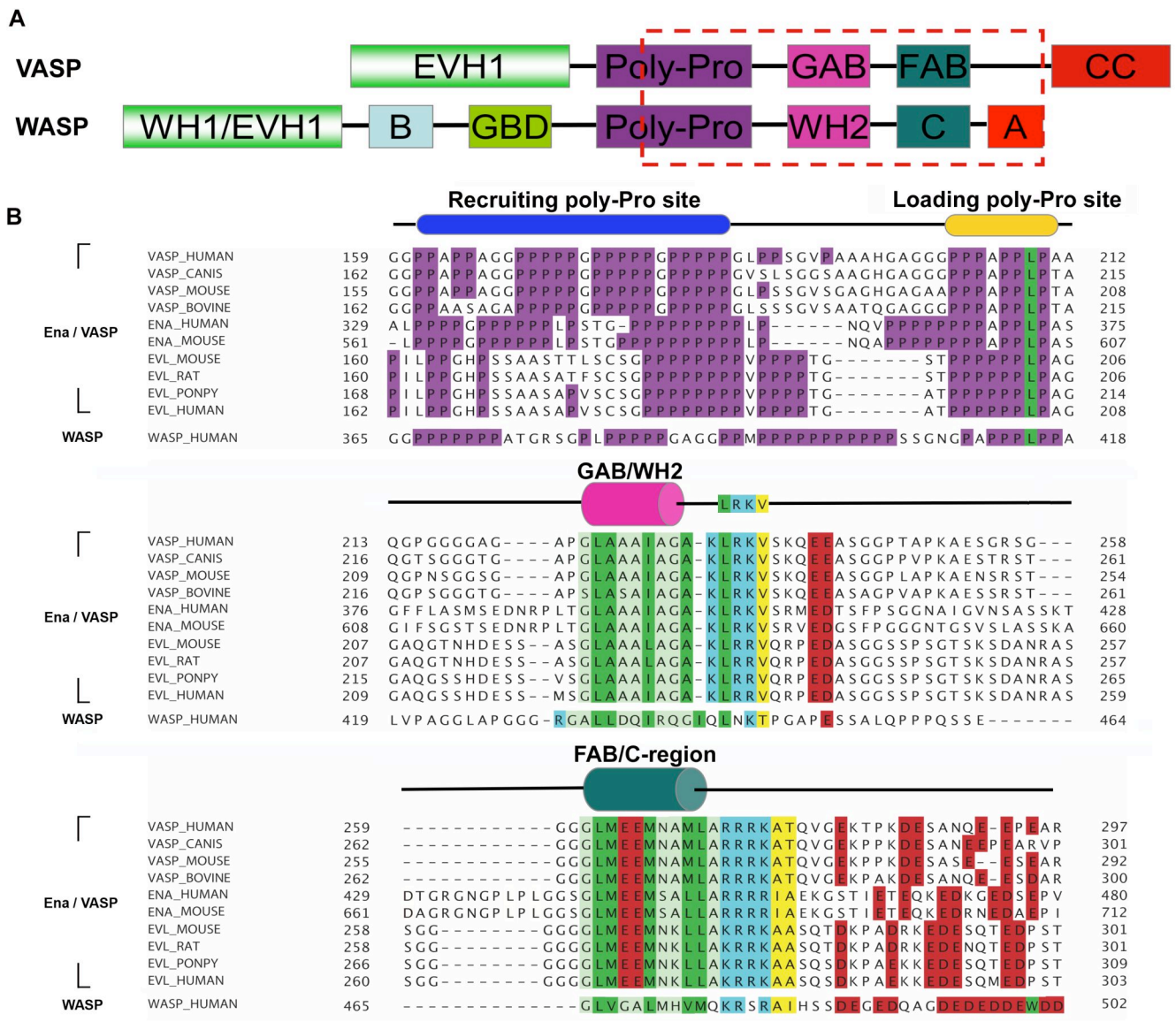
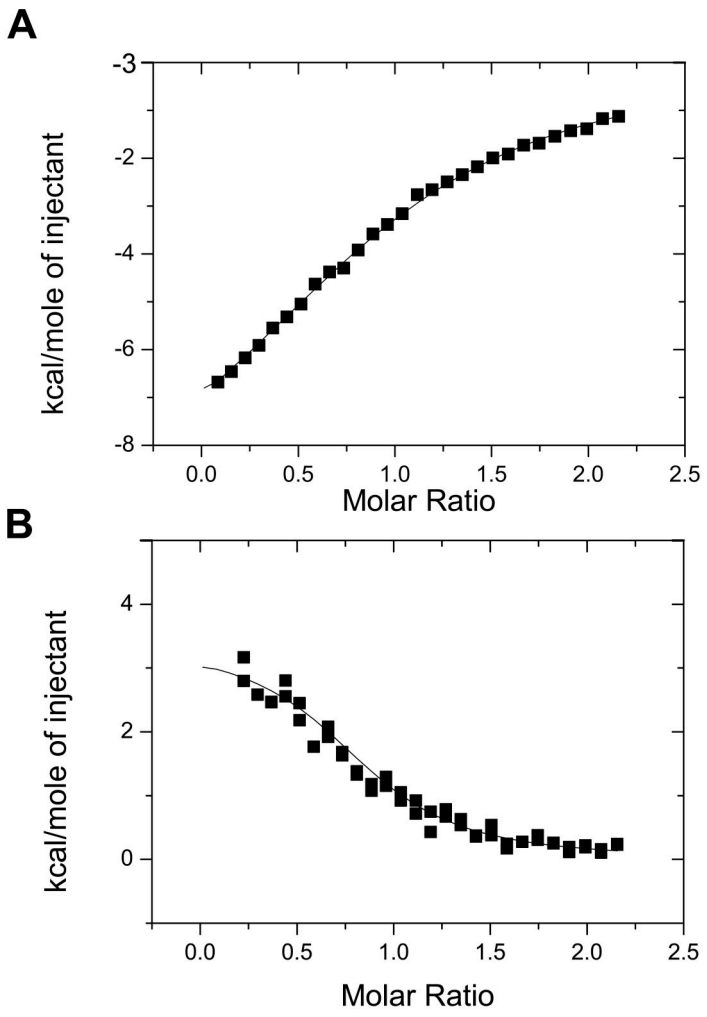
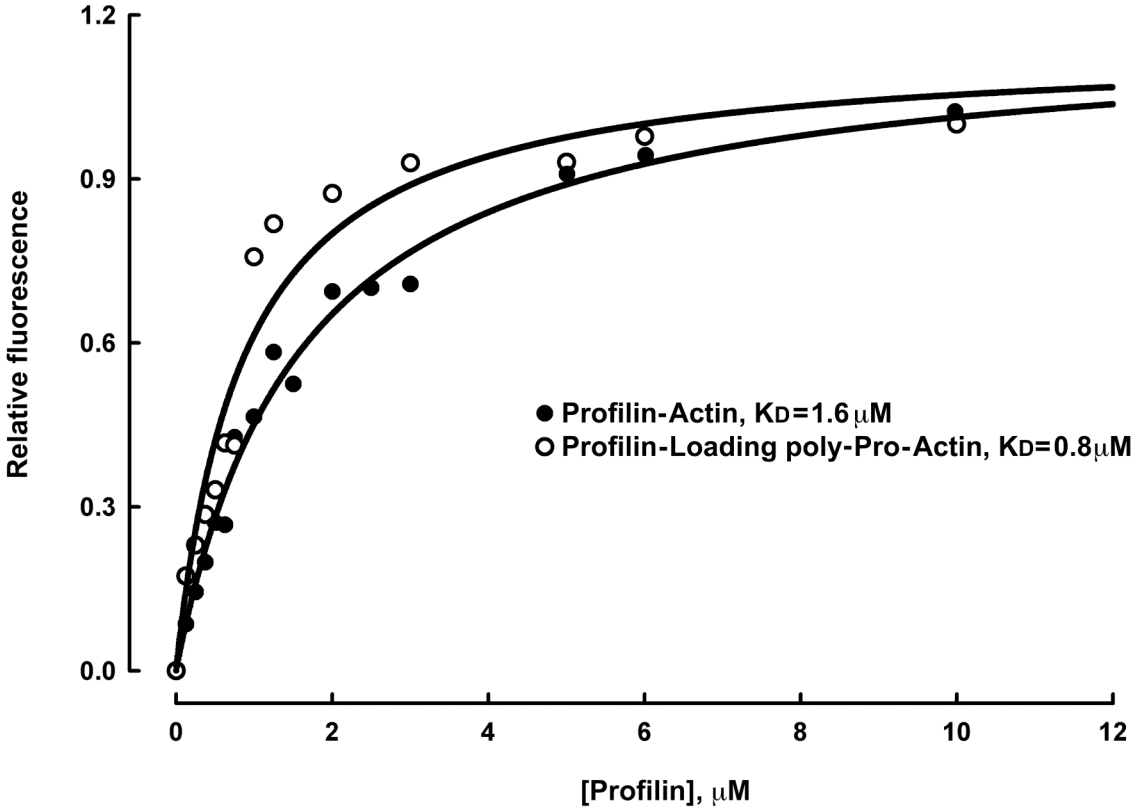


Supplementary Figure 1. Conserved domain architecture of the poly-Pro and actin-binding regions of Ena/VASP and WASP. (**A**) Domain organization of Ena/VASP (EVH1, Ena/VASP homology domains 1; poly-Pro sequence; GAB and FAB, globular- and filamentous-actin binding domains; CC, coiled-coil) and WASP (WH1, WASP-homology domain 1; B, basic region; GBD, G-protein-binding domain; poly-Pro sequence; WH2, WASP-homology domain 2; C, central-region; A, acidic-region). A similar color scheme was used to stress the analogy existing between the various domains of Ena/VASP and WASP. (**B**) Sequence alignment of the region highlighted by a red dashed rectangle in part **A** of this figure (accession numbers are: VASP_HUMAN, P50552; VASP_CANIS, P50551; VASP_MOUSE, P70460; VASP_BOVINE, Q2TA49; ENA_HUMAN, Q8N8S7; ENA_MOUSE, O03173; EVL_MOUSE, P70429; EVL_RAT, O08719; EVL_PONPY, Q5R896; EVL_HUMAN, Q9UI08; WASP_HUMAN, P42768). Conserved amino acids are colored according to their chemical characteristics and role in the interactions