

Variant	Actin Binding (30 μ M)	Actin Bundling (20 μ M)
WT Vt	65.5 \pm 7.8	74.1 \pm 6.4
Y1065N	64.1 \pm 7.4	42.2 \pm 6.6
Y1065D	71.0 \pm 4.1	51.1 \pm 9.4
Y1065H	57.6 \pm 2.3	30.2 \pm 2.6

Table S1. Summary of F-actin binding and bundling by additional Vt Y1065 variants. The percent of Vt that pellets with F-actin at 30 μ M F-actin is reported as a measure of F-actin binding, whereas the percent of F-actin bundled at 20 μ M F-actin is a measure of F-actin bundling. Vt concentration for these experiments is 10 μ M.

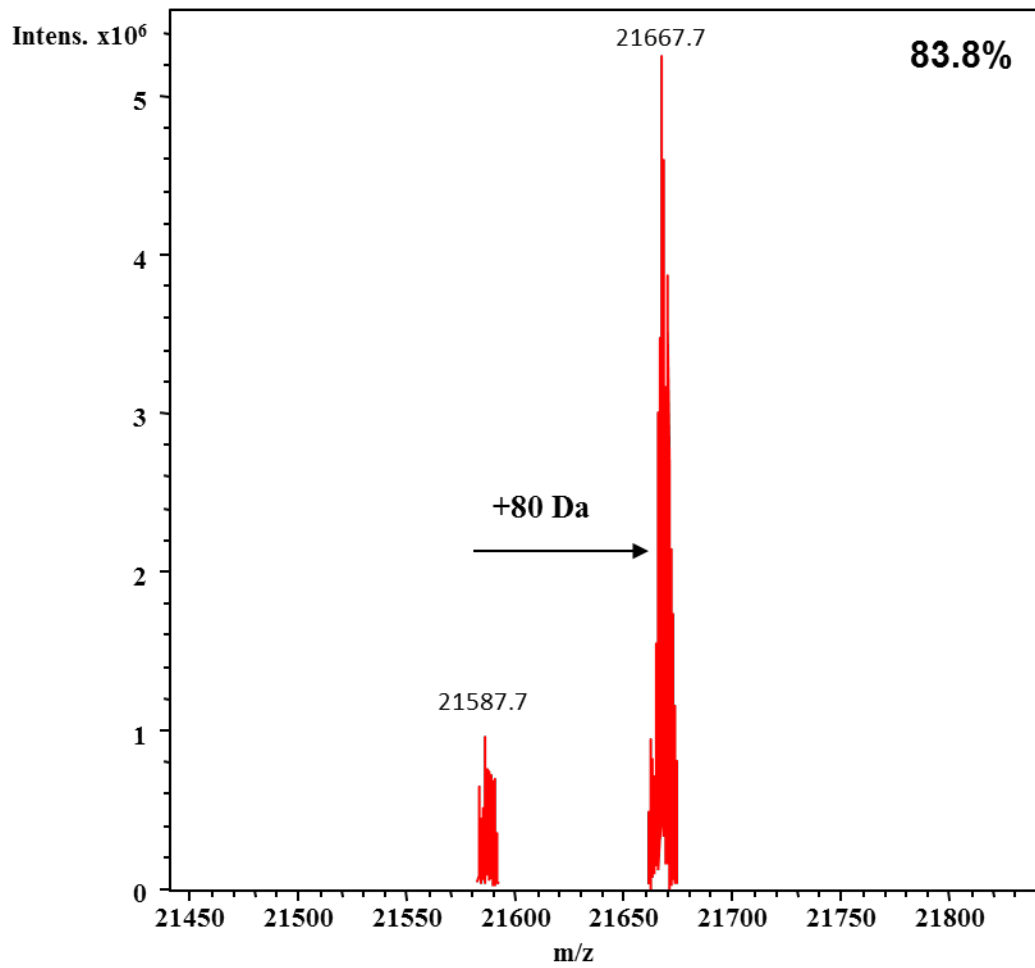


Figure S1. FTICR-MS verification of Vt 1065 phosphorylation by Src. To determine the purity of our phosphorylated Vt samples, we analyzed the samples by FTICR-MS. These analyses demonstrated high levels of Vt phosphorylation (84%).

