Supplementary Materials and Methods

High-content image processing

Images were quantified using CellProfiler (1) or MetaXpress software (Molecular Devices). For CellProfiler, briefly, for all image analysis pipelines, nuclei were identified using the 'Identify Primary Objects' module using an Otsu threshold followed by one of the following methods. For the FAK mutant combination screen, the phalloidin and HCS Cell Mask channels were combined together and used to derive a 'cell' object using the 'propagate' function in the 'Identify Secondary Objects' module. Finally, a mask was created using an Otsu threshold on the HCS Cell Mask channel, which was subtracted from the 'cell' mask to give cellular 'protrusions'. For YAP localization, YAP immunostaining was used to derive a 'cell' object by expanding the nuclear object by 50 pixels without joining any other object in the 'Identify Secondary Objects' module. The 'nuclear' object was subtracted from the 'cell' object to give the 'cytoplasm'. The fluorescent intensity of anti-YAP immunocytochemistry in the nuclear and cytoplasmic regions was measured per cell. For cell cycle analysis, images of Hoechst-labeled nuclei were analyzed using the 'Cell Cycle' module in the MetaXpress software.

High-content data analysis

High-content imaging data were analyzed using the TIBCO Spotfire High Content Profiler application (PerkinElmer). For the FAK mutant combination screen, cellular measurements were aggregated to image median values and then to well level. Plates were median normalized to the DMSO controls of each individual plate and then each plate was normalized to the global median of the DMSO wells and *Z*-scores were calculated. Wells that contained fewer than 50 cells were excluded. To identify differential effects between FAK-WT and FAK-mutant (FAK-G431A,F433A or FAK−/−) cells, the FAK-WT value for each treatment was subtracted from each FAK mutant value. Data were then analyzed using principal component analysis, and a Mahalanobis distance was used to identify differentially active compounds. Hit compounds were selected as having a Mahalanobis distance *Z*-score > 2.

FAK turnover profiling

FAK synthesis and degradation curves were determined from normalized SILAC ratio profiles (heavy label/light label and medium label/light label, respectively) by nonlinear regression and plotted as means ± SEM with best fit curves and 95% confidence interval bands using Prism (GraphPad). We modeled FAK synthesis as a one-phase association curve for which $y = y_0 + (y_\infty - y_0) \times (1 - e^{-kt})$, where y_0 is the normalized SILAC ratio (heavy label/light label) at time $t = 0$, y_∞ is the normalized SILAC ratio (heavy label/light label) at $t = \infty$, and k is the rate constant. We modeled FAK degradation as a one-phase decay curve for which $y = (y_0 - y_\infty) \times e^{-kt} + y_\infty$, where y_0 is the normalized SILAC ratio (medium label/light label) at $t = 0$, y_∞ is the normalized SILAC ratio (medium label/light label) at $t = \infty$, and k is the rate constant.

Inferred FAK turnover times were calculated as the intersections between the respective synthesis and degradation curves. Statistical significance of synthesis and degradation curves was determined by extra sum-of-squares F test ($\alpha = 0.025$; degradation, $F = 0.225$, $DF_n = 6$, $DF_d = 320$; synthesis, $F = 0.170$, $DF_n = 6$, $DF_d = 319$). Statistical significance of turnover times was determined by two-tailed Mann–Whitney U test ($\alpha = 0.05$).

Supplementary References

1. McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, et al. CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol 2018;16:e2005970.