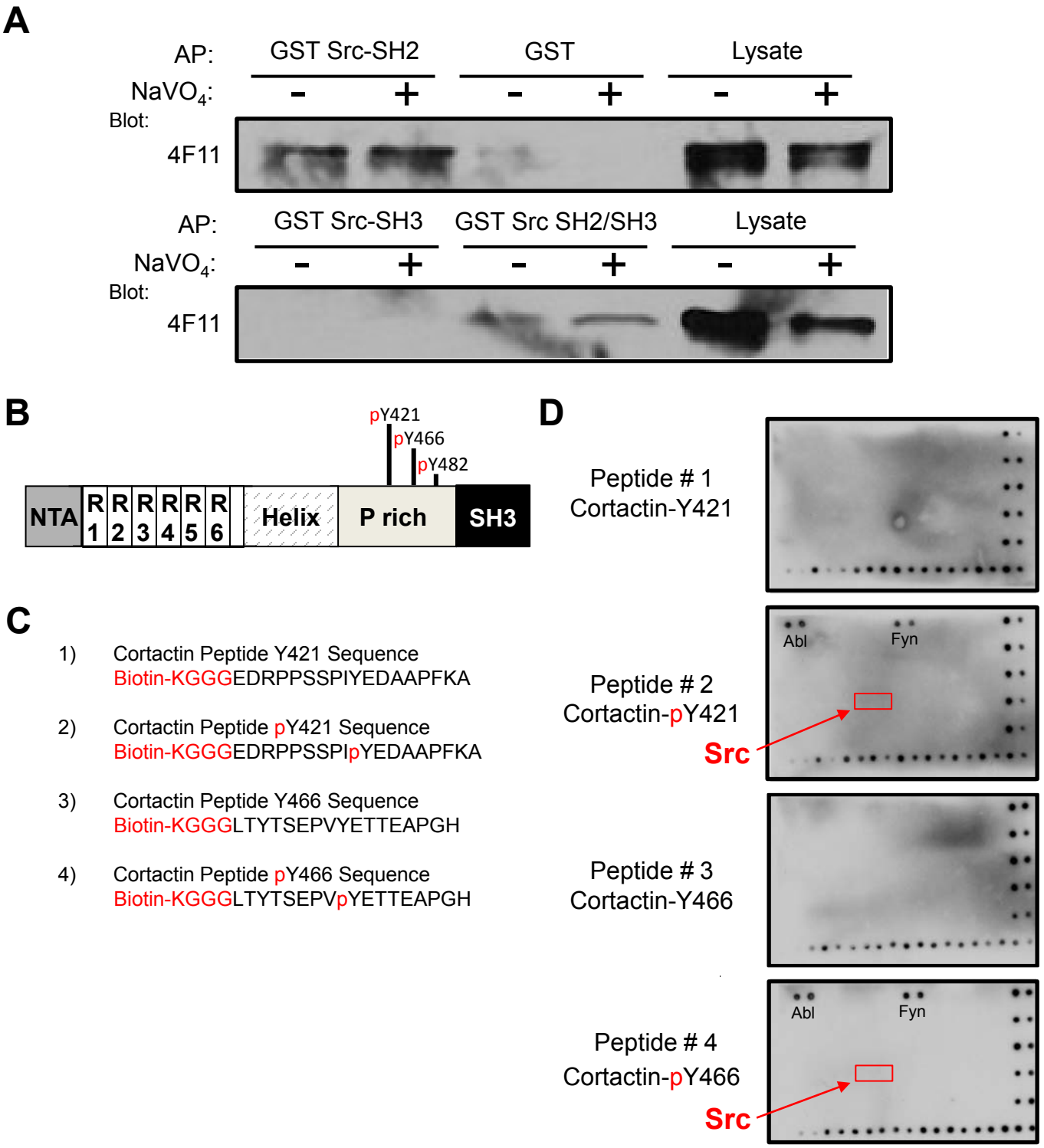

Fig. S1. Src binding to cortactin requires the SH2 Domain and is phosphotyrosine independent. (A) Affinity precipitation (AP) assays from MTLn3 cells with immobilized GST Src-SH2, -SH3 or tandem SH2-SH3 fusion proteins. Cortactin was detected with mAb 4F11. (B) Cortactin schematic showing protein domains and tyrosine phosphorylation sites. (C) Sequence of cortactin peptides used for SH2 domain array screening. (D) SH2 domain arrays (Panomics #MA3040) screened with cortactin peptides.