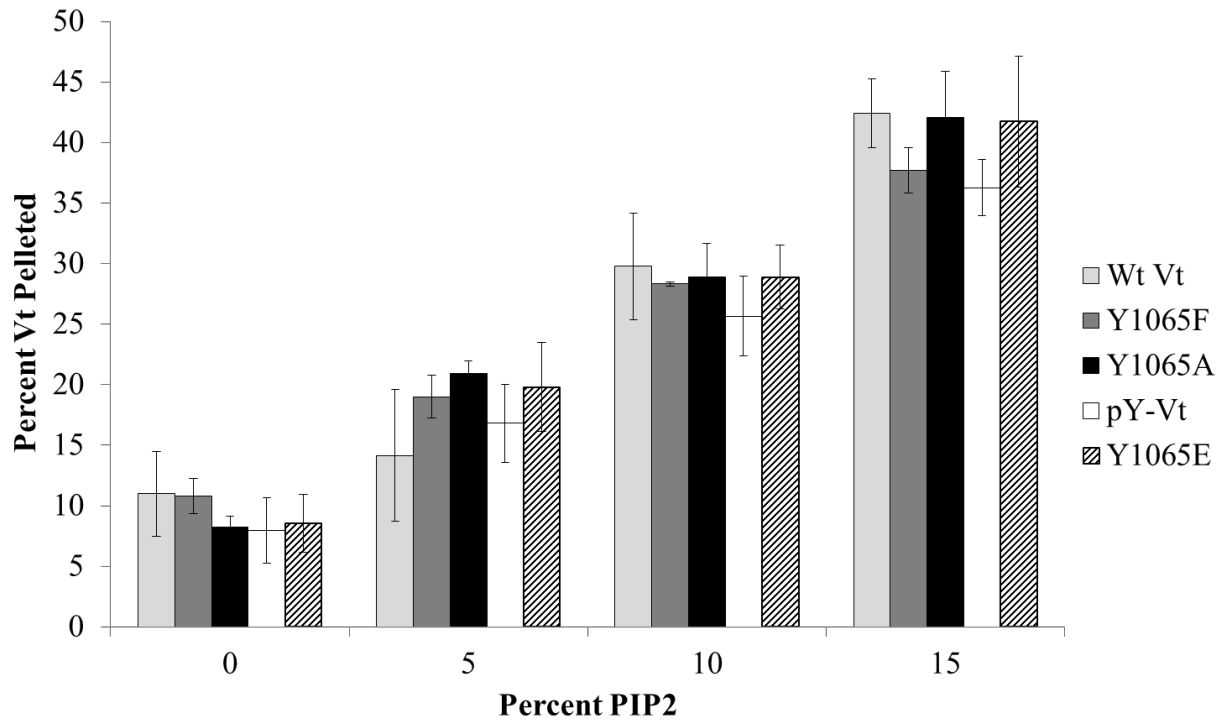


Figure S2. Vinculin Y1065 variants and pY-Vt do not affect PIP₂ association. Similar to previous results with a hairpin deletion mutation, Vt Δ C5 (55), phosphorylation (pY-Vt) or point mutations at Y1065 do not alter binding to liposomes containing PIP₂.

