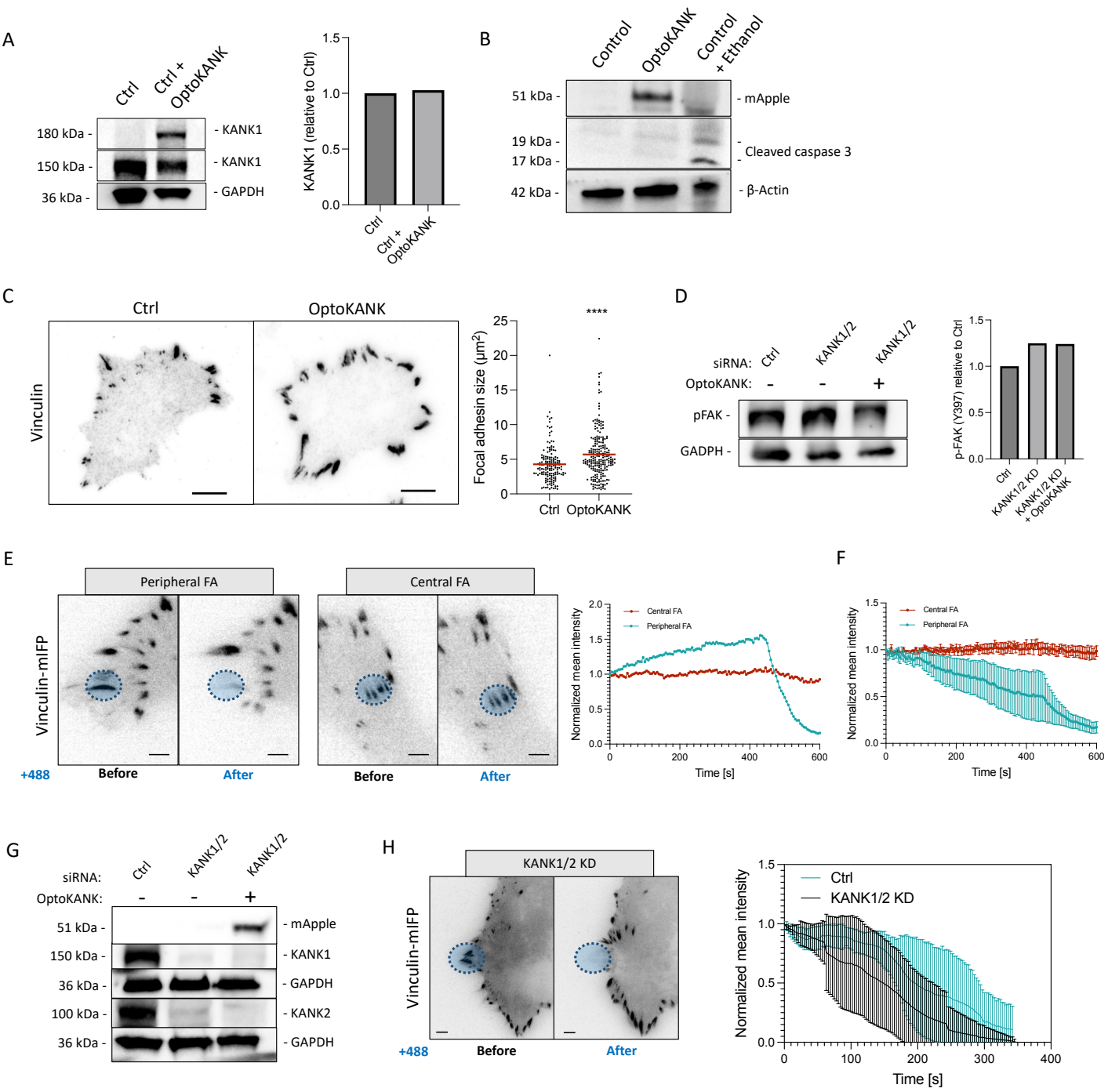
Appendix

Appendix Figure

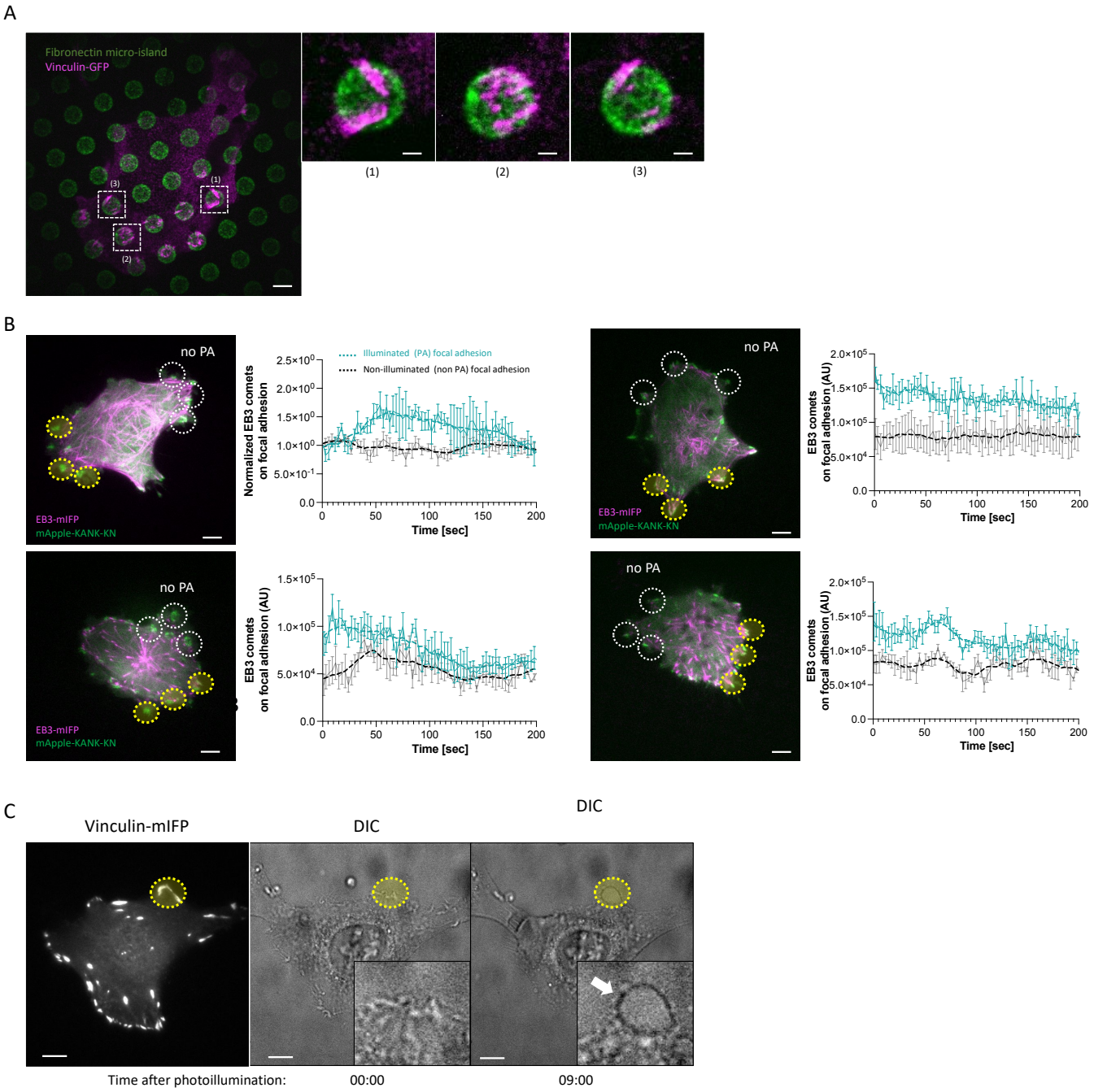


Appendix Figure S1

(A) Western Blots showing Control vs OptoKANK transfected HT1080 cells and probed for endogenous KANK1 (anti-KANK1), exogenous KANK1 (SSpB-ΔKN-mEmerald with anti-KANK1), and GAPDH loading control. The graph shows the KANK1 blot densitometries in control and exogenous KANK1 in OptoKANK transfected cells, normalized to GAPDH and relative to control.

(B) Western blots showing Control, OptoKANK transfected HT1080 cells and positive control (ethanol treated HT1080 cells) and probed for endogenous KANK1, mApple-KN-LOV2-ssrA and β-actin loading control and an indicator of cell death Cleaved Caspase 3.

(C) Representative images of HT1080 cells in control and after transfection with OptoKANK and stained for Vinculin. Graph shows the focal adhesion sizes in these conditions (**** p<0,0001 t-test, n = 18 cells for KANK1/2, 10 cells for Ctrl, both from 3 independent experiments; scale bar 10 µm).
(D) Western Blots showing Control, KANK1/2-depleted HT1080 cells and KANK1/2-depleted HT1080 cells transfected with OptoKANK and probed for p-FAK (Y397) and GAPDH loading control. The graph shows the p-FAK blot densitometries in these conditions, normalized to GAPDH and relative to control.
(E) Representative images of HT1080 cells in which OptoKANK has been activated (blue dotted line) in central focal adhesion vs peripheral focal adhesion with the corresponding vinculin intensity on the right graph (scale bar 5 µm).
(F) Graph shows the normalized mean vinculin intensity of illuminated focal adhesions on central vs peripheral focal adhesions over the time in these two conditions (mean ± s.e.m; n = 5 cells).
(G) Immunoblots of mApple, KANK1, KANK2 and GAPDH loading controls in control (Ctrl), in KANK1/2-depleted HT1080, and in KANK1/2-depleted HT1080 carrying the OptoKANK constructs.
(H) Representative images of Vinculin-mIFP-transfected HT1080 cells, depleted for KANK1/2 and carrying the OptoKANK constructs before and after blue light illumination on the encircled focal adhesion (blue dotted line. Graph shows the normalized mean vinculin intensity of illuminated focal adhesions over the time (Data are presented as mean ± s.e.m; n = 20 cells minimum from three independent experiments; p<0,0001 between Ctrl and KANK 1/2 KD; two-way ANOVA test. scale bar 5 µm).