with actin and profilin (green, hydrophobic; blue, basic; red, acidic; yellow, small conserved amino acid, purple, poly-Pro region). The GAB (WH2) was aligned with the FAB (C region) to stress the relationship between their sequences, both of which form amphiphilic α-helices. Although the acidic region of WASP is not conserved in Ena/VASP, a high density of negatively charged amino acids in this region of Ena/VASP further suggests a common evolutionary origin of these two protein families.



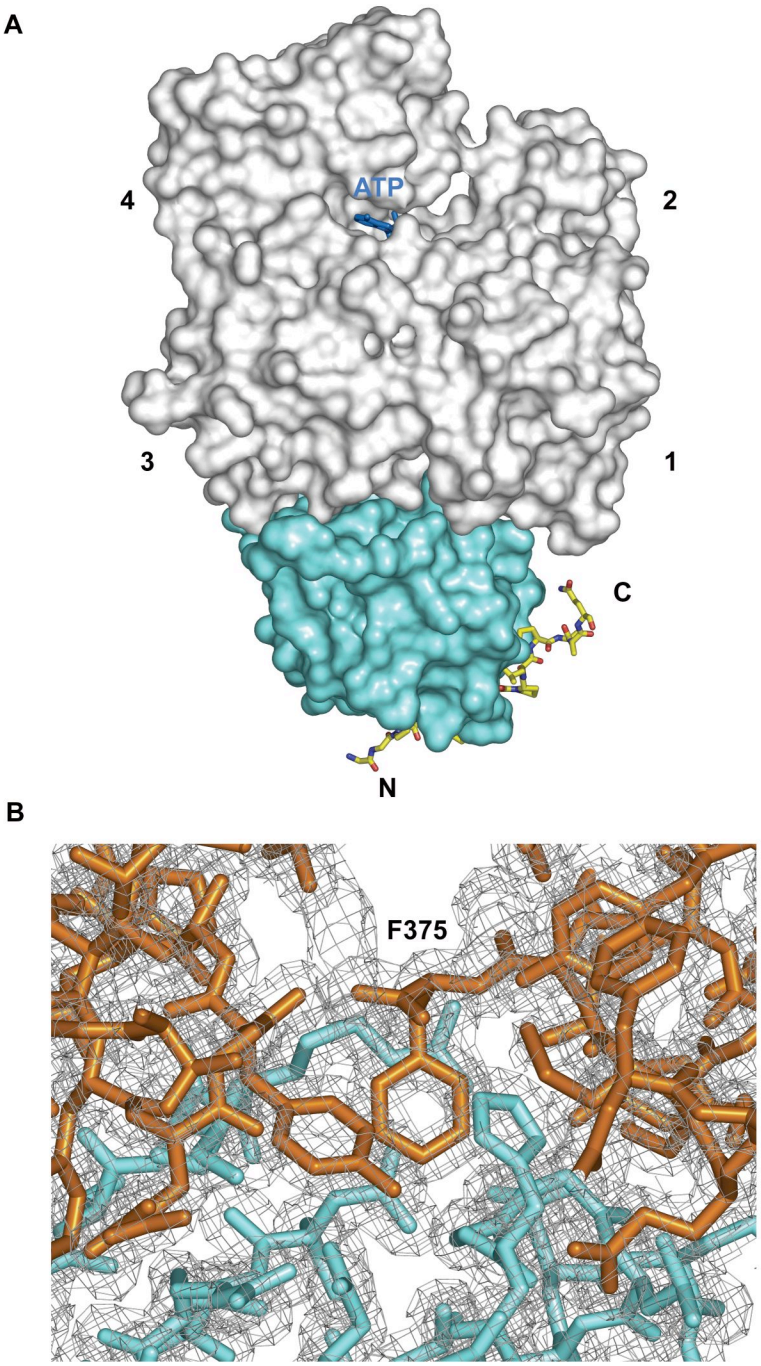
Supplementary Figure 2. Binding to profilin and profilin-actin of a peptide comprising the loading poly-Pro site of human VASP (amino acids 198-213) measured by isothermal titration calorimetry. (**A**) Binding isotherm produced by integration of the heat of 10 μL injections of a 1 mM solution of the VASP peptide into a 100 μM solution of profilin. The experiment was performed at 25°C in 10 mM sodium phosphate pH 7.4 and 150 mM NaCl. The line represents a non-linear least squares fit to the data using a single-site binding model. For this experiment, n = 0.93, K_D = 50 μM peptide, and $\Delta H°_{bind}$ = –10.15 