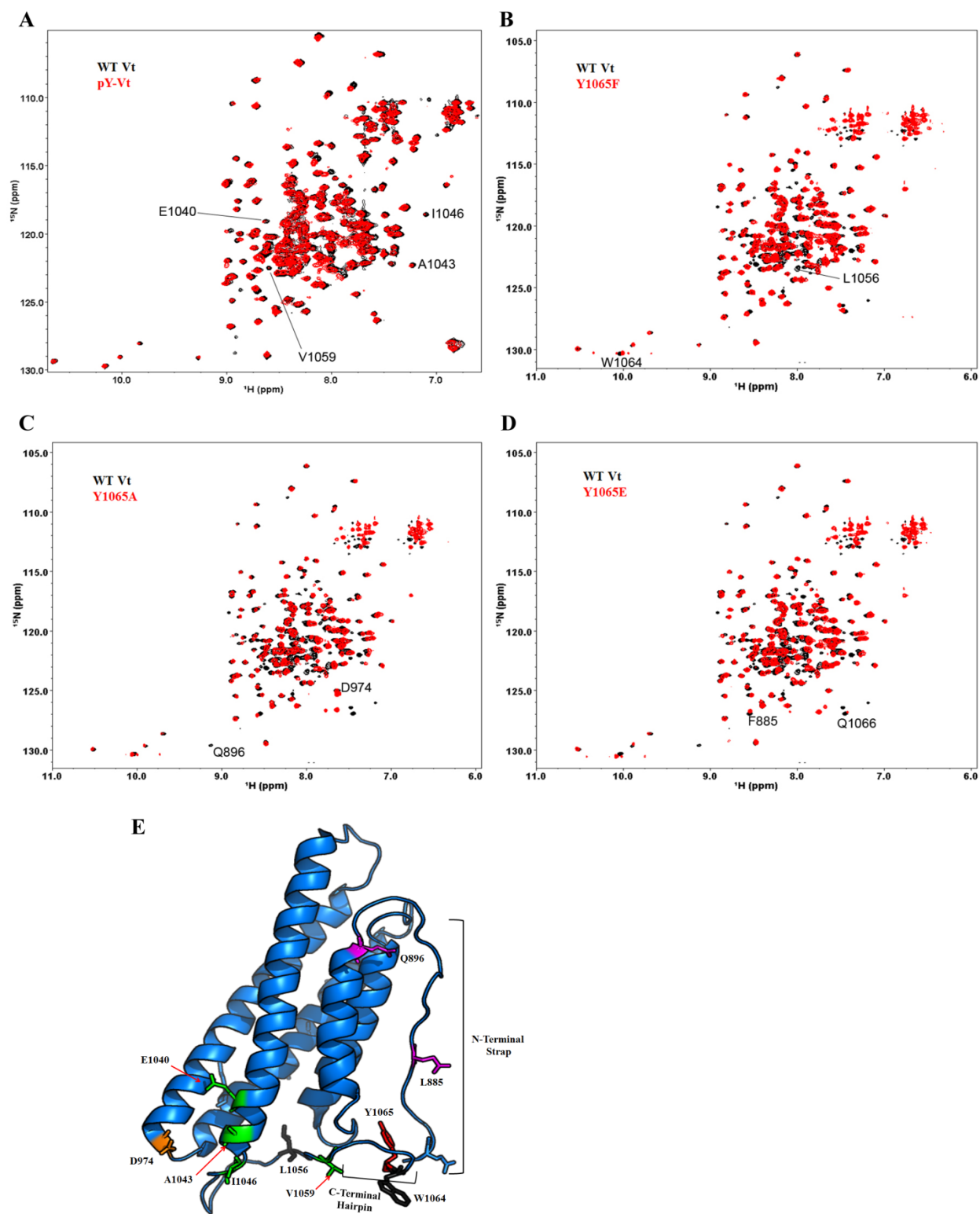


Figure S3. 2D NMR ^1H - ^{15}N HSQC spectral overlay of WT Vt and Vt variants. WT Vt is in black, with each of the variants in red. (A) Although pY-Vt overlays well with most of the NH peaks in WT Vt, line broadening is observed for E1040, A1043, I1046, and V1059. (B) Vt

Y1065F overlays well with WT Vt and displays changes in peaks corresponding to L1056, V1059, and W1064. (C) Vt Y1065A and (D) Vt Y1065E showed greater differences, with shifts in the above peaks, as well as Q896, F885, and Q1066, and D974 for Y1065A. (E) Changes in the HSQC spectra have been mapped onto the Vt ribbon diagram (PDB: 1ST6) with chemical shift differences for WT Vt relative to Vt Y1065A alone (orange), Vt Y1065A and Vt Y1065E (pink), Vt Y1065A, Vt Y1065E, and Vt Y1065F (black), Vt Y1065 and pY-Vt (yellow), and pY-Vt alone (green) shown.