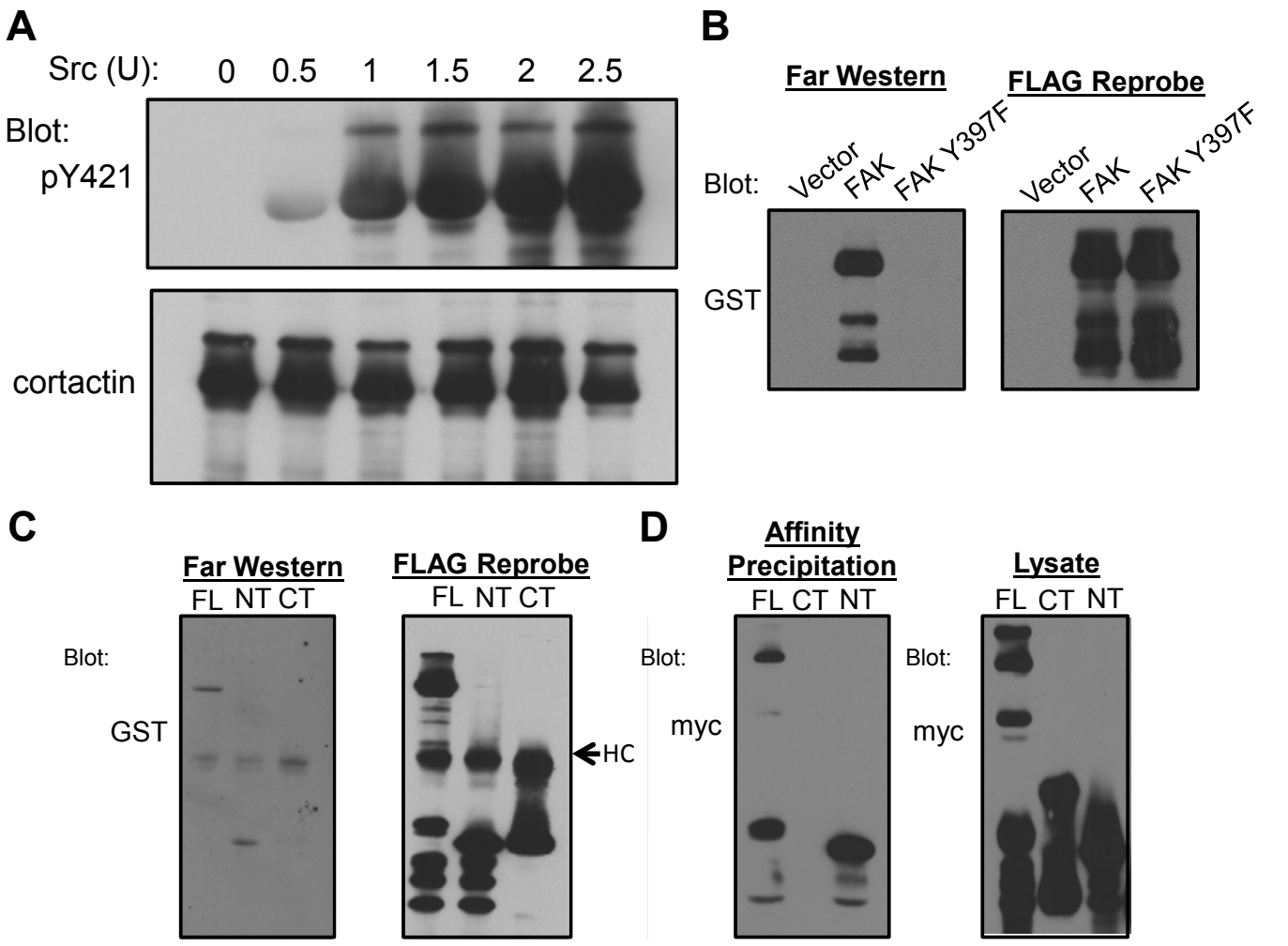

Fig. S2. Phosphorylation of recombinant cortactin by Src and Mapping of Src SH2 binding to the cortactin N-terminal domain. (A) Phosphorylation of purified recombinant murine cortactin with activated Src monitored by Western blotting with anti-cortactin pY421 antibodies. (B) Far Western blotting of immunoprecipitated FAK proteins with GST-Src SH2 domain. (C) Far Western analysis of cortactin full length (FL), amino terminal (NT) and carboxyl terminal (CT) binding to Src SH2 domain. Far Westerns were probed with anti-GST antibodies to detect bound GST-Src SH2 domain. The position of immunoglobulin heavy chain (HC) is indicated on the right. (D) Affinity precipitation of cortactin proteins with GST-Src SH2 domain. Co-precipitated and total (lysate) myc-tagged FL, CT and NT cortactin proteins were identified by anti-myc Western blotting.