kcal mol^{-1}). (**B**) Binding isotherm produced under similar conditions by integration of the heat of injection of the VASP peptide (at 0.8 mM) into a solution of purified profilin-actin complex (at 80 μM). The binding was endothermic in character and resulted in estimated n = 0.86, K_D = 8 μM peptide, and $\Delta H°_{bind}$ = 3.369 kcal mol^{-1}.



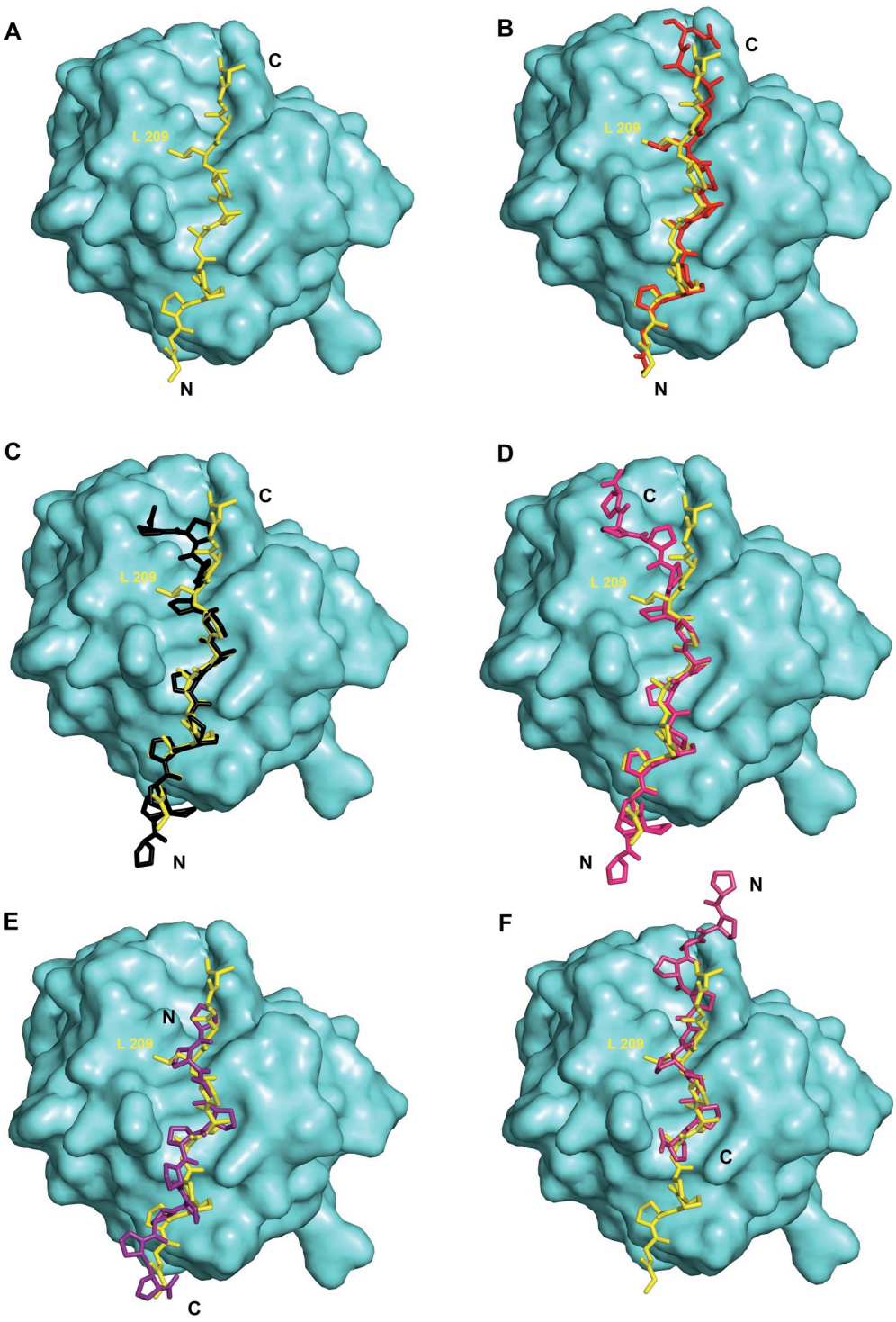
Supplementary Figure 3. Binding to actin of profilin alone and the complex of profilin with the loading poly-Pro peptide of VASP. The binding affinities were measured by the change of intrinsic tryptophan fluorescence using a Cary Eclipse Fluorescence spectrophotometer (Varian). The measurements and the analysis of raw data were carried out according published procedures (Perelroizen et al., 1994; Vinson et al., 1998). The excitation wavelength was set to 295 nm and the emission spectra were recorded from 300 to 400 nm. The measurements were performed at 20°C in 2 mM Tris pH 7.4, 0.2 mM $CaCl_2$ and 0.2 mM ATP. The concentration of actin in the cell was fixed to 1 μM. Profilin alone and complexed with 2-fold molar excess of the loading poly-Pro