



This is a repository copy of *Pressure-Dependent Chemical Shifts in the R3 Domain of Talin Show that It Is Thermodynamically Poised for Binding to Either Vinculin or RIAM.*

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/124596/>

Version: Accepted Version

Article:

Baxter, N.J., Zacharchenko, T., Barsukov, I.L. et al. (1 more author) (2017)
Pressure-Dependent Chemical Shifts in the R3 Domain of Talin Show that It Is Thermodynamically Poised for Binding to Either Vinculin or RIAM. *Structure*. ISSN 0969-2126

<https://doi.org/10.1016/j.str.2017.10.008>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Pressure-dependent chemical shifts in the R3 domain of talin show that it is thermodynamically poised for binding to either vinculin or RIAM

Nicola J. Baxter,¹ Thomas Zacharchenko,^{2,3} Igor L. Barsukov,² and Mike P. Williamson^{1,4,*}

¹Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

²School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, United Kingdom

³Current address: School of Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

⁴Lead contact

*Correspondence: m.williamson@sheffield.ac.uk (M.P.W.)

SUMMARY

Talin mediates attachment of the cell to the extracellular matrix. It is targeted by the Rap1 effector RIAM to focal adhesion sites, and subsequently undergoes force-induced conformational opening to recruit the actin-interacting protein vinculin. The conformational switch involves the talin R3 domain, which binds RIAM when closed and vinculin when open. Here, we apply pressure to R3 and measure ¹H, ¹⁵N and ¹³C chemical shift changes, which are fitted using a simple model, and indicate that R3 is only 50% closed: the closed form is a four-helix bundle, while in the open state helix 1 is twisted out. Strikingly, a mutant of R3 that binds RIAM with an affinity similar to wild-type but more weakly to vinculin is shown to be 0.84 kJ mol⁻¹ more stable when closed. These results demonstrate that R3 is thermodynamically poised to bind either RIAM or vinculin, and thus constitutes a good mechanosensitive switch.

KEYWORDS hydrostatic pressure, cell adhesion, talin, focal adhesion complex, vinculin, singular value decomposition, chemical shift

1 INTRODUCTION

2 The adhesion of cells to the extracellular matrix usually makes use of the cell surface receptors known
3 as integrins. The intracellular tail of an activated integrin forms part of a complex that links the receptors
4 to the actin cytoskeleton, and thus enables communication between extracellular ligands and the
5 cytoskeleton. The assembly of this complex is tightly regulated, and requires a number of proteins,
6 including talin, vinculin and the Rap1-GTP-interacting adaptor molecule (RIAM). Talin is a large 2541-
7 residue protein, consisting of a head that binds to integrins and to phospholipids, and a long rod-like tail
8 that binds to F-actin and vinculin (Calderwood et al., 2013). Talin is recruited to the membrane by RIAM
9 and subsequently undergoes a force-dependent conformational change that exposes cryptic vinculin
10 binding sites. Vinculin recruitment by talin reinforces the connection to the actin cytoskeleton, constituting
11 a force-sensing mechanism that regulates formation of the focal adhesion complex. The key element of
12 the force sensor in the talin rod is the R3 domain, which binds both RIAM and vinculin (Atherton et al.,
13 2015). The interaction of R3 with RIAM and vinculin is mutually exclusive, with RIAM binding to the closed
14 form of R3, while vinculin interacts with the open form (Fillingham et al., 2005). This opening occurs as
15 a result of mechanical stretching (del Rio et al., 2009; Yao et al., 2014), leading to the displacement of
16 RIAM by vinculin (Goult et al., 2013). The R3 domain is located in a compact region of the talin rod
17 formed by three sequential four-helix bundle domains R2-R4 that are particularly susceptible to
18 mechanical unfolding (Yan et al., 2015). R3 has the lowest stability of the three and was observed to fold
19 and unfold reversibly on a sub-second timescale at 5 pN applied force, within the range of a single
20 actomyosin contraction (Yan et al., 2015; Yao et al., 2014; Yao et al., 2016). These observations have
21 led to a model (Atherton et al., 2015; Calderwood et al., 2013; Yao et al., 2014) which proposes that
22 RIAM initially binds to and recruits talin, but that a force-induced conformational change in talin (caused
23 by the flow of actin filaments past the talin complex) opens up the R3 domain, weakening the interaction
24 with RIAM and permitting an interaction with vinculin. The newly formed vinculin complex then stabilizes
25 the focal adhesions by strengthening interactions with actin (Figure 1).

