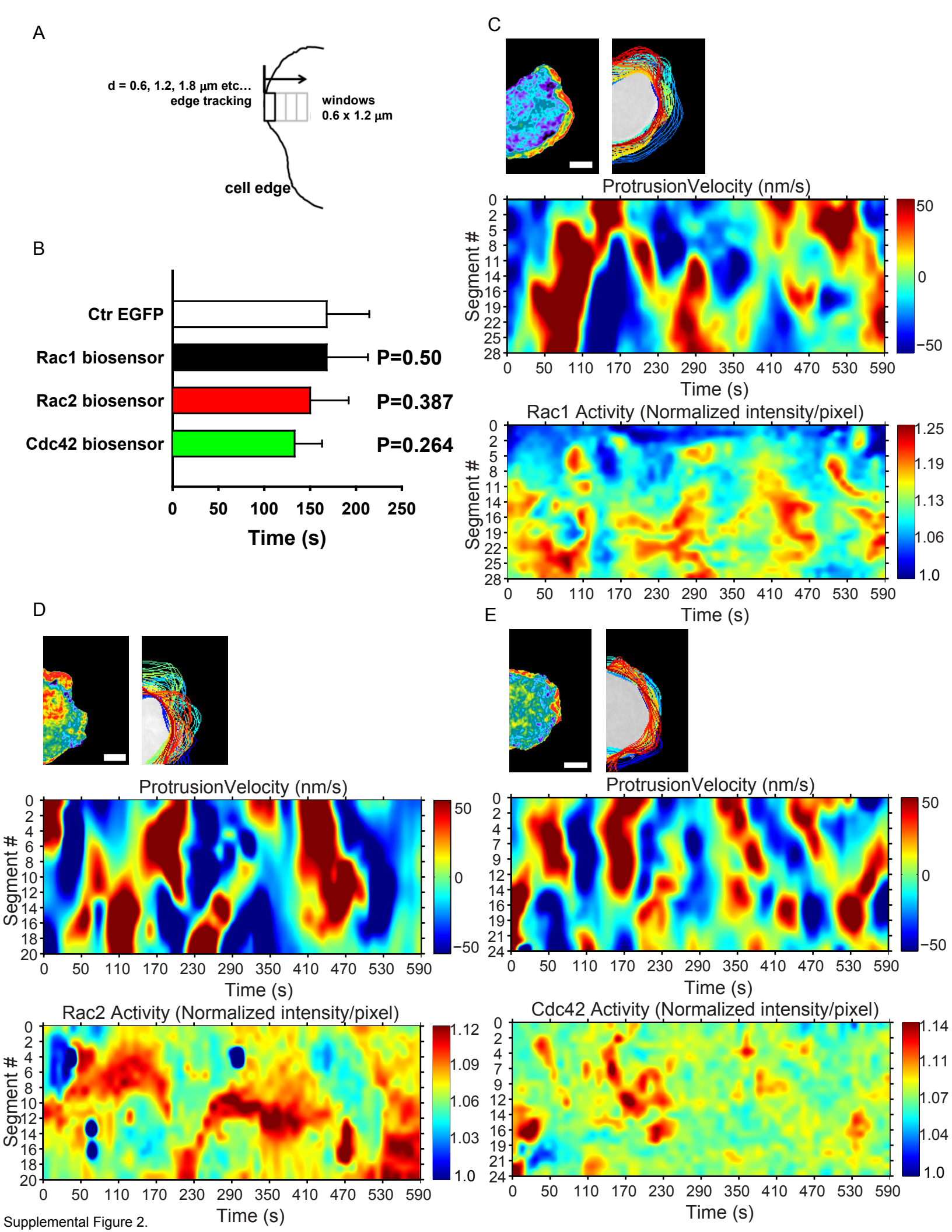
B

```
Syn.Mod.v2 PBD2   AAGGAAAGGCCCGAAATCAGCCTGCCCAGCGACTTCGAGGATACCATCGACG
Original PBD2     AAAGAGCGGCCAGAGATTTCTCTCCCTTCAGATTTTGAAGACACAATTGATG
                  ** **  **** ** **    ** **    ** ** ** ** ** ** ** *

Syn.Mod.v2 PBD2   TGGGCTTCGACGCCGTGACCGGCGAGTTTACCGGCATGCCCGAACAGTGGGC
Original PBD2     TCGGTTTTGATGCTGTCACAGGGGAGTTTACGGGAATGCCAGAGCAGTGGGC
                  * ** ** ** ** ** ** ** ******** ** ***** ** ********

Syn.Mod.v2 PBD2   TAGGCTGCTGCAGACCAGCAACATCACCAAAGCGAACAGAAAAAGAACCCC
Original PBD2     CCGCTTGCTTCAGACATCAAATATCACTAAGTCGGAGCAGAAGAAAAACCCG
                    *  **** *****    ** ***** **    ** ***** ** *****

Syn.Mod.v2 PBD2   CAGGCCGTGCTGGACGTGCTGGAGTTCTATAATAGCAAAAAGACCAGCAATT
Original PBD2     CAGGCTGTTCTGGATGTGTTGGAGTTTTACAACTCGAAGAAGACATCCAACA
                  ***** ** ***** *** ******* ** **    ** *****   ***

Syn.Mod.v2 PBD2   CCCAGAAGTATATGTCCTTCACCGACAAAAGC
Original PBD2     GCCAGAAATACATGAGCTTTACAGATAAGTCA
                   ****** ** ***  *** ** ** **
```