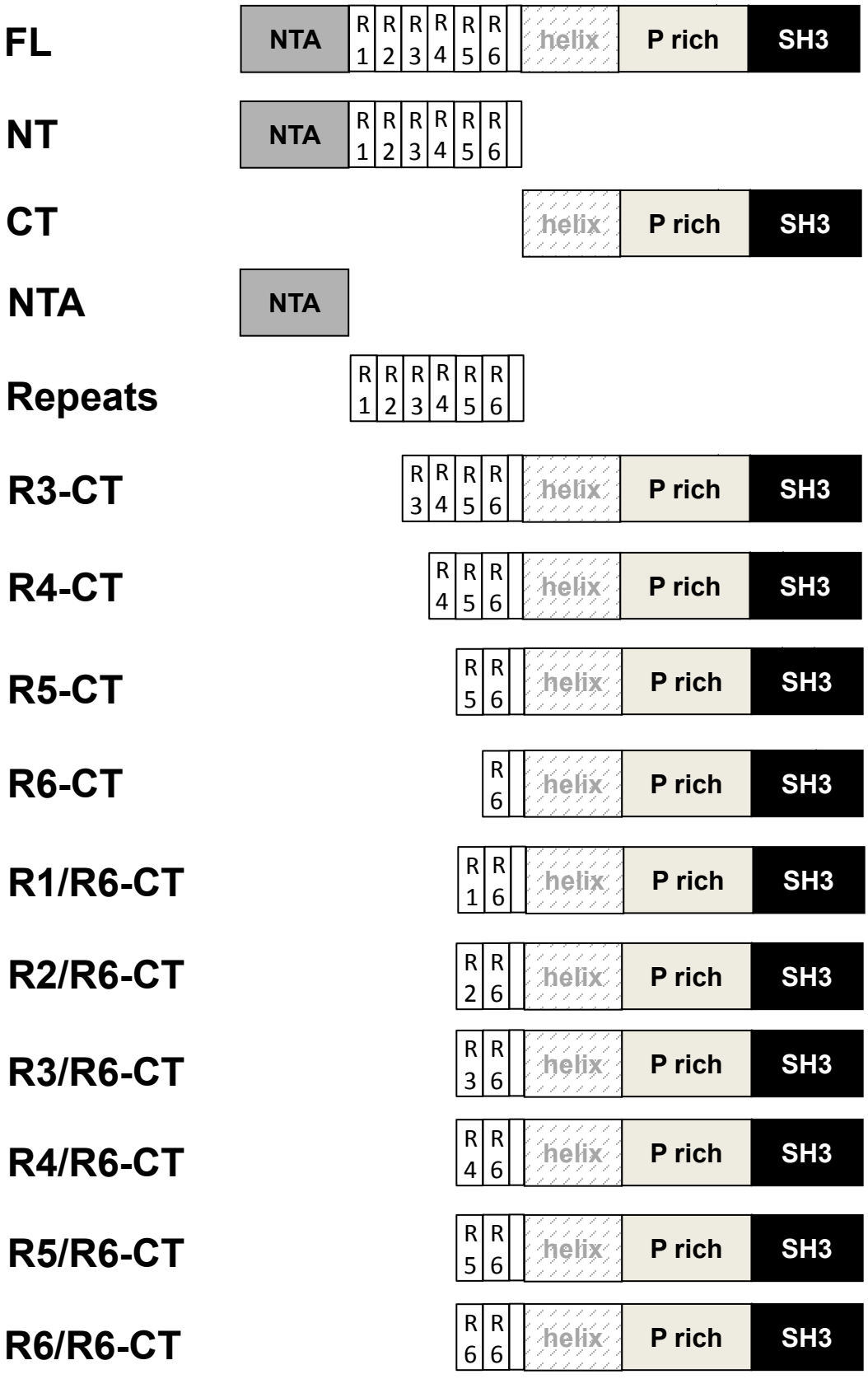

Fig. S3. Schematic diagram of cortactin constructs used in this study. Deletion and chimeric constructs are shown. NTA; amino terminal acidic domain, R; individual cortactin repeat units within the repeats domain, helix; predicted alpha helical domain, P rich; proline-rich domain, SH3, Src homology 3 domain.