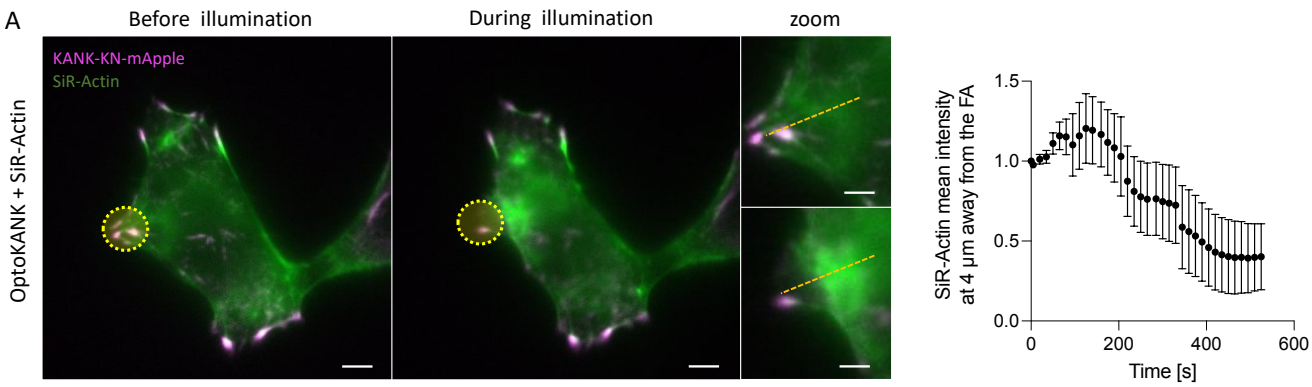


Appendix Figure S2

(A) Example of HT1080 cell transfected with vinculin-GFP, plated on microislands pattern labelled in far red (Alexa 647). Typically, the cell displays 2 to 4 focal adhesions par island where the microtubules visualized thanks to TAU protein (yellow) are passing by the focal adhesion. (Scale bar 5 µm)

(B) Graphs show the integrated EB3 comet fluorescence after processing with U-Track2 for photoactivated (yellow dotted line) and non-photoactivated (white dotted line) focal adhesions over the time. (Data are presented as the mean ± s.e.m of integrated EB3 fluorescence; n= 3 focal adhesions per cell; scale bar 5 µm). See also movie EV4.

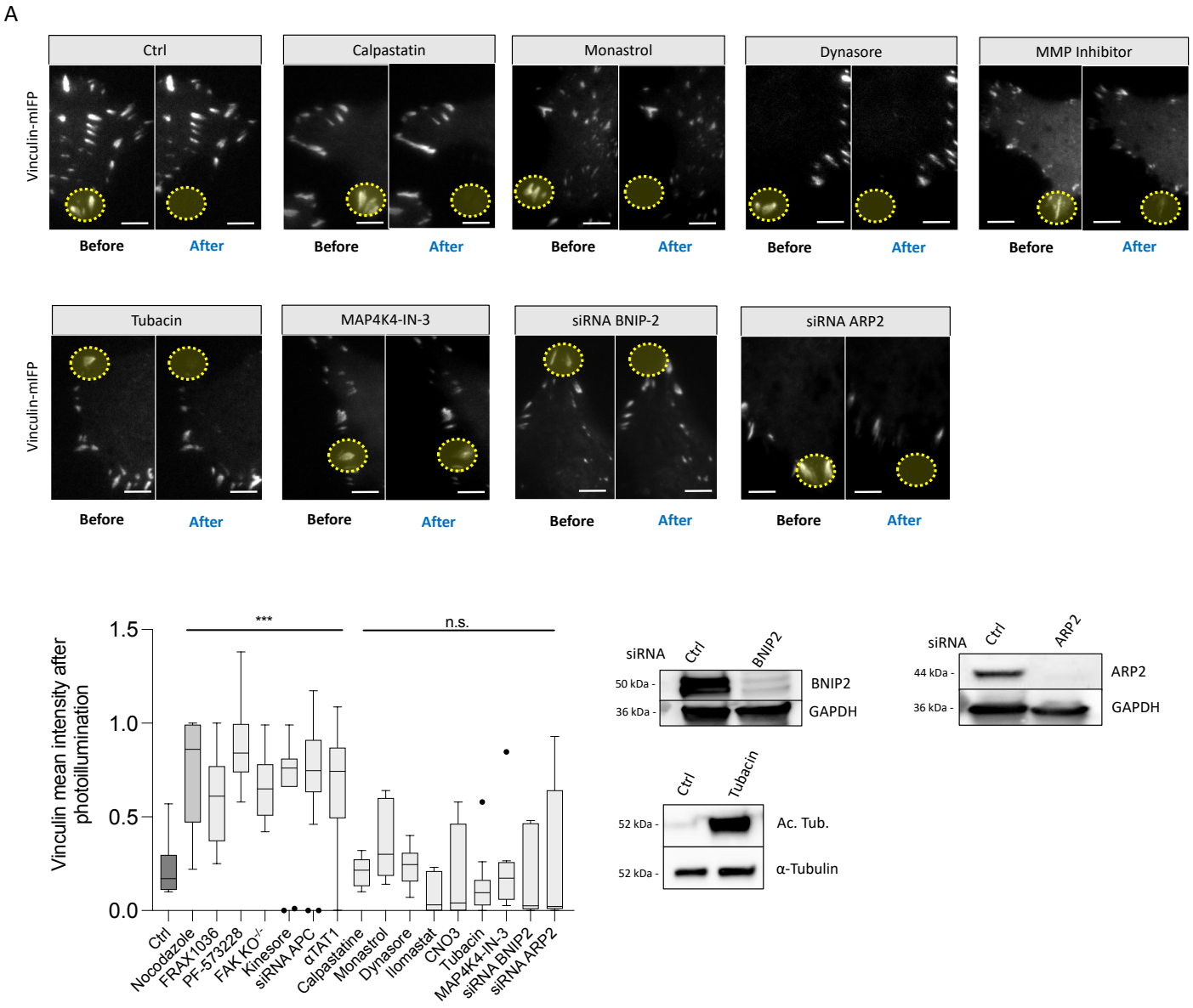
(C) Representative images of Vinculin-mIFP-transfected HT1080 cells carrying the OptoKANK constructs after treatment with dynasore. Images show the illuminated focal adhesion (yellow dotted circle) using