C

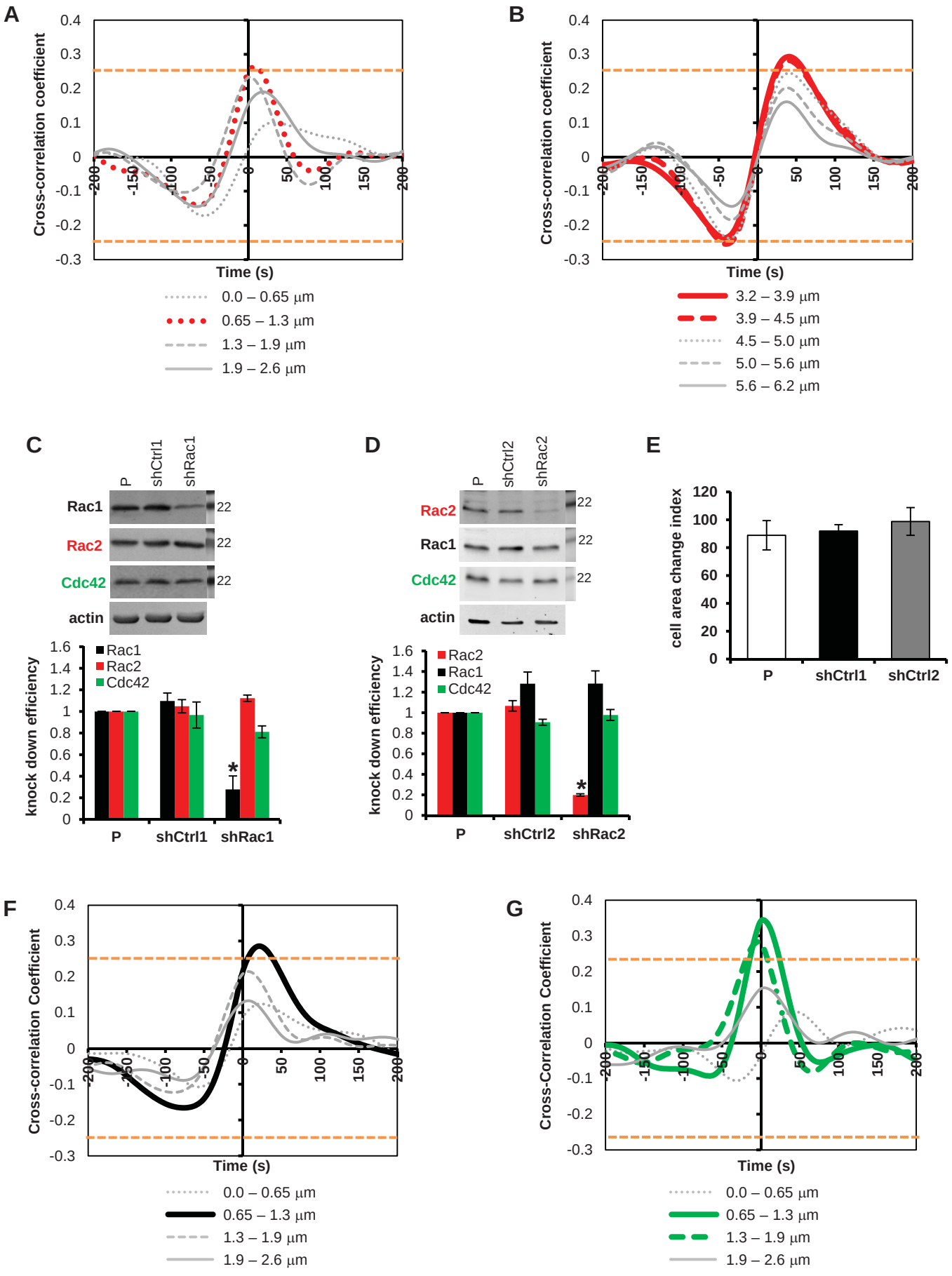
```
Syn.Mod.v2 mCer1  ATGGTGTCCAAAGGAGAAGAACTGTTTACAGGAGTGGTCCCTATTCTGGT
Original mCer1    ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGT
                  ******  *** ** ** ** ***** ** ** ***** ** ** *****

Syn.Mod.v2 mCer1  GGAACTGGATGGAGATGTGAATGGACATAAATTTTCCGTGAGCGGAGAAG
Original mCer1    CGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG
                   ** ***** ** ** ** ** ** ** ** **    ****  *** ** *

Syn.Mod.v2 mCer1  GAGAAGGAGACGCTACATATGGAAAACTGACACTGAAGTTTATTTGTACA
Original mCer1    GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC
                  * ** ** ** ** ** ** ** ** ** ***** ******** ** ** **

Syn.Mod.v2 mCer1  ACAGGAAAACTGCCTGTGCCTTGGCCTACACTGGTGACCACACTGACATG
Original mCer1    ACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTG
                  ** ** ** ***** ***** ***** ** ** ******** ***** **

Syn.Mod.v2 mCer1  GGGAGTCCAGTGTTTTGCTAGGTATCCTGATCATATGAAACAGCATGATT
Original mCer1    GGGCGTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACT
                  *** ** ***** ** **  * ** ** ** ** ***** ***** ** *

Syn.Mod.v2 mCer1  TCTTTAAAAGCGCTATGCCTGAGGGATATGTGCAGGAAAGGACAATTTTC
Original mCer1    TCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC
                  **** **   *** ***** ** ** ** ** *****  * ** ** ***

Syn.Mod.v2 mCer1  TTTAAAGATGATGGAAATTATAAAACAAGGGCTGAAGTGAAATTTGAAGGG
Original mCer1    TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGG
                  ** ** ** ** ** ** ** ** ** **  * ** ** ***** ** ** **

Syn.Mod.v2 mCer1  AGATACACTGGTGAATAGGATTGAACTGAAAGGAATTGATTTTAAAGAAG
