SUPPLEMENTAL DATA

Supplemental Table 1. Deletion of PAK1 N-terminus improved FRET efficiency of YFP-PAK1-CFP constructs
The emission YFP/CFP ratios of the indicated YFP-PAK1-CFP fusion constructs were measured by *in vitro* spectroscopy on 293T cell lysates as previously described (1). The fluorescence spectrum was obtained upon excitation at 433 nm with an FP-750 spectrofluorometer (Jasco, Tokyo, Japan).

Supplemental Table 2. Acceptor photo-bleaching experiment
Living Cos1 cells expressing YFP-PAK70-CFP were used. Single cells were YFP bleached by illumination at 510 nm for few minutes through a pin-hole without neutral density filter. YFP, CFP and FRET fluorescence intensities of 10 cells were measured before and after bleaching and normalized to the fluorescence prior to bleaching. Bleaching of the YFP acceptor led to the expected increase of the CFP donor fluorescence and decrease of the FRET fluorescence (YFP emission upon CFP excitation). The apparent FRET efficiency (E) was estimated roughly 9% from the donor emission before (Fd) and after (Fd pb) acceptor photo-bleaching using the formula E=1-(Fd/Fd pb).
During the development of the project we found that a construct lacking the first 64 residues (YFP-PAK65-CFP, than named Pakabi) behaved as YFP-PAK70-CFP in all the tested conditions and it was preferred because of its shorter deletion.

Supplemental Table 3. FRET of Pakabi is mainly intra-molecular
Since donor CFP and acceptor YFP of Pakabi are fused in a single-molecule and Pakabi forms dimers, the observed FRET could occur both intra-molecularly and inter-molecularly, i.e. from one CFP moiety either to the YFP moiety of the same Pakabi molecule, or to the YFP moiety of the other Pakabi molecule in the dimer. To evaluate the possible contribution of the inter-molecular component, the following constructs were expressed in Cos-7 cells: 1) YFP-PAK65 (PAK1 lacking the first 64 residues), acceptor-only condition; 2) PAK1-CFP (PAK1 full-length), donor-only condition; 3) YFP-PAK65 and PAK-CFP, inter-molecular FRET condition; 4) YFP-PAK65-CFP (i.e. Pakabi). All of these PAK1 constructs carried the kinase-inactivating mutation K299R. We followed the method of Sorkin et al. (2) in order to measure the FRET "corrected" for cross-over components (spectral bleedthrough and cross-excitation) (FRETc):
FRETc = FRET - (Fd/Dd x Dfd) - (Fa/Aa x Afa)
We determined in our microscope setting the ratios Fd/Dd (0.433 ± 0.121) and Fa/Aa (0.174 ± 0.055) using the first and second conditions, i.e. cells expressing only donor or only acceptor, respectively. We measured the FRET of the inter-molecular condition and of Pakabi as following: for each cell we acquired FRET (FRET), CFP (Dfd) and YFP (Afa) images; after back-ground subtraction, we calculated the FRETc according to the above formula using average intensities on whole cell; "normalized" corrected FRET (FRETNc) is obtained by dividing FRETc by the mean fluorescence of both CFP and YFP ((Dfd+Afa)/2) of each cell. N≥20 for each condition.

Supplemental Figure S1. Depletion of PAK1 inhibits cells spreading.
The experiment was performed as for Figure 3A, but using RPE1 cells instead of HEK-HT cells. Depletion of PAK1 was >65% and that of PAK2 >80%, according to quantification with ImageJ software.

Supplemental Figure S2. Pakabix expression does not perturb cell spreading.
Cos-7 cells expressing YFP or Pakabix were treated as described in legend of Figure 3B.

Supplemental Figure S3. Spatiotemporal Cdc42 activity during cell spreading (Raichu-Cdc42)
Cos-7 cells expressing Raichu-Cdc42 were imaged by FRET wide-field video-microscopy during spreading on fibronectin-coated dishes. Time 0 is the beginning of the video recording, approx. 15 min after re-plating. Selected time points are shown as indicated. Scale bar, 10 μm. The outlined region is shown enlarged below. For the entire time-lapse video sequence, see Supplemental Movie 6. Cdc42 activity is monitored by the biosensor's FRET, which is measured as YFP/CFP ratio and represented

using the 8-colour scale code shown on the right with the upper and lower limits. Red corresponds to high Cdc42 activity and blue to low Cdc42 activity.

REFERENCES OF SUPPLEMENTAL DATA

1. Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001) *Nature* **411**(6841), 1065-1068
2. Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000) *Curr Biol* **10**(21), 1395-1398

SUPPLEMENTAL VIDEOS

Supplemental Movie 1. PAK1 activity during Cos-7 cell spreading (Pakabix)

Supplemental Movie 2. Control mutant Pakabix KD

Supplemental Movie 3. PAK1 activity during NRK cell motility (Pakabix)

Supplemental Movie 4. Pakabix during ruffling

Supplemental Movie 5. Pakabix at isolated protrusion

Supplemental Movie 6. Cdc42 activity during Cos-7 cell spreading (Raichu-Cdc42)