vinculin-mIFP staining and the DIC images showing the bleb formation (see white arrow in the box) after 9 min of OptoKANK activation (scale bar 10 µm). See also movie EV6.

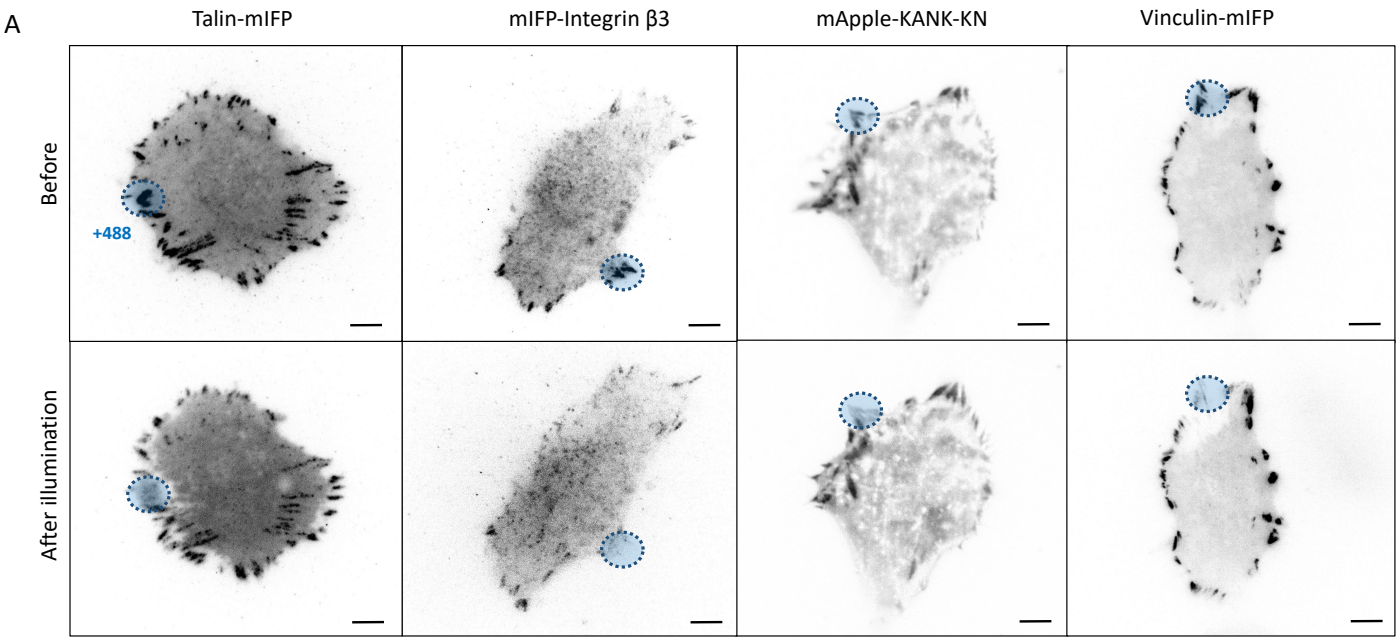
Appendix Figure S3

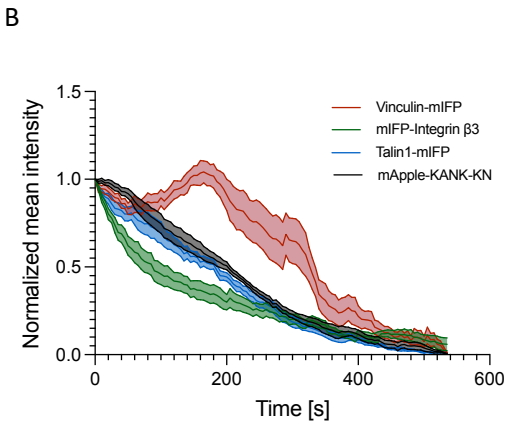
Representative image of OptoKANK-transfected HT1080 cell before and during OptoKANK activation of the selected focal adhesion (yellow dotted line). SiR-Actin was used to assess the myosin-II dynamics upon OptoKANK activation. The line scan was used to measure the SiR-Actin intensity in the vicinity (4 µm away in centripetal direction) of the proximal end of the photoactivated focal adhesion shown. The graph shows the SiR-Actin mean intensity at this distance upon OptoKANK activation (Data are presented as mean ± s.e.m; n = 7 cells; scale bar 5 µm)). See also movie EV8.



Appendix Figure S4

Representative images of Vinculin-mIFP-transfected HT1080 cells carrying the OptoKANK constructs before and after blue light illumination of the focal adhesion (yellow dotted line) for control cells, cells treated with a calpain inhibitor, calpastatin, a kinesin-V inhibitor, monastrol, a dynamic inhibitor, dynasore, a MMP inhibitor, Ilomastat, a MAP4K4 inhibitor, MAP4K4-IN-3, and depleted for BNIP2 and ARP2. Graph shows the