Original mCer1    CGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
                   ** ** ********  * ** ** ***** ** ** ** ** ** ** *

Syn.Mod.v2 mCer1  ATGGAAATATTCTGGGACATAAACTGGAATATAATGCTATTAGCGACAAT
Original mCer1    ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACGCCATCAGCCACAAC
                  * ** ** ** ***** ** ** ***** ** ** ** ** *** ****

Syn.Mod.v2 mCer1  GTGTACATTACAGCTGATAAACAGAAAAATGGAATTAAGGCTAATTTTAA
Original mCer1    GTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAA
                  ** ** ** ** ** ** ** ** ***** ** ** ** ***** ** ** **

Syn.Mod.v2 mCer1  AATTAGGCATAATATTGAAGATGGAAGCGTGCAGCTGGCTGATCATTATC
Original mCer1    GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
                   **  * ** ** ** ** ** ** ** ************ ** ** ** ** *

Syn.Mod.v2 mCer1  AGCAGAATACACCTATTGGAGATGGACCTGTGCTGCTGCCTGATAATCAT
Original mCer1    AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCAC
                  ******* ** ** ** ** ** ** ** ** ************ ** ** **

Syn.Mod.v2 mCer1  TATCTGTCCACACAGAGCAAACTGTCCAAAGATCCTAATGAAAAAGGGA
Original mCer1    TACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGA
                  ** ***  *** ***  *** ***  ****** ** ** ** **  * **

Syn.Mod.v2 mCer1  CCATATGGTGCTGCTGGAATTTGTGACAGCTGCCGGCATTACCCTGGGAA
Original mCer1    TCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA
                   ** ***** ******** ** ***** ** ***** ** ** ** ** *

Syn.Mod.v2 mCer1  TGGATGAACTGTATAAA
Original mCer1    TGGACGAGCTGTACAAG
                  **** ** ***** **
```