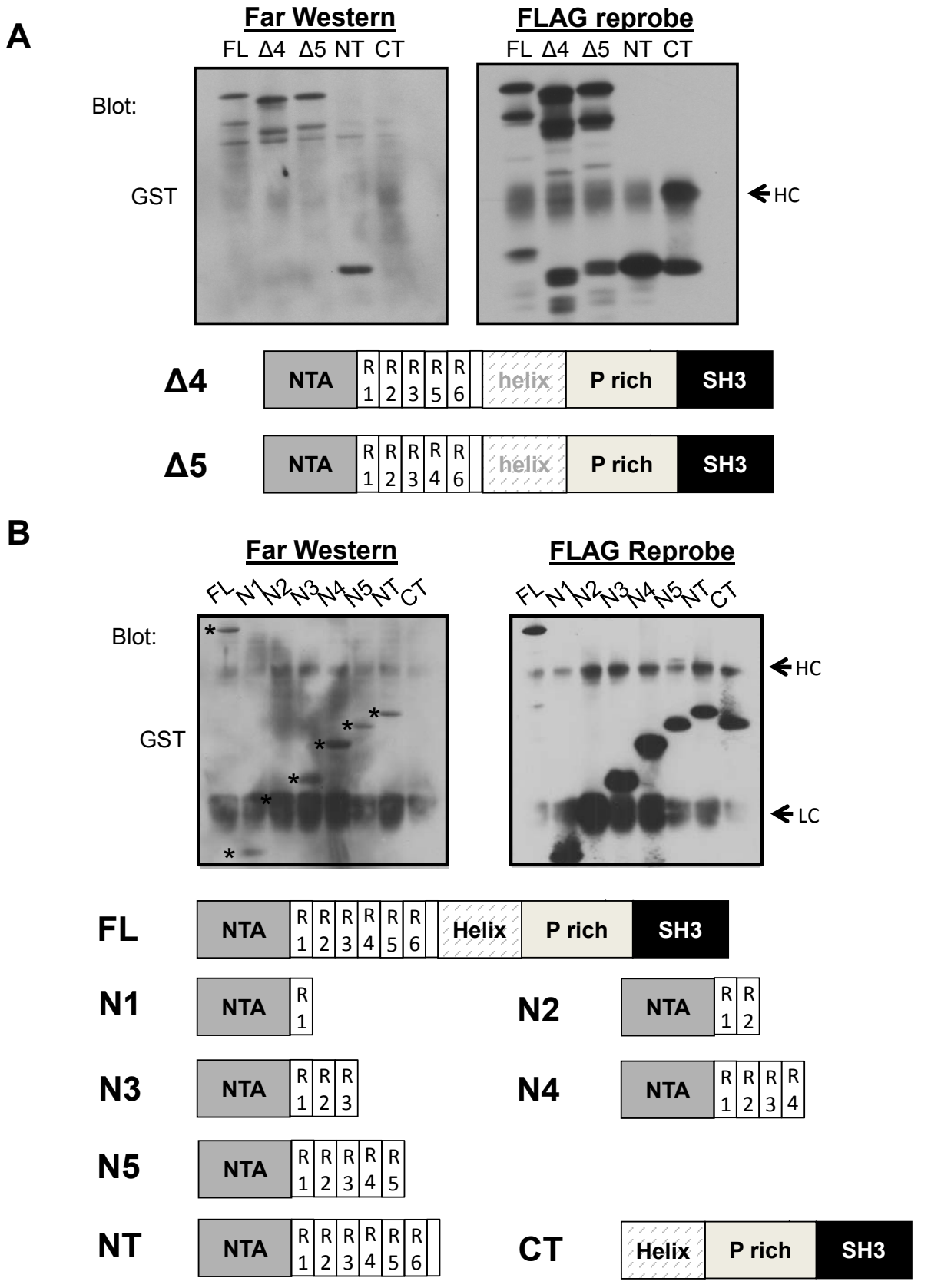

Fig. S4. Src SH2 domain does not bind to the fifth cortactin repeat. (A) Far Western binding analysis of the GST-Src SH2 domain with FLAG-WT full-length cortactin (WT), lacking the 4th cortactin repeat (D4), lacking the 5th cortactin repeat (D5), amino terminal (NT) and carboxyl terminal (CT) proteins. (B) Far Western analysis of GST-Src SH2 with the indicated FLAG-cortactin