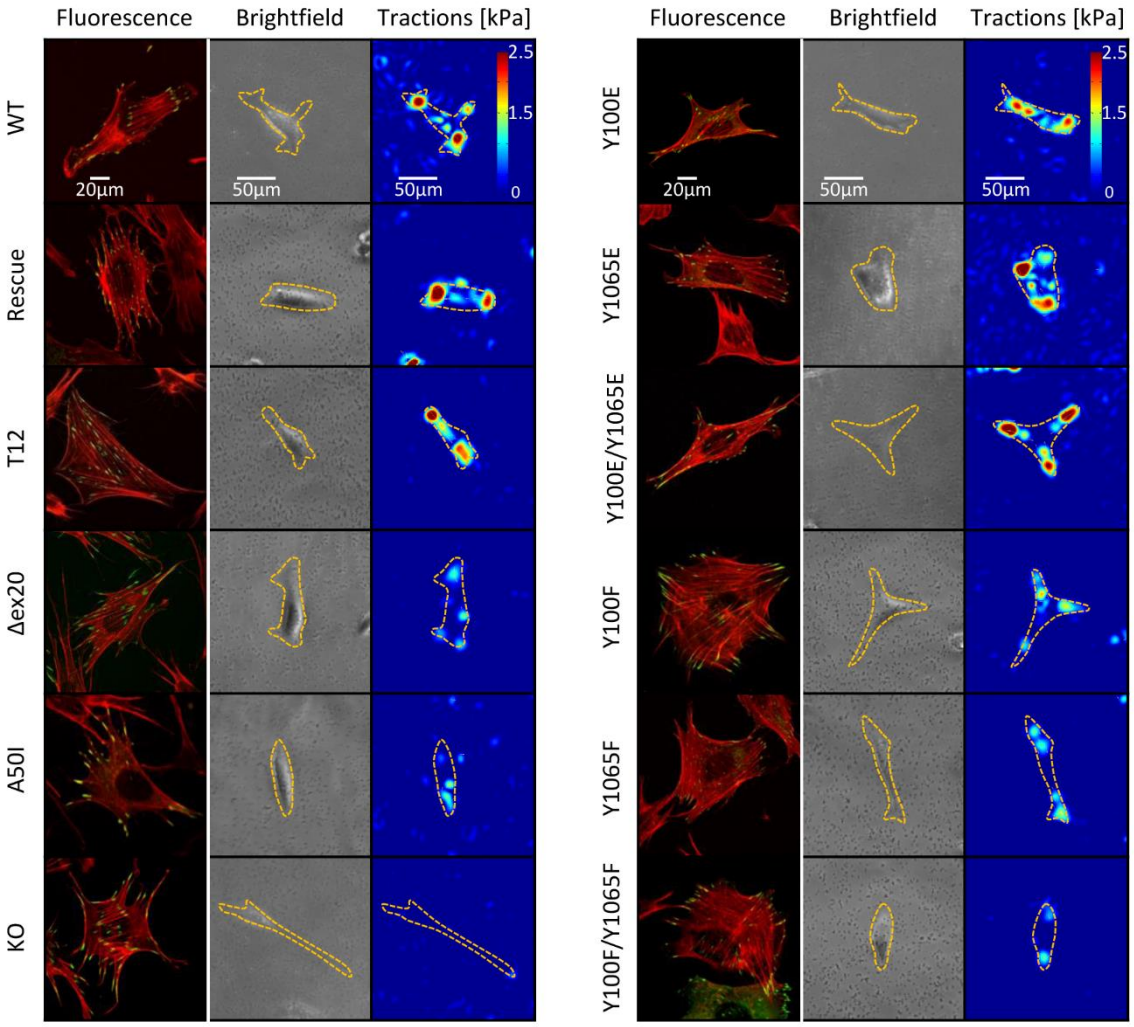

Fig. S1. Representative fluorescence, brightfield images, and force maps for all vinculin forms.

On the left, representative images of fixed MEF cells. F-actin is stained with phalloidin in red, and focal adhesions are marked in green in transfected eGFP-tagged vinculin mutants, and paxillin-stained WT and KO cells, respectively. The scale bar represents 20 µm. In the middle, brightfield images of cells attached to traction gels, and on the right, the corresponding force maps are shown. The dashed lines indicate the cell's outline, and the scale bars represent 50 µm. The color bar indicates tractions in kPa.