SUPPLEMENTAL FIGURE 1. Biosensor construction and sequence alignments. **A**) schematic of Rac biosensor including cloning restriction sites. **B**) original *vs* synonymously-modified PBD2, where p21 binding domain of the PAK1 was codon optimized to achieve Codon Adaptation Index of 0.93 (mouse; Genscript) and 66% sequence homology with the original PBD. **C**) original *vs* synonymously-modified mCer1, where mCer1 was codon optimized to achieve Codon Adaptation Index of 0.82 (mouse; Genscript) and 66% sequence homology against the mcp229Ven (original and the linker optimized versions), and 70% homology against the original mVen, used in the single-chain biosensors.



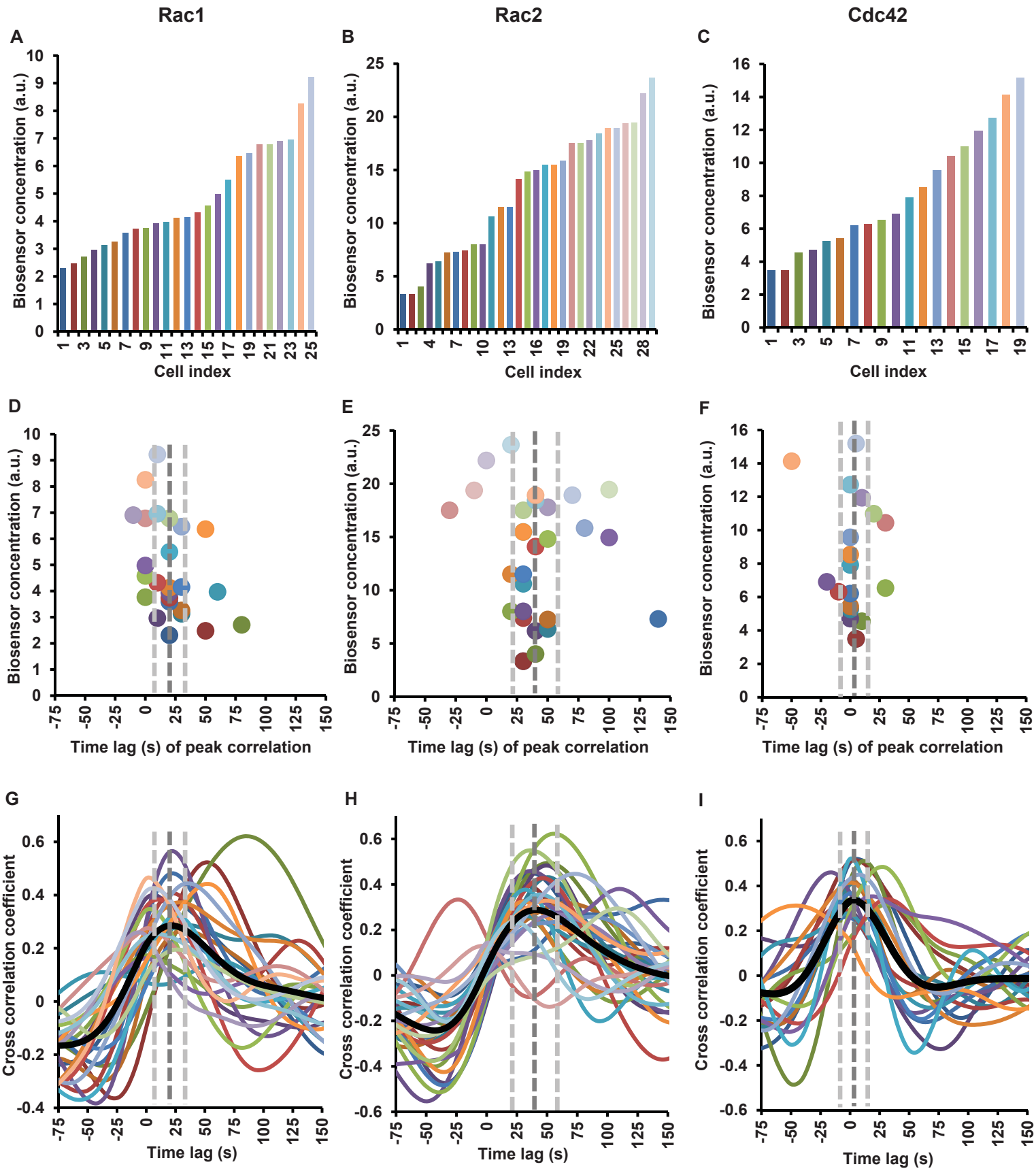
Supplemental Figure 2.

SUPPLEMENTAL FIGURE 2. Morphodynamic mapping of Rac1, Rac2 and Cdc42 activation during serum-stimulated random protrusions in macrophages. **A**) Schematic of measurement windows constructed along the edge of the protrusion. **B**) Protrusion cycling periodicity, determined from the auto-correlation function of leading edge protrusion velocities. Correlation coefficient pattern indicates that cells expressing the control EGFP, Rac1, Rac2 or Cdc42 biosensor have the same protrusion cycle periodicity. The protrusion cycling period was taken as the temporal width between the first inflection points after the functional zero-crossing. Control-EGFP: 168 +/- 46.5sec; Rac1 biosensor: 168 sec +/- 45sec; Rac2 biosensor: 150 sec +/- 42 sec; and Cdc42 biosensor: 133 sec +/- 30 sec. (+/- S.D. derived from the 95% confidence intervals determined from the 2000x bootsrap average calculations). The p-values between the control versus the biosensor conditions are shown. See text for additional statistical values for morphodynamic analysis. Representative example of RAW/LR5 cell with inducible expression of **C**) Rac1, **D**) Rac2 and **E**) Cdc42 showing ratiometric image of a protrusion (left) and its corresponding computational edge tracking (right); activity map of edge movement of the protrusion shown with red shades indicating protrusive and blue shades indicating retraction event (middle), and matching GTPase activation (bottom). Scale bar = 5 μm.



Supplemental Figure 3.