peptide of VASP were added at varying concentrations (0, 0.125, 0.375, 0.5, 0.6, 0.75,1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6 and 10 μM). The raw data were corrected to account for the intrinsic fluorescence of profilin and actin alone as described (Vinson et al., 1998). Each data point represents the average of 6 independent measurements. Least-square fitting of the data, using a single-site binding model, resulted in dissociation constants (K_D) of actin for profilin and profilin-poly-Pro peptide of 1.6 μM and 0.8 μM, respectively.

Perelroizen, I., Marchand, J.B., Blanchoin, L., Didry, D. and Carlier, M.F. (1994) Interaction of profilin with G-actin and poly(L-proline). *Biochemistry*, **33**, 8472-8478.

Vinson, V.K., De La Cruz, E.M., Higgs, H.N. and Pollard, T.D. (1998) Interactions of Acanthamoeba profilin with actin and nucleotides bound to actin. *Biochemistry*, **37**, 10871-10880.



Supplementary Figure 4. Crystal structure at 1.8 Å resolution of the ternary complex of profilin-actin with the loading poly-Pro site of human VASP. (**A**) View according to the conventional orientation of actin. (**B**) Electron density map contoured at 1.0 s, at the profilin (cyan) actin (gold) interface of the complex. The C-terminal actin residue Phe 375 is at the center of the binding interface between the two proteins.



Supplementary Figure 5. Comparison of the binding of the loading poly-Pro site of VASP in the structure of its complex with profilin-actin with those of plain poly-Pro sequences crystallized with profilin alone (Mahoney et al., 1997; Mahoney et al., 1999). (**A**) Loading poly-Pro (yellow) bound to profilin (cyan) from the higher resolution structure of the loading poly-Pro-GAB of VASP. (**B,C,D,E,F**) Comparisons with the structures of the loading poly-Pro site alone (red), chain A of the pentadecameric L-Pro peptide (black), chain B of the pentadecameric L-Pro peptide (pink), first orientation of the decameric L-Pro peptide (purple), and second orientation of the decameric L-Pro peptide (magenta).

Supplementary Movie 6. Structure at 1.5 Å resolution of profilin-actin with the loading poly-Pro-GAB region of human VASP. The linker region (blue), which is not seen in the structure, was modeled following a grove along the actin-profilin interface.