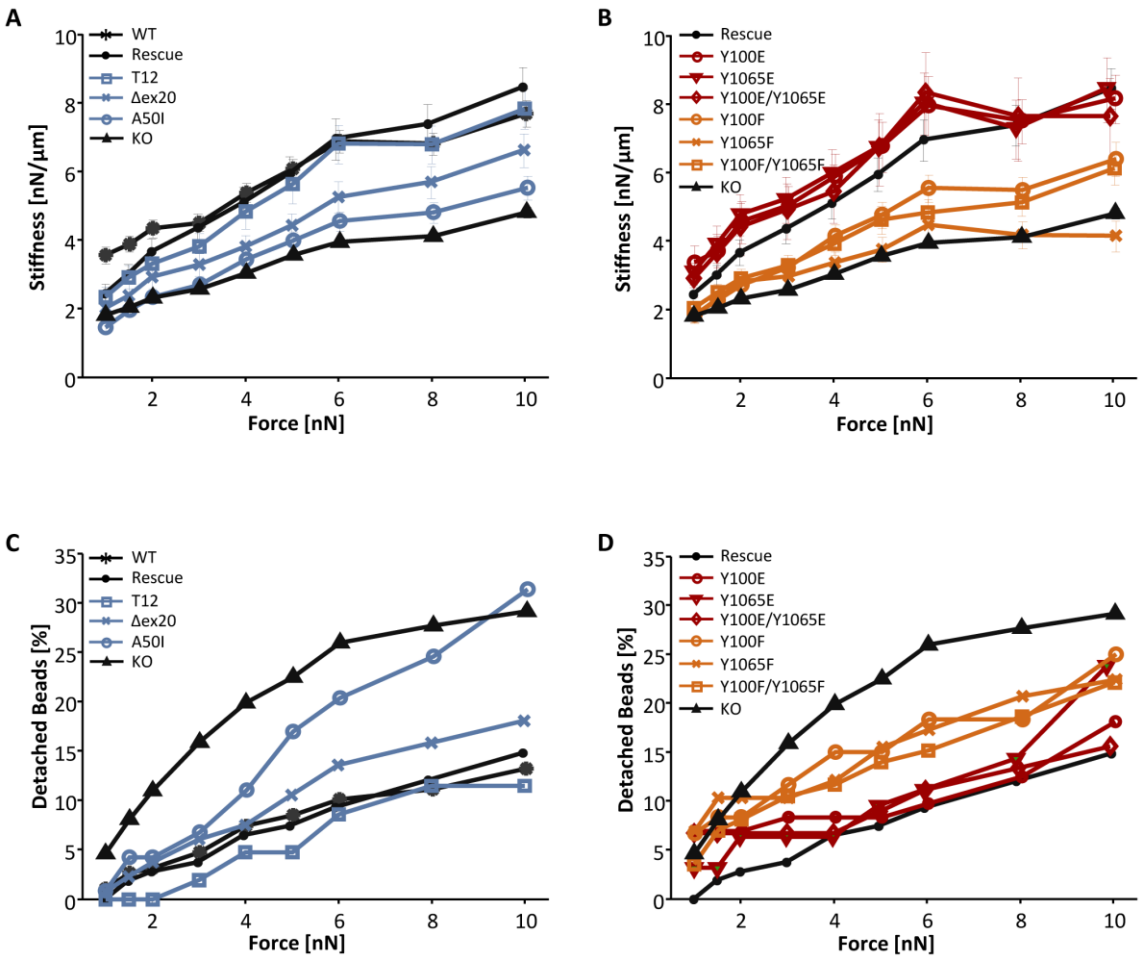

Fig. S2. Cell stiffness and binding strength at increasing force steps.

Plot of stiffness values with standard errors for A) conformational vinculin mutants (T12, Δex20, and A50I in blue) and B) for phospho-mimicking (red) and non-phosphorylatable (orange) vinculin mutants at increasing force steps during magnetic tweezer measurements compared to WT, rescue, and KO cells (black). C) Plot of detached beads (%) for conformational vinculin mutants and (D) for phospho-mimicking (red) and nonphosphorylatable (orange) vinculin mutants compared to WT, rescue, and KO cells (black) at increasing force steps.

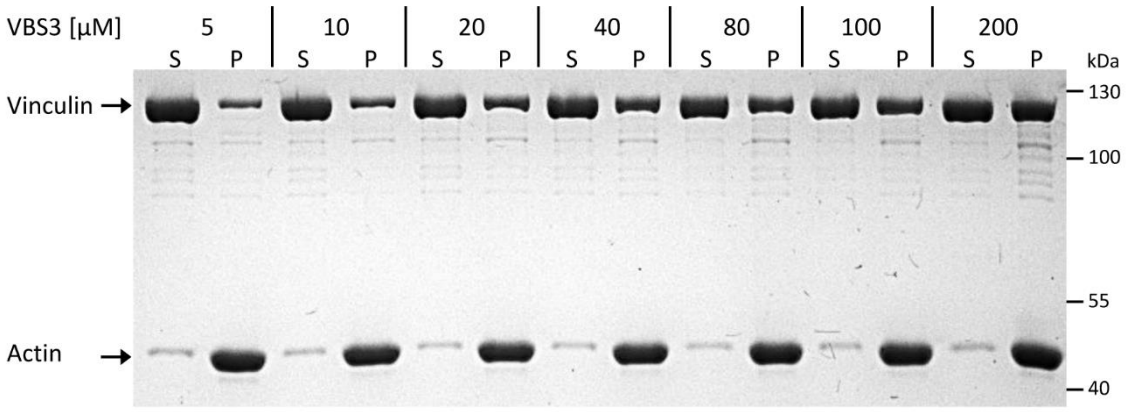

Fig. S3. Co-sedimentation of vinculin with F-actin using increasing VBS3 concentrations.

SDS-PAGE gel loaded with the supernatant and pellet fractions after ultracentrifugation. Vinculin binding to F-actin was tested, adding 5-200 µM VBS3 peptides to the reaction. With increasing VBS3 concentration, the percentage of co-sedimented vinculin increased in the pellet fraction.