SUPPLEMENTAL FIGURE 3. Morphodynamic analysis of Rac1, Rac2 and Cdc42 activation dynamics of random protrusions of macrophages grown in serum. **A**) Temporal cross-correlation of GTPase activation relative to edge velocity for front-associated Rac2, **B**) distal Rac2. **C**) Western blot analysis of shRNA-mediated reduction of Rac1 and **D**) Rac2 in RAW/LR5 cells; P = parental RAW/LR5, shCtrl1 = empty pSuper.retro.puro vector, shCtrl2 = empty pGIPZ vector; quantitation of reduction of Rac1, Rac2 and Cdc42 protein levels with data mean +/- SEM, $p < 0.005$. **E**) Cell area change index for parental RAW/LR5 cells (P, 46 cells) , control for shRac1 cells (shCtrl1, 127 cells) and control for shRac2 cells (shCtrl2, 69 cells); data mean +/- SEM, $p > 0.5$ **G**) Temporal cross-correlation of GTPase activation relative to edge velocity for front-associated Rac1, **H**) front-associated Cdc42 activation.



Supplemental Figure 4.

SUPPLEMENTAL FIGURE 4. Sensitivity of image measurements on GTPase biosensor concentration. **A**) Rac1, Rac2 or Cdc42 biosensor (left to right) was stably and inducibly expressed in RAW/LR5 cells, and each cell analyzed is color-coded based on the absolute biosensor expression level (calculated from the whole-cell averaged pixel intensities measured in FRET-acceptor YFP channel, taking into account the exposure times and the neutral density filters used to attenuate the excitation light). Fold-difference values in the lowest to highest expressors are: Rac1: 3.99-fold; Rac2: 7.08-fold; and Cdc42: 4.35-fold. **B**) Scatterplots showing the time lag of the peak correlation curve versus the biosensor expression level in cells expressing the Rac1, Rac2 or Cdc42 biosensor (left to right: color-coded as in **A** to correspond to specific cells and expression levels). The time lags are from the spatial position that showed the highest average cross-correlation coefficient values in each biosensor condition (Rac1: 0.65-1.3μm; Rac2: 3.2-3.9μm; and Cdc42: 0.65-1.3μm). Dashed dark gray line indicates the average time lag of the peak cross-correlation at this spatial position (95% confidence intervals are shown with dashed light gray lines). **C**) Raw data sets showing the cross-correlation functions between the biosensor activity and edge velocity for the Rac1, Rac2 or Cdc42 biosensor. The expression level of the biosensor for each cross-correlation curve is color-coded as in **A** to correspond to specific cells and expression levels. Bootstrap-averaged trace of the cross-correlation functions is also shown in black. Dashed dark gray line indicates the average time lag of the peak cross-correlation at this spatial position (95% confidence intervals are shown with dashed light gray lines).

Movie legends

Movie S1. Rac1 activation during fMLP stimulation of macrophages. Time lapse of FRET/mCer ratio and corresponding DIC image. Duration of original sequence: 15 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 5 μm. * = start of fMLP stimulation.

Movie S2. Rac2 activation during fMLP stimulation of macrophages. Time lapse of FRET/mCer ratio and corresponding DIC image. Duration of original sequence: 15 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 5 μm. * = start of fMLP stimulation.

Movie S3. Rac1 activation during serum-induced random protrusions in macrophages. Time lapse of FRET/mCer ratio and corresponding DIC image. Duration of original sequence: 10 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 10 μm.

Movie S4. Rac2 activation during serum-induced random protrusions in macrophages. Time lapse of FRET/mCer ratio and corresponding DIC image. Duration of original sequence: 10 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 10 μm.

Movie S5. Cdc42 activation during serum-induced random protrusions in macrophages. Time lapse of FRET/mCer ratio and corresponding DIC image. Duration of original sequence: 10 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 10 μm.

Movie S6. Serum-induced random protrusions in Rac2-reduced macrophages. Time lapse of DIC images of control (shCtrl2, left) and shRac2 (right). Duration of original sequence: 10 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 10 μm.

Movie S7. Serum-induced random protrusions in Rac1-reduced macrophages. Time lapse of DIC images of control (shCtrl1, left) and shRac1 (right). Duration of original sequence: 10 min. Magnification 60X, 2X2 binning. Frame interval: 10 s. Replay: 5 frames/s. Scale bar = 10 μm.