26 The four-helix bundle that comprises the R3 domain of talin is held together by hydrophobic
27 interactions. The hydrophobic interior includes a ‘threonine belt’ composed of four threonine residues,
28 one from each helix in the bundle, that reduces the stability of the domain (Fillingham et al., 2005). We
29 previously constructed a quadruple mutant of R3, referred to as R3-IVVI, in which these four threonine
30 residues were replaced by hydrophobic residues (T809I/T833V/T867V/T901I) (Goult et al., 2013). This
31 mutant is more resistant to unfolding by both mechanical force (Yao et al., 2014) and thermal unfolding
32 (Goult et al., 2013), and binds much more weakly to vinculin than R3, consistent with the coupling of
33 unfolding to productive vinculin binding (Figure 1).

34 There is thus ample evidence that the activity of R3 as a mechanosensitive switch is due to a
35 reversible opening of the four-helix bundle. Structural characterization of the open state has so far
36 remained challenging. Here, we use high pressure as a tool to allow us to characterize the energetics
37 and structures of the two conformations.

38 High hydrostatic pressure has proved to be an effective way to reveal alternative protein
39 conformations that are close in energy to the ground state (ie low-lying excited states) (Akasaka, 2003,
40 2006). Elevated pressure leads to a general compression of the protein, which to a good approximation
41 is a linear effect. NMR chemical shift changes with pressure are therefore often linear (Kitahara et al.,
42 2013). However, pressure also stabilizes alternative conformational states with lower partial molar
43 volumes. Such states become increasingly populated as pressure increases, leading to non-linear
44 chemical shift changes in the vicinity of the structural change. Because folded proteins always contain
45 small cavities and packing defects, they always have a larger partial molar volume than unfolded
46 proteins, and therefore pressure leads to local, and ultimately to global, unfolding (Roche et al., 2012).
47 The low-energy excited state conformations revealed in this way can be functionally important: for
48 example, a locally unfolded form of ubiquitin has been identified as the conformation seen when ubiquitin
49 binds to the E2 ubiquitin-conjugating enzyme (Kitazawa et al., 2014). We therefore applied elevated
50 hydrostatic pressures up to 2.5 kbar (250 MPa) to the R3 domain of talin to characterize the equilibrium

51 between closed and open forms, making the assumption that the excited state produced by high pressure
52 is likely to be similar to the state produced by mechanical force.

53 Application of elevated pressure usually leads to linear or smoothly curved plots of chemical shift
54 vs pressure. The analysis of pressure-dependent NMR chemical shifts therefore most commonly
55 proceeds by fitting ¹⁵N-HSQC chemical shift changes to a quadratic expression, $\delta = a + bp + cp^2$, where
56 p is pressure (Akasaka, 2006; Kitahara et al., 2013). The linear term b is related to hydrogen bond
57 strength and other local geometrical effects, and is often difficult to interpret. By contrast, the non-linear
58 component c arises from the equilibrium between the native ground state and an excited state, and
59 therefore tends to report on conformational changes in the transition to excited states, for example
60 around cavities, and is much more useful and protein-specific (Kitazawa et al., 2014). However, although
61 a quadratic expression is a convenient functional form, it cannot be related in any simple way to physical
62 phenomena, and the terms b and c have no simple physical meaning. We therefore propose a more
63 physically meaningful equation.

64 Pressure causes a change in the free energy difference between two conformations. We therefore
65 expect the states to be populated according to a pressure-dependent Boltzmann expression:

$$66 \quad P_2/P_1 = \exp\left(-(\Delta G + p\Delta V)/RT\right) \quad [1]$$

67 We assume that R3 can exist in two states, 1 and 2 (ground state and excited state, respectively),
68 populated in a pressure-dependent way as above. Each of these states can also undergo a linear
69 pressure-dependent compression, giving rise to a linear change in chemical shift. We can therefore
70 model the observed pressure-dependent chemical shift δ in a more physically meaningful way as:

$$71 \quad \delta = \frac{(\delta_1^0 + p\Delta\delta_1) + (\delta_2^0 + p\Delta\delta_2)\exp(-[\Delta G + p\Delta V]/RT)}{1 + \exp(-[\Delta G + p\Delta V]/RT)} \quad [2]$$

72 where δ_1^0 and δ_2^0 are the chemical shifts of forms 1 and 2 at ambient pressure; $\Delta\delta_1$ and $\Delta\delta_2$ are the linear
73 pressure-dependent changes in chemical shift; p is the pressure; ΔG is the difference in free energy

74 between the two states at ambient pressure; and ΔV is the change in volume between the two states.

75 This equation makes some assumptions, which are discussed in Supplementary Material.

76 This more complex analysis of pressure-dependent chemical shifts is more difficult to fit than the
77 standard quadratic approximation, because it has one more variable. We therefore used singular value
78 decomposition (SVD) to help analyze the chemical shift changes. SVD is a well-established statistical
79 technique for reducing the dimensionality of fitting problems by identifying the minimum number of
80 components needed to generate the experimental data patterns. In the process, it can also be used to
81 identify and remove non-correlated noise, thus dramatically reducing experimental random noise in the
82 data. This analysis showed that fitting of the pressure-dependent chemical shifts only requires four
83 components, despite the complexity of the chemical shift changes. The four components are identified
84 as those required by Eq. 2, namely the ground state at ambient pressure, a compressed ground state,
85 an excited state (whose population increases with pressure) and a compressed excited state.