normalized mean vinculin intensity after the illumination of cells treated as indicated (Data are presented as mean ± s.e.m; Ctrl, n =18 cells; Nocodazole, n = 12 cells; FRAX1036, n = 15 cells; PF-573228, n = 10 cells; FAK KO$^{-/-}$, n = 8 cells; Kinesore, n = 11 cells; siRNA APC, n = 18 cells; αTAT1, n = 10 cells; n = 12 cells; Calpastatin, n = 10 cells; Monastrol, n = 8 cells; Dynasore, n = 10 cells; MMP inhibitor, n = 7 cells ; CNO3, n = 8 cells; Tubacin, n = 12 cells, MAP4K4-IN-3, n = 9 cells; siRNA BNIP2, n = 16 cells; siRNA ARP2, n = 10 cells; one-way ANOVA, *** p < 0,001, N.S. not significant; the center line denotes the median value (50th percentile) while the box contains the 25th to 75th percentiles of dataset. The black whiskers mark the minimum and maximum percentiles, and values beyond these upper and lower bounds are considered outliers; scale bar 5 µm). Immunoblots of BNIP2, ARP2, acetylated tubulin and GAPDH are shown in the black box.

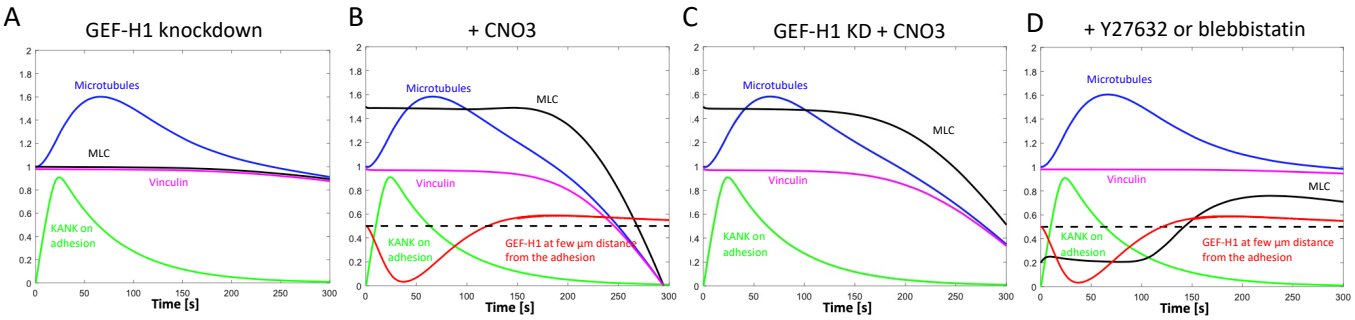




Appendix Figure S5

(A) Representative images of HT1080 cells carrying the OptoKANK constructs before and after blue light illumination of areas (blue dotted line) containing focal adhesion visualized using Talin-mIFP, mIFP-Integrin β3, mApple-KANK-KN and Vinculin-mIFP labeling (scale bar 10 µm).