Supplemental Movie 7. Pakabix + Cdc42

Supplemental Movie 8. Mutant Pakabix CRIB

Supplemental Table 1

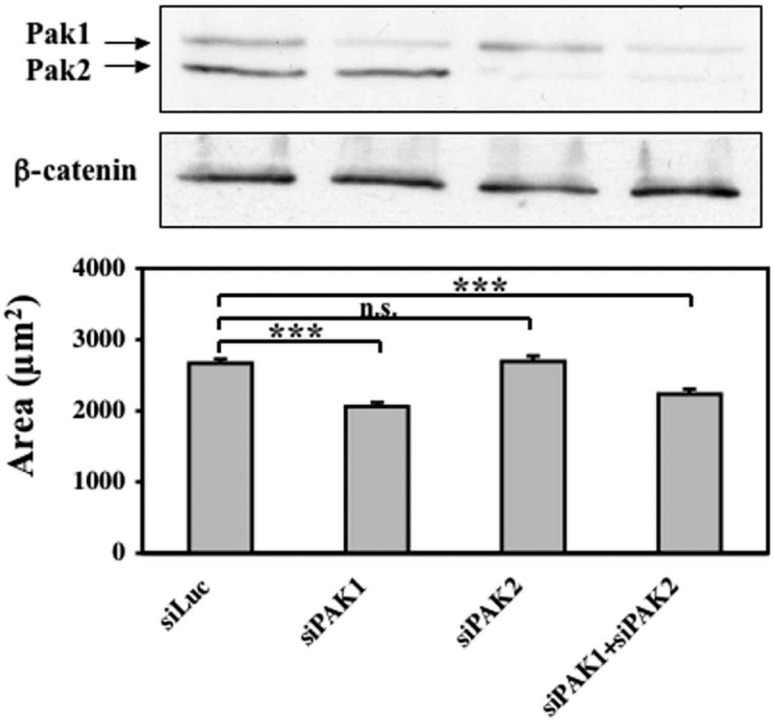
Constructs	Emission Ratio YFP/CFP
YFP-PAK-CFP full-length PAK1 (K299R)	0.783
YFP-PAK30-CFP deletion of 29 N-ter residues	0.804
YFP-PAK50-CFP deletion of 49 N-ter residues	0.955
YFP-PAK70-CFP deletion of 69 N-ter residues	1.017

Supplemental Table 2

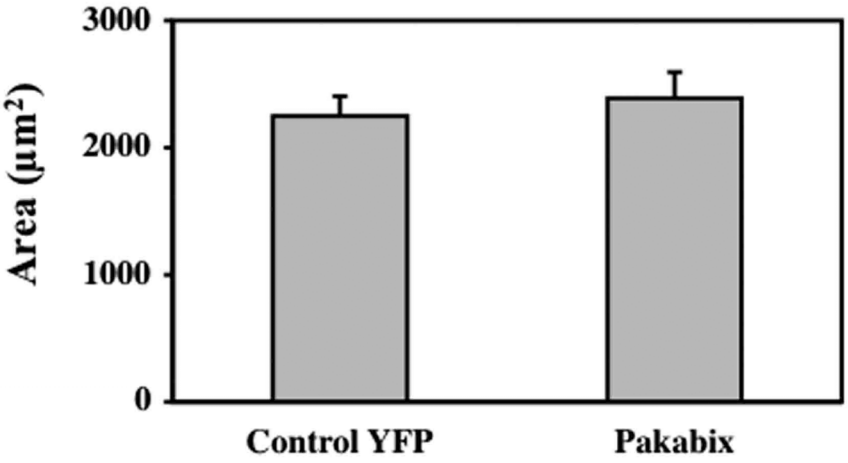
		Channel		
		Acceptor YFP Ex 510 nm Em 560 nm	**Donor CFP Ex 420 nm Em 477 nm**	**FRET Ex 420 nm Em 525 nm**
YFP-PAK70-CFP	before YFP bleaching	100%	100%	100%
	after YFP bleaching	19.1% ± 8.5%	109.6% ± 5.6%	66.3% ± 7.2%

Supplemental Table 3

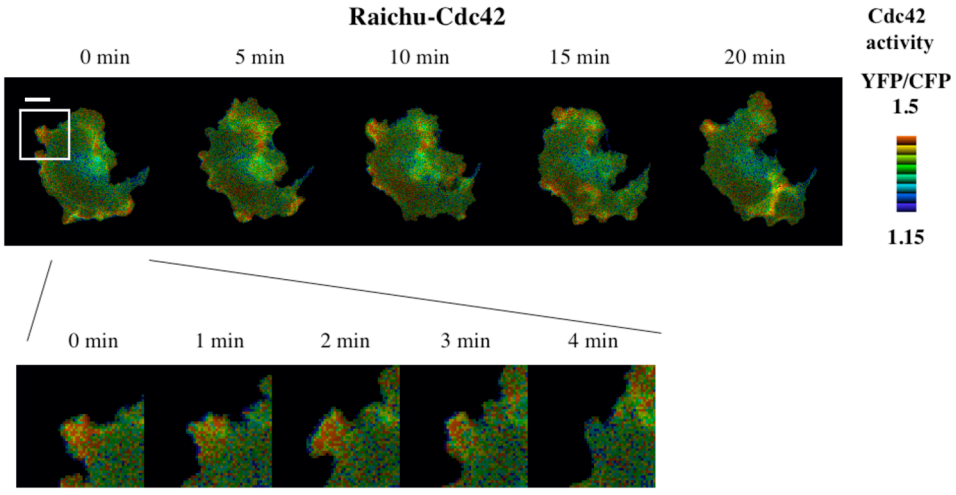
Constructs	Normalized corrected FRET (A.U.)
YFP-PAK65 + PAK-CFP	2.3 ± 1.6
YFP-PAK65-CFP (Pakabi)	27.1 ± 6.2



Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3