constructs. The membrane was reprobed with an anti-FLAG monoclonal antibody to verify protein expression.

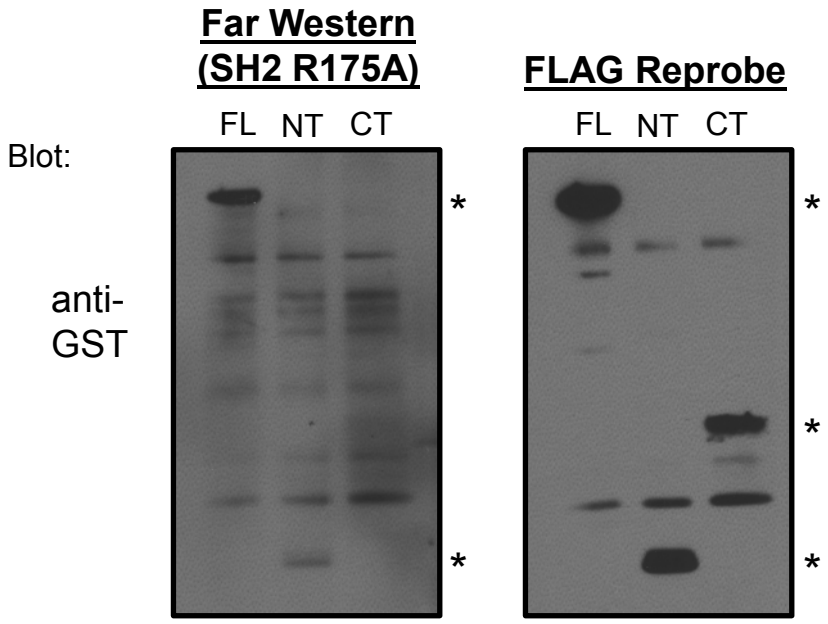

Fig. S5. Src SH2 R175A retains binding to cortactin. Far Western binding analysis of GST-Src SH2 R175A with full-length (FL),NT and CT cortactin proteins. The membrane was stripped and reprobed with anti-FLAG antibody to verify cortactin fusion protein expression.

