Supporting Information

Disease-associated mutations in p150^{Glued} destabilizes CAP-gly domain[#]

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Materials:

All chemicals and reagents were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise stated. Tubulin, taxol and GTP were purchased from Cytoskeleton Inc. For ^{15}N labeling, $^{15}NH_4Cl$ was purchased from Cambridge Laboratories Inc.

Methods:

Cloning expression and purification of WT and mutated CAP-gly domain of p150^{Glued}:

The CAP-gly domain (residues 19-107) was cloned into pET28b-His6-SMT3 vector as described previously (*1, 2*). Site directed mutagenesis (G59A, G71A, T72P and Q74P) was performed by a standard protocol using CAP-gly 19-107. Each construct was confirmed by DNA sequencing (City of Hope Comprehensive Cancer Center Core facility). Cloned vectors were transformed into *E. coli* BL21(DE3) strain for protein expression. WT-CAP-gly was expressed in LB (Luria-Bertani) media following standard protocol of induction at OD_{600} = 0.5-0.7 with 250 mM of IPTG and grown at 37 °C for 4 h while the domains harboring the point mutations were expressed at 18 °C overnight to increase solubility.

For ^{15}N labeling the cells were first grown in 4 L LB media to OD_{600} ~ 0.7. At this point cells were harvested, washed thrice with minimal media and then resuspended in 1 L minimal media (1X M9 salts, 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 10 mg/L of $^{15}NH_4Cl$ (*1*). The cells were then revived for 1 h at 37 °C prior to induction with 250 mM IPTG. After induction, the WT-CAP-gly was grown for 3 h at 37 °C, while the mutated domains were grown at 18 °C for overnight.

For each of the CAP-gly constructs, cells were harvested and resuspended in phosphate buffered saline (PBS). For protein purification, the cells were lysed using a French press and clarified at 40,000 rpm at 4 °C for 40 min. The clarified supernatant was loaded on a Ni-NTA agarose (Qiagen) column and eluted with a gradient of imidazole (5 to 500 mM). The SMT-tag was removed by Ulp-1 enzyme and buffer was exchanged to 50 mM Tris pH-8.0, 100 mM NaCl, 1 mM EDTA and 1 mM DTT. The size exclusion chromatography was performed with Hiload 26/60 Superdex 75 (GE Health Sciences) column in the same buffer. The eluted protein was concentrated and buffer was exchanged with PBS buffer, aliquoted, flash frozen and stored at -80 °C.

Cloning expression and purification of EB1:

The C-terminal end of EB1, comprising residues 194-268, was synthesized by assembly PCR of 8 primers:

1. 5'-GATCGAGGATCCGCGGAACTGATGCAGCAGGTGAACGTGCTG-3'
2. 5'-CGCGCTCCTTCTCCAGATCTTCCACGGTCAGTTTCAGCACGTTCACCTGC-3'
3. 5'-CTGGAGAAGGAGCGCGATTTCTATTTCGGCAAACTGCGCAACATTGAACTGATTTGCC-3'
4. 5'-CCGGATCGTTTTCGCCTTCGTTTTCCTGGCAAATCAGTTCAATGTTGCGC-3'
5. 5'-GGCGAAAACGATCCGGTGCTGCAGCGCATTGTGGATATTCTGTATGCG-3'

6. 5'-CGCCTTCATCCGGAATCACAAAGCCTTCATCGGTCGCATACAGAATATCC-3'
7. 5'-CCGGATGAAGGCGGCCCGCAGGAAGAACAGGAAGAATAT-3'
8. 5'-GATCGACTCGAGTCAATATTCTTCCTGTTC-3'

The synthesized gene was cloned into pET28b-His6-SMT vector between the *BamHI* and *HindIII* restriction sites. The construct was confirmed by DNA sequencing. Expression was carried out in LB media at 37 °C. Purification was performed following the same procedure as the CAP-gly proteins.

Protein Concentration:

Using Beer's law, the concentration of each protein was determined by measuring its absorption at 280 nm. The molar extinction co-efficient was calculated from the sequence. In each case, the protein sample was concentrated or diluted to fall within the linear range (0.1-1.0 OD) of spectrophotometer.