(B) Normalized mean intensity of Vinculin (vinculin-mIFP), Integrin β3 (mIFP-Integrin β3), Talin1 (Talin1-mIFP) and KN (mApple-KANK-KN) over the illuminated focal adhesion of cells carrying OptoKANK.



Appendix Figure S6

Model-predicted time series for microtubule, vinculin, KANK, GEF-H1 and myosin densities for the following simulated perturbations: GEF-H1 knockdown **(A)**, treatment with CNO3 **(B)**, GEF-H1 knockdown + CNO3 **(C)** and treatment with Y27632 or blebbistatin **(D)**. Model parameters used in the simulations are described in the Supplemental Text.

Appendix details of computational model

The conceptual model is shown schematically in **Figure 6**. Model variables and parameters are listed in Supplemental Tables 1 and 2, respectively. Below, we discuss the dimensions and parameter values in detail. We discuss the model assumptions along with describing mathematical terms in the model equations. The first three model equations introduce the dynamics of the number of microtubules (MTs) on the focal adhesion (FA), number of KANK molecules on the FA, and active myosin density proximal to the FA, respectively:

$$\frac{dT}{dt} = 1 - c\exp\left(-K\right)T \qquad (1)$$

$$\frac{dK}{dt} = i\left(t\right) - kH\left(T-1\right)K \qquad (2)$$

$$\frac{dM}{dt} = m_1 + m_2 f_1\left(G\right) - m_3 M \qquad (3)$$

Here, T is the number of MT plus ends on the FA, K is the number of KANK molecules on the FA, and M is the active myosin density proximal to the FA. The model does not describe explicit spatial molecular distributions, and so we only follow the temporal dynamics. Furthermore, even though MT and KANK numbers are not large, we approximate these numbers with continuous variables and from here on calling them 'densities' (these densities can be thought of as the respective numbers divided by the FA area). Also, we neglect stochasticity of these numbers and consider deterministic continuous model. Lastly, all densities in the model are non-dimensional, measured in units of characteristic observed scales. In the conceptual model, in the absence of the data on density dependence of relevant chemical rates, the dimensional numbers for the variables are not crucial.

Left-hand-sides of Equations 1-3 are the rates of change of the respective variables; we now turn to description of the right-hand-sides' terms. The first term in Equation 1 describes arrival of the polymerizing growing MTs at a constant rate at the FA. This non-dimensional rate is chosen to be equal to unity because of the scaling discussed in the previous paragraph. The constancy of this rate is reasonable based on our observations suggesting that near the cell margins, the overall MT number and average dynamic instability parameters away from but proximal to adhesions do not exhibit noticeable variations. The second term is responsible for MTs leaving the FA with the rate equal to $c\exp\left(-K\right)$, where c is the inverse pause time for MT prior to MT disassembly, before KANK activation. This parameter value can be estimated based on the observation of the MT pauses on the order of tens of seconds on the FAs (Azoitei et al 2019). The factor $\exp\left(-K\right)$is responsible for the increase of the MT pause on the FA (and so the rate of leaving decrease) with growing KANK

number (Bouchet2016). The exact functional form of this dependence is unknown, and the exponential form we use is a reasonable assumption. Implicitly, we use another assumption – that this rate decreases a few-fold when KANK is fully loaded onto the FA upon the illumination – which is based on the observed 1.5-fold increase of the MT number on the FA after the illumination. Note also that in principle there could be an effect of the MT catastrophe being promoted by the attachment of microtubule to the FA (Efimov and Kaverina 2009), but this effect can be effectively accounted for by the constant parameter c.

In Equation 2, the first term describes loading of KANK to the FA upon the illumination, as the following function of time:

$$i(t) = K_1 \frac{\exp\left(s_1\left(t_0 - t\right)\right)}{1 + \exp\left(s_1\left(t_0 - t\right)\right)} \qquad (4)$$

This function is a smoothed step function equal to constant, K_1, before time t_0, and zero after this time. Parameter K_1 (Supplemental Table 2) is chosen so that by the end of the loading, KANK density reaches a characteristic unit; for t_0 (Supplemental Table 2), we use the observed time of 20-30 seconds; smoothing parameter s_1 (Supplemental Table 2) is chosen to account for a sharp step-function-like transition. The second term in this equation accounts for the observed dissociation of KANK molecules from the FA on the scale of tens of seconds (constant parameter k (Supplemental Table 2)). We also assume that MTs bring with them an adhesion-weakening molecule (Yue et al 2014), which triggers dissociation of integrins and talins, and because KANK binds to talin, of KANK molecules. This effect is accounted for by the factor $H\left(T-1\right)$, where H is Heaviside function equal to zero when the MT density is less or equal to unity, and to one otherwise. Thus, we assume that this effect has a threshold character. Another possibility is that KANK proteins themselves, without the MTs, diminish the talin-actomyosin linkage, which curbs force transmission across integrins, leading to reduced integrin–ligand bond strength, slippage between integrin and ligand, and sliding (Sun et al 2016). In that case, the factor $H\left(T-1\right)$ must be absent from Equation 2, and instead the dissociation rate simply equals constant k. The model's results compare to the observations equally well with both choices.