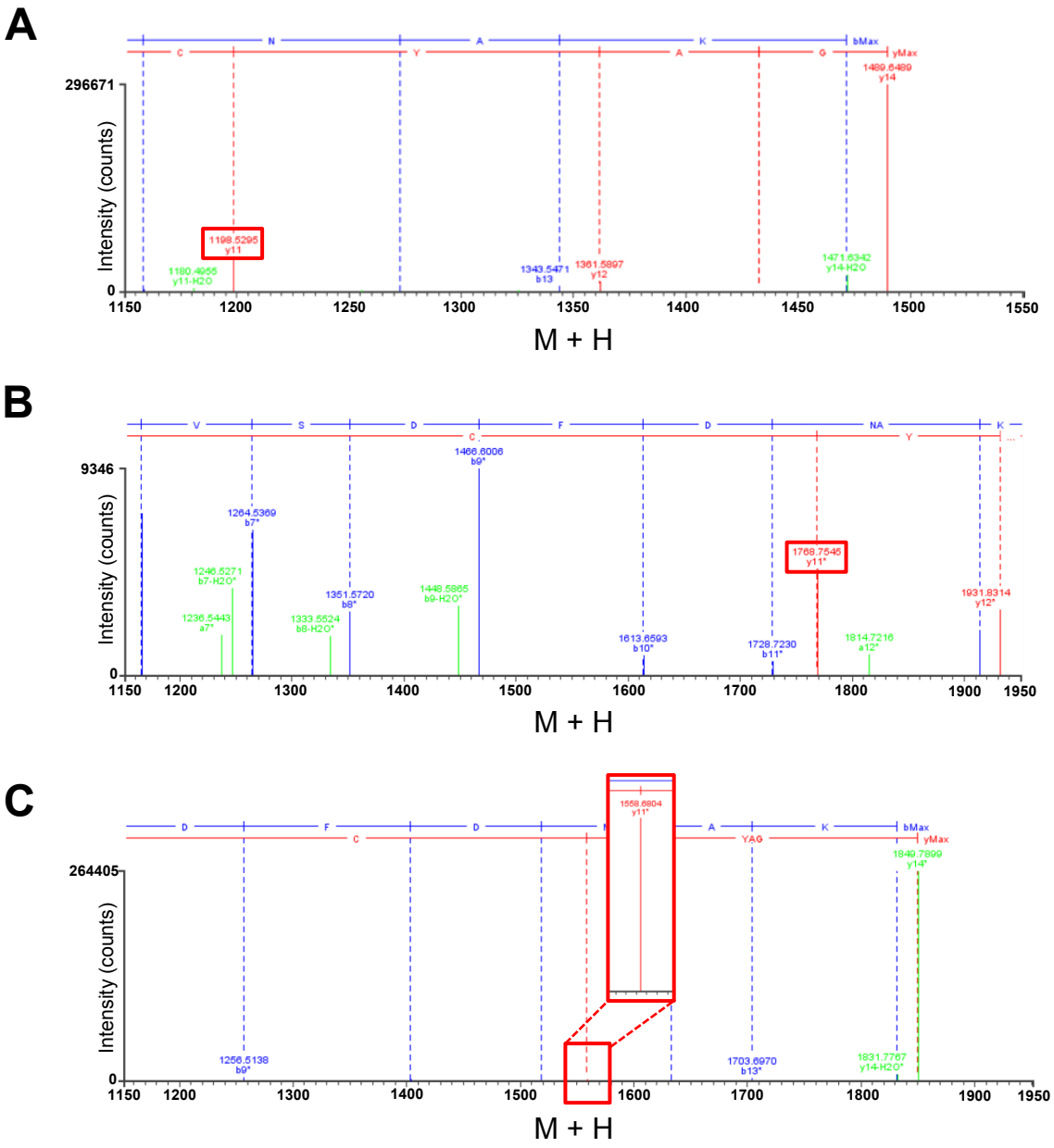

Fig. S6. Y-ion analysis of cystine bonding between Src C185 and cortactin C112 and C246. (A) LC-MS/MS ion fragmentation spectra of the GST-Src SH2 domain showing the position of y11. (B) Position of y11 in the GST-SH2 domain with the cortactin C112 peptide. (C) Position of Y11 in the GST-SH2 domain with cortactin C246 peptide.