Analytical Size Exclusion Chromatography of WT and Mutated CAP-gly:

Size exclusion chromatography was performed on an analytical Superdex75-S10/300GL column (GE Health Sciences). The column was pre-equilibrated in 50 mM Tris, pH - 8.0, 100 mM NaCl, 1 mM EDTA and 1 mM DTT. Protein samples were centrifuged at 21,000 x G for 15 min, before loading on the column (Fig S2A). The fractions at ~12.5 mL were pooled. These monomeric fractions were further analyzed by size exclusion chromatography to ensure that they remain monomeric (Fig S2B).

For the interaction studies between WT or mutated CAP-Gly domains and EB1, 80 μM of the CAP-gly domain was incubated with 65 μM of EB1 (WT or mutated CAP-gly domains used in 1.2 molar excess of EB1) at room temperature for 5 min, centrifuged at 20,000 x G for 10 min, and loaded on an analytical Superdex75 column.

NMR Spectroscopy:

^{1}H-^{15}N HSQC spectra of the WT CAP-gly and CAP-gly mutated domains (G59A, G71A and Q74P) were acquired at 298 K on a 14.1 T Bruker Avance spectrometer (^{1}H frequency of 600.13 MHz) shown in Figures 2 and S1B. Chemical shifts were referenced to 2,2-dimethylsilapentene-5-sulfonic acid (*3*). All spectra were processed in NMRpipe (*4*) and analyzed in Sparky (*5*). Chemical shift assignments of the wild type CAP-gly (19-107) have been reported before (*1*). Detailed acquisition and processing parameters for each individual experiment are tabulated in supplementary table S1.

Table S1. Acquisition and processing parameters for HSQC experiments of wild type CAP-gly and CAP-gly point mutations

Sample	Acquisition		Processing	
	ω2	ω1	ω2	ω1
Wild type CAP-gly (480 μM)	1024 complex 16 scans	128 complex, States-TPPI	90-degree shifted sine bell apodization;	90-degree shifted sine bell apodization;
G59A (110 μM)	1024 complex 64 scans	128 complex, States-TPPI	90-degree shifted sine bell apodization;	90-degree shifted sine bell apodization;
G71A (110 μM)	1024 complex 64 scans	128 complex, States-TPPI	90-degree shifted sine bell apodization;	90-degree shifted sine bell apodization;
Q74P (110 μM)	1024 complex 64 scans	128 complex, States-TPPI	90-degree shifted sine bell apodization;	90-degree shifted sine bell apodization;

Circular Dichroism:

CD spectra were collected with a J-810 spectropolarimeter (Jasco) using a 1 nm bandwidth and 4 sec response time. Molar ellipticity experiments were performed with 5 μM protein using a 0.1 cm cuvette. Scans were collected from 280-195 nm at 4 °C, at 80 °C and again at 4 °C after cooling (Fig. S3). Ellipticity is reported as mean residue ellipticity [θ] in deg cm^2 $dmol^{-1}$ using the equation: $[\theta] = \theta_{obs}$ x MRW/(10*l*c), where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight (molecular mass/number of residues), *l* is the path-length in cm, and c is the concentration in mg/mL. Temperature scans for thermal denaturation, were performed with 5 μM protein in a 1 cm cuvette with constant stirring from 4-80 °C at a fixed wavelength of 223 nm with a data pitch of 1 °C, a 45 sec delay, and a temperature slope of 40 °C/h. All protein was in PBS buffer and 1mM TCEP.