86 The high quality of the 'noise-free' data allowed us to fit Eq. 2 globally, producing the result that
87 in the wild-type R3 the free energy difference between the ground and excited states is very close to
88 zero, ie R3 is 50% closed and 50% open at ambient pressure. The nature of the conformational change
89 can be identified by analyzing the chemical shifts, δ_1° and δ_2° , and the difference in the pressure-
90 dependent gradients, $\Delta\delta_1 - \Delta\delta_2$, on a per-residue basis. The main change is localized to helix 1, which is
91 part of the four-helix bundle in the ground state (closed conformation), but which becomes twisted out in
92 the excited state (open conformation), thereby explaining the effect of shear force on modulating the
93 availability of binding surfaces. Finally, a similar analysis of R3-IVVI showed that in this mutant the ground
94 state is 0.84 kJ mol^{-1} more stable than the excited state, implying that the mutant accesses the open
95 conformation less readily than R3, and explaining its lower affinity for vinculin. These results show that
96 R3 is delicately poised between open and closed states, and is thus well placed to act as a
97 mechanosensitive switch, able to exchange easily between binding RIAM in the closed state or vinculin
98 in the open state in response to appropriate stimuli.

99

100 RESULTS

101 Backbone assignments and pressure-dependent chemical shift changes

102 The NMR spectra of R3 and R3-IVVI are similar (Figure S1). The signals of R3-IVVI were assigned using
103 standard three-dimensional (3D) heteronuclear experiments. Backbone chemical shifts of R3-IVVI are
104 very similar to those of wild-type R3, except in the immediate locations of the mutations, strongly
105 suggesting that the structure of R3-IVVI is very similar to that of R3 (Figure S2). 2D ¹⁵N-HSQC spectra
106 of both R3 and R3-IVVI at variable pressures up to 2.5 kbar show extensive chemical shift changes due
107 to pressure (Figure 2). Shift changes of this magnitude are common (Kitahara et al., 2013), and as is
108 normally observed, most N and HN nuclei move to higher resonance frequencies at elevated pressure.
109 For most amide groups, compression results in an increased polarization of the H-N bond, causing the
110 chemical shifts of HN and N to move to higher frequencies as the amide proton becomes more
111 deshielded and the amide nitrogen becomes more shielded. In most proteins, the majority of residues
112 show approximately linear pressure-dependent chemical shift changes, whilst a few residues have
113 curved quadratic shapes. However here, some resonances have very unusual curved pressure titration
114 data (Figure 2 inset), indicating an unusually strong pressure dependence, particularly in R3. The
115 curvature strongly suggests that an alternative conformation is present with a high population. Since it is
116 widely agreed that R3 is in equilibrium between closed and open states, we identify this alternative
117 conformation as the open state. The R3 talin domain thus provides an interesting test case for analysis
118 of pressure-dependent data. Chemical shifts at variable pressure were also measured for C α , C β and C'
119 nuclei, using 2D HN(CO)CACB and 2D HNCO spectra. Most nuclei were in fast exchange at all
120 pressures, although a few C α and C β nuclei broadened at higher pressures. Large-scale conformational
121 exchange typically slows at high pressures (Williamson, 2015), implying that this broadening is due to
122 pressure-dependent conformational exchange. This result implies a conformational exchange rate of
123 around 1-2000 s⁻¹ at ambient pressure, consistent with a large-scale hinge bending.

124 As a first step, the chemical shift vs pressure data were fitted to a quadratic expression, following
125 common practice (Kitahara et al., 2013). Neither R3 nor R3-IVVI gave a good fit to the data, with a large

126 number of both amide proton and nitrogen chemical shifts giving a poor fit (Figure S3). Mapping these
127 residues onto the structure gave no perceptible pattern (Figure S4A). We also tried fitting the data to a
128 cubic equation, this being the most obvious progression from a quadratic, as it includes the next term of
129 the Taylor expansion. The fit was much better, but there remained a significant number of peaks
130 systematically not fitting well (Figure S3), that again showed no obvious pattern of distribution when
131 mapped onto the structure of the protein.

132 The pressure-dependent chemical shifts were processed using SVD in order to reduce the
133 amount of experimental noise in the peak positions. The resulting singular values are plotted in Figure
134 3A for the combined fitting of the backbone amide NH and N signals of R3. Other data are similar. It is
135 clear that the first three singular values contribute to the spread of data, but it is not obvious whether the
136 fourth is also needed. In other words, at least three chemical