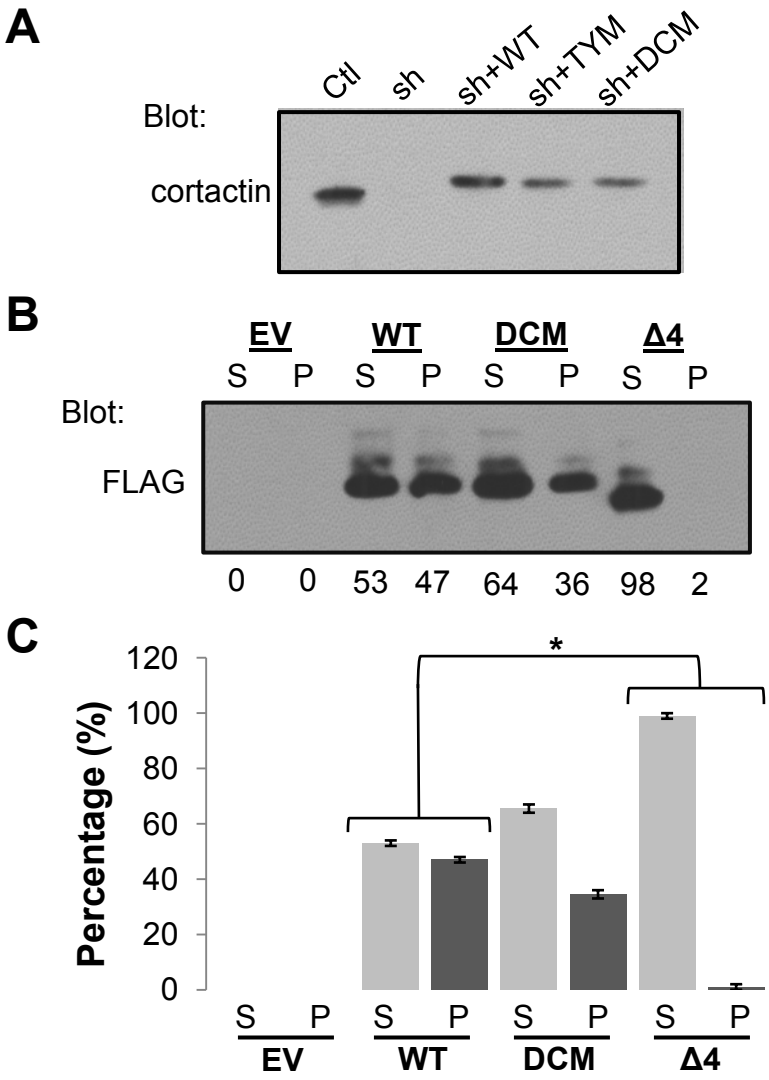

Fig. S7. Expression of cortactin mutants in cortactin knockdown cells and F-actin co-sedimentation of cortactin mutants. (A) Total cell lysate from 1483 cells (Ctl), cells with stable lentiviral-transduced shRNA cortactin knock-down (sh), and sh cells transfected with FLAG-cortactin WT (WT), TYM, and DCM mutants were analyzed for knockdown efficacy and construct expression by cortactin immunoblotting. (B) Western blot analysis F-actin co-sedimentation from 293T cells transfected with empty vector (EV), cortactin WT, DCM and Δ4 constructs. Constructs in supernatant (S) and pellet (F) fractions were detected with anti-FLAG and percentages of constructs in each fraction determined by densitometry. (C) Quantitation of F-actin co-sedimentation assays. Data are shown from two independent experiments. Bars indicate standard error, asterisk indicate p-value < 0.05.