Co-sedimentation assay with Microtubules:

For the MT co-sedimentation assays, we dialyzed each CAP-gly construct into 1 x PEM buffer (100 mM PIPES (Piperazine-N,N′-bis(2-ethanesulfonic acid)), pH-7.0, 1 mM $MgCl_2$, 1 mM EGTA (Ethylene-bis(oxyethylenenitrilo)tetraacetic acid) per manufacturer's recommendation. To generate MTs, tubulin (15 μM) was incubated with taxol (45 μM) in 1 x PEM buffer with 1 mM GTP and incubated at 37 °C for 45 min. Polymerized MTs, 5 μM, were incubated with 75 μM (1:15) of WT or mutated CAP-gly domains in a volume of 25 μL for 5 min, followed by centrifugation for 40 min at 200,000 x G in a Beckman Optima L-80 XP ultracentrifuge (*6*). Supernatants were carefully separated from pellet. Pellet was resuspended in 50 μL 2 X SDS-PAGE sample buffer while 25 μL of each supernatant was mixed with 25 μL of 2 X SDS-PAGE sample loading buffer. An 18 μL aliquot of each sample was loaded on a 15% SDS-PAGE for analysis.

ImageJ (NIH) Quantification:

To quantitate the binding of WT-CAP-gly and mutated domains with MTs, we used ImageJ software from NIH. In each case, the software was used to quantify bands on the

scanned SDS-PAGE, both in presence and absence of MT in the pelleted fractions. The experiment was repeated thrice for each CAP-gly construct, and the standard error was calculated.

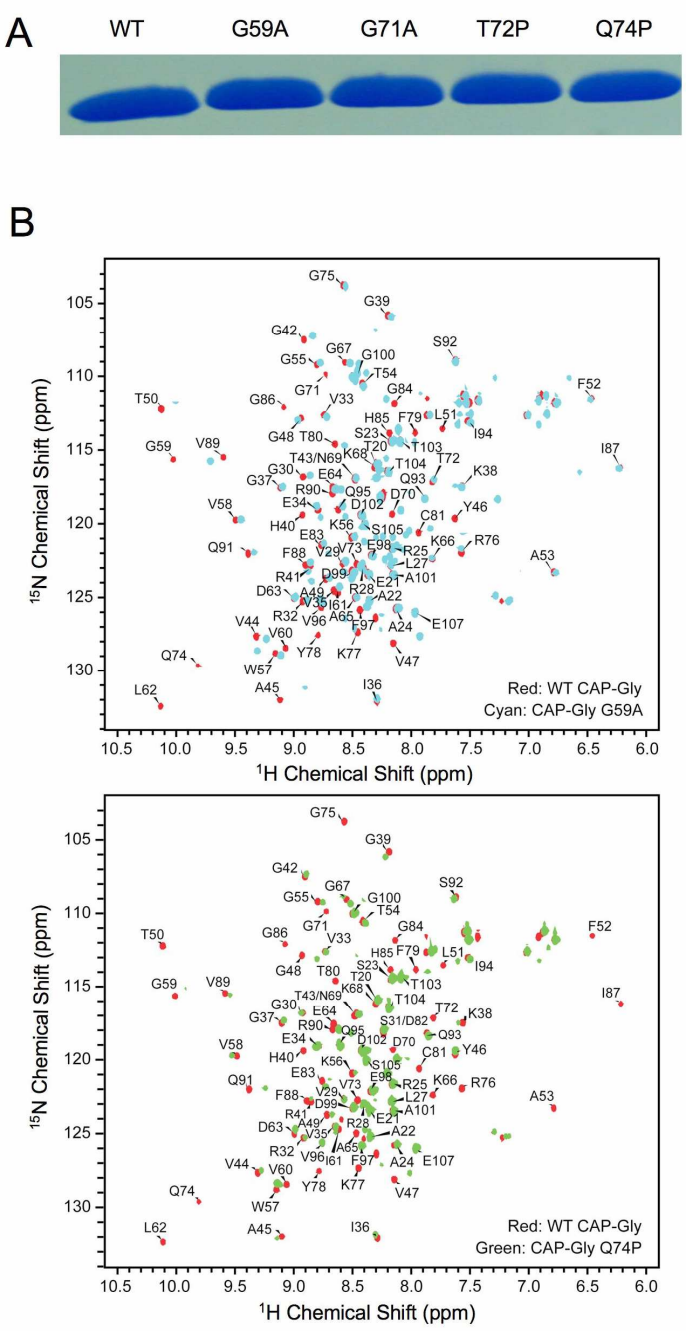

Figure S1. Protein Purification and NMR

A) SDS-PAGE showing the WT CAP-gly and the mutated domains purified to homogeneity.