In Equation 3, the first term is the constant (m_1 (Supplemental Table 2)) basal, GEF-H1-independent activation rate of myosin, and the third term is the respective deactivation rate (which is assumed to be the first order chemical reaction with rate m_3 (Supplemental Table 2)). The constant m_1 is chosen

to bring the basal active myosin density to unity; the constant m_3 is chosen based on MLC phosphorylation cycle on the order of tens of seconds (Amano et al 1996). Note that in order to not overwhelm the model with molecular complexity, we do not explicitly describe the intermediate steps, like action of RhoGTPases, activation of ROCK, which are relatively fast, on the order of seconds (Bolado-Carrancio et al 2020) and can be lumped together with MLC phosphorylation rate. We also do not explicitly describe myosin clusters' assembly. In the model, the myosin density is assumed to be proportional to the effective traction force and is a harbinger of the observed pMLC density.

The second term in Equation 3 is the GEF-H1-dependent rate of myosin activation. Rate m_2 (Supplemental Table 2) characterizes the relative effect of GEF-H1-dependent compared to GEF-H1-independent rate. This parameter is unknown and chosen to fit the data. Function $f_1\left(G\right)$ accounts for assumed threshold character of the GEF-H1 action:

$$f_1\left(G\right)=\frac{\exp\left(s_2\left(G-G_0\right)\right)}{1+\exp\left(s_2\left(G-G_0\right)\right)} \qquad (5)$$

Here G_0 is the characteristic threshold density of activated GEF-H1 in the cytoplasm in the vicinity of the assembling actomyosin array proximal to the FA. Below this threshold, there is no effect, above the threshold there is a saturated effect. The threshold value is unknown and is chosen to fit the data (Supplemental Table 2). Parameter s_2 (Supplemental Table 2) characterizes the sharpness of the threshold effect; it is unknown from the experiment and is chosen to fit the data.

To close the system of model equations, we need GEF-H1 density as a function of time. The following equation provides such density at distance L from the FA:

$$G\left(L,t\right)=G_0-g\int_0^t\frac{dT}{dt}\left(\tau\right)\times\frac{\exp\left(-L^2/\left(4D\left(t-\tau\right)\right)\right)}{\sqrt{4\pi D\left(t-\tau\right)}}\times d\tau \quad (6)$$

This formula is based on the following assumptions and estimates. First, MTs are effectively a sink for GEF-H1 molecules (Krendel2002). Thus, we assume that any time a MT arrives at the FA, a certain number of GEF-H1 molecules, equal to g, is locally absorbed from the cytoplasm. The number of MTs arriving per time Δt is $\frac{dT}{dt}\Delta t$, and so $g\ \frac{dT}{dt}\Delta t$ molecules are absorbed during this time interval. This expression assumes that the rate of absorption is independent of the local GEF-H1 concentration in the cytoplasm, so the limiting factor in the absorption is the local MT

length/number and not GEF-H1 diffusion. Value of parameter g (Supplemental Table 2) is unknown and chosen to fit the data. Similarly, if MTs are leaving the FA but not arriving, $g\,\frac{dT}{dt}\Delta t$ molecules are released during this time interval, assuming that GEF-H1 molecules are released from tubulin instantly upon the MT disassembly, so there is then a local source of GEF-H1 molecules on the FA. Neglecting local sinks and sources elsewhere in the vicinity, the spatially explicit concentration of GEF-H1 molecules in the cytoplasm can be described by the following reaction-diffusion equation:

$$\frac{\partial G}{\partial t} = \frac{dT}{dt}\delta(x) + D\Delta G$$

Here $\delta(x)$ is the delta-function responsible for the location of the source/sink at the FA location, Δ is the Laplacian operator, and D is the diffusion coefficient of GEF-H1 in the cytoplasm. We use a one-dimensional approximation of the geometry where x becomes the distance inward from the cell margin. In this approximation, the exact analytical solution for this reaction-diffusion equation is Equation 6 (Strauss 2007), where now L is the distance between the FA and proximal actomyosin array. Parameter L (Supplemental Table 2), based on our data is several microns. Value of parameter D (Supplemental Table 2), on the order of square microns per second, can be indirectly estimated from the data reported in (Azoitei et al 2019). To not overwhelm the model with molecular complexity, we do not explicitly include the step of activation of the released GEF-H1 molecules (Azoitei et al 2019), however, such process will not qualitatively change the conclusions from the model. One possible variant of the model is that we can in principle include the acetylation of MTs at the FA, and subsequent release of GEF-H1 from the MTs independent of the MT disassembly, since GEF-H1 has a low affinity to the acetylated MTs (Seetharaman et al 2022). Such variant of the model will lead to the same conclusions as our basic model.