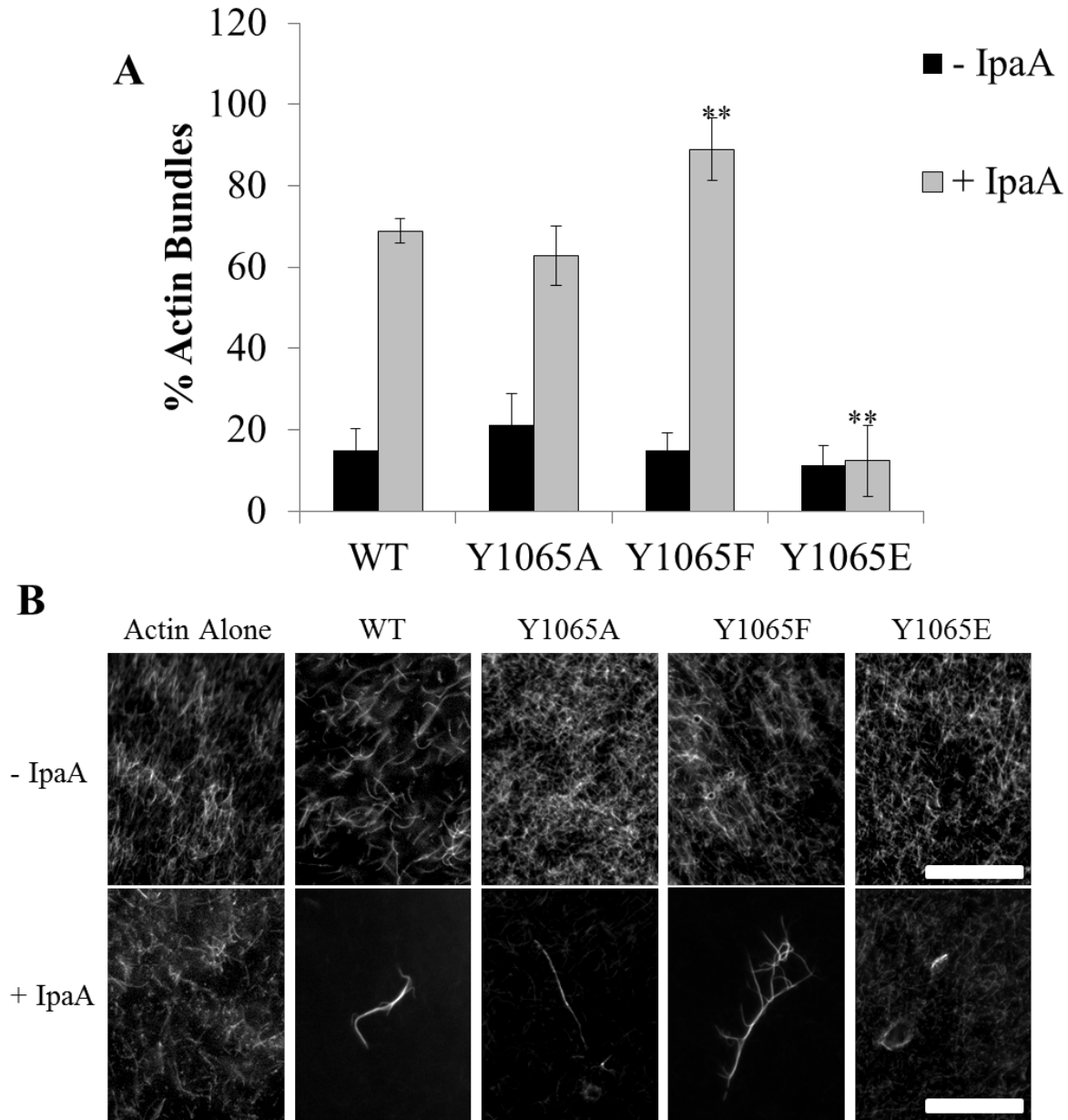


Figure S4. Y1065 mutants in full-length vinculin alter actin bundling. (A) Actin bundling assays for the vinculin Y1065 mutations and (B) fluorescence microscopy of actin bundles with or without IpaA. WT vinculin and Y1065 variants bundle actin only in presence of IpaA. ** $p \leq 0.001$ comparing Y1065 variants to WT vinculin. Error bars show standard deviation. Scale bar: 25 μm .