B) ^{1}H-^{15}N HSQC spectra of G59A (cyan) and Q74P (green) mutated domains overlaid on the WT CAP-gly spectrum (red). The spectra from both samples are well resolved, but a indicate a large number of resonance shifts, indicating the point mutations perturb the domain globally.

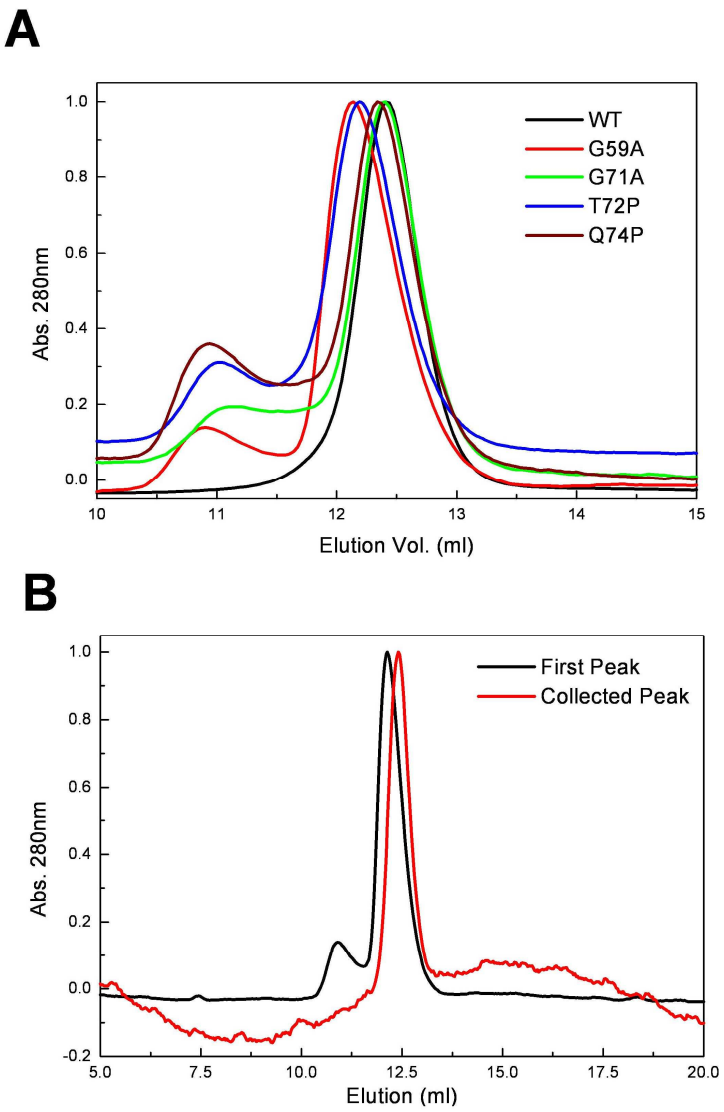

Figure S2. Analytical Size Exclusion Chromatography

A) Analytical Size Exclusion Chromatography indicates that the mutated domains have similar hydrodynamic properties compared to WT domain. However, each mutated domain also produced another small peak at a higher elution volume.

B) The monomeric fraction of G59A remains monomeric. The peak corresponding to a monomer (12.5 mL) from first run (black trace) was concentrated and reloaded on the column (red trace).

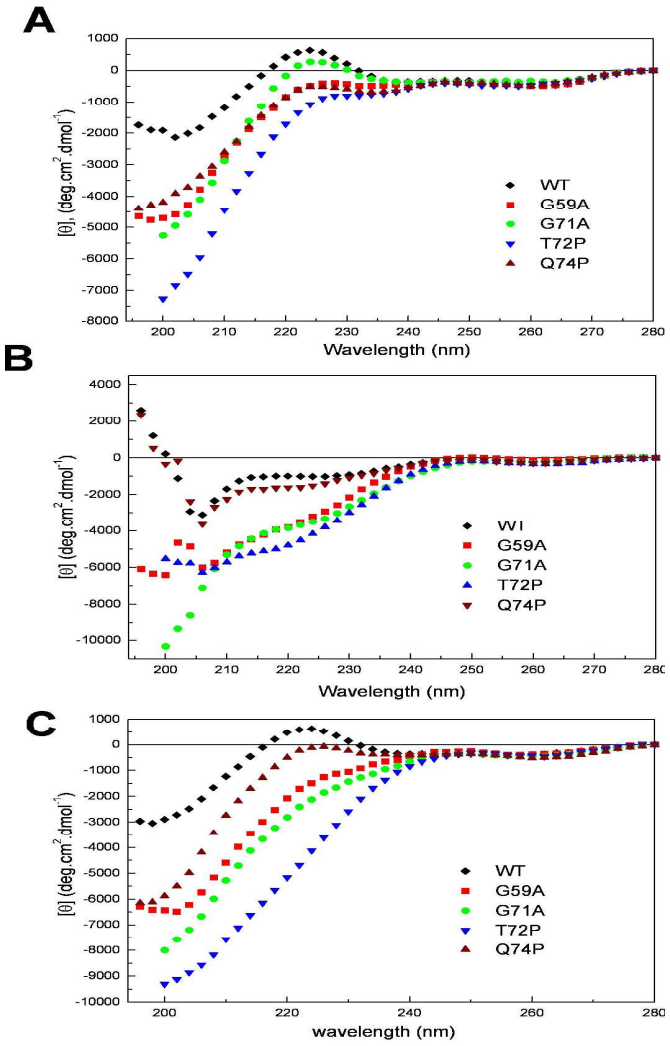

Figure S3. Structural Comparison of WT and Mutated CAP-gly domains

A) Far UV-CD spectra was collected for both the mutated and WT CAP-gly domains. The WT CAP-gly produces a positive maxima at 223 nm as reported earlier (*1*) which is a characteristic of anti-parallel β-strands (*7*). A positive maximum was not observed for any of the point mutants. Consistent with the HSQC NMR spectra of G59A, G71A and Q74P domains, the differences observed in the CD spectra indicate changes in the secondary and potentially tertiary structure of the mutated CAP-gly domains.

B) CD spectra collected at 80 °C.

C) CD spectra collected at 4 °C after heating the samples to 80 °C.

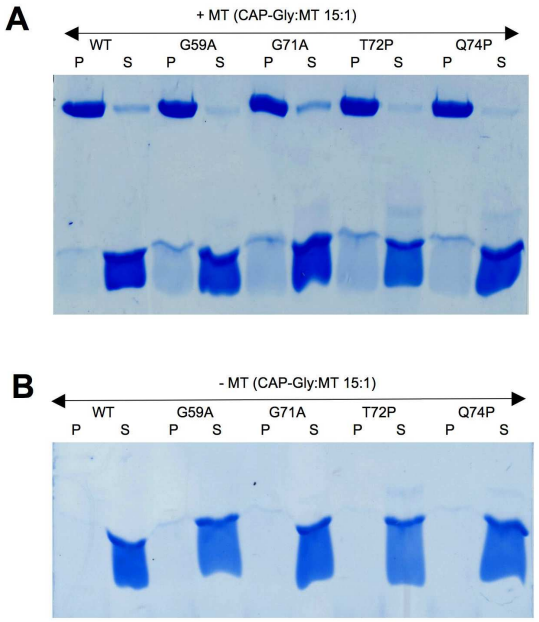

Figure S4. Representative SDS-PAGE from MT co-sedimentation experiment

A) Precipitation of WT and mutated CAP-gly domains by MTs. Taxol stabilized MTs (5 μM tubulin) were incubated with the WT or individual CAP-gly point mutants (75 μM) and precipitated by centrifugation. The pellet (denoted "P") and supernatant (denoted "S") were then analyzed by SDS-PAGE and quantified by ImageJ. The experiment was repeated three times.

B) As a control, the WT CAP-gly and each mutated domain was centrifuged to identify precipitation in the absence of MTs.

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