Lastly, the mechanics of the FA slippage is accounted for in the model as follows. We assume that the pulling force applied to the FA, and therefore the traction force is directly proportional to the myosin density in the model. Next, we assume that the FA is 'gripping' if the ratio of the pulling force to adhesive strength is below a threshold, and slipping, if this ratio is above the threshold. We assume that the adhesive strength is proportional to the KANK density on the FA (not that KANK directly contributes to the adhesive strength, but rather that its adhesive molecular partners do, and their numbers are proportional to the KANK numbers), and that there is some basal, KANK-independent part of the strength. Mathematically, this translates into the assumption that the adhesive strength is proportional to the expression $(K_0 + K)$, where K_0 is a constant parameter.

Then, the ratio of the pulling force to adhesive strength is proportional to the expression $\left(M/\left(K_0+K\right)\right)$, and we define the velocity of slippage as follows:

$$V = V_0 f_2\left(M/\left(K_0+K\right)\right) \qquad (7)$$

Here V_0 is the characteristic magnitude of the slippage velocity (Supplemental Table 2) that can be estimated from our data. Threshold function f_2 is defined as follows:

$$f_2(f) = \frac{\exp\left(s_3\left(f-f_0\right)\right)}{1+\exp\left(s_3\left(f-f_0\right)\right)} \qquad (8)$$

Here f_0 is the threshold force to adhesion strength ratio and s_3 is the sharpness of the threshold parameter. The values of parameters K_0, f_0 and s_3 are unknown and chosen to fit the data (the results are insensitive to the value of s_3). The shift of the FA due to the slipping is given by the formula:

$$X = \int_0^t V(\tau)\, d\tau \qquad (9)$$

There is a gradual physical slipping of MTs, KANK and myosin, together with the slipping FA, and so we assume that the measured MT, KANK and myosin densities on the area of the initial adhesion scale with the initial adhesion length, on the order of one micron, minus the shift. Therefore, when the shift becomes equal to the initial adhesion length, the measured densities approach zero. Thus, as a result, we plot expressions $\left(1-X\right)\times T, \left(1-X\right)\times M$ for the measured MT and myosin density. KANK density decreases so significantly by the time the slippage starts, that factor $\left(1-X\right)$ does not affect the result. Finally, we assume that the measured vinculin density is effectively a marker for the slipping adhesion area and approximate the measured vinculin density with expression $A = 1-X$.

Results: The model equations are integrated by using standard Euler numerical scheme. The results are shown in Figure 6F. Here is the qualitative explanation for the predicted molecular densities as functions of time. KANK is rapidly loaded on the FA upon illumination, and then, as a part of the gradual, MT-induced weakening of the FA, starts to decrease exponentially. The transient accumulation of KANK causes longer pauses of the MTs on the FA, so the MT density on the FA increases at first, up to ~ 1.5-fold its value before the illumination, but after ~ 60 sec starts to decrease because the diminishing KANK number leads to shorter MT pauses on the FA.

Table S1: model variables

T	Number of microtubule tips on the adhesion
K	Number of KANK molecules on the adhesion
M	Number of activated MLCs proximal to the adhesion
G	Activated GEF concentration proximal to the adhesion
V	Rate of slippage of the adhesion
X	Displacement of the adhesion
A	Number of vinculin molecules in the adhesion

Table S2: model parameters

c	Rate of microtubules' leaving the adhesion	1/30 sec
k	Rate of KANK dissociation	1/60 sec
m_1	Basal rate of MLC activation	1 (control,GEFKD), 1.5 (CNO) 0.2 (BlebY)
m_2	GEF-H1-dependent rate of MLC activation	5 (control), 0.2 (BlebY), 0.1 (CNO), 0 (GEFKD)
m_3	Basal rate of MLC deactivation	1/30 sec
G_0	Basal GEF-H1 concentration in the cytoplasm	0.5
g	Sequestered/released GEF-H1 amount per microtubule	0.01
L	Distance between the adhesion and myosin assembly site	4 μm
D	GEF-H1 diffusion coefficient in the cytoplasm	1 μm^2/sec
V_0	Characteristic slippage velocity	0.5 μm/sec
K_0	Basal strength of a weak adhesion	0.2
f_0	Slippage force/adhesion ratio	7.5
K_1	Rate of KANK-adhesion association upon illumination	2.2
t_0	Characteristic time of KANK loading upon illumination	20 sec
s_1	Inverse threshold width for KANK loading function	0.1
s_2	Inverse threshold width for GEF-H1-dependent rate of MLC activation	0.05
s_3	Inverse threshold width for force-dependent rate of adhesion slippage	1

Appendix OptoKANK plasmids information

pmApple-KN-LOV2ssrA

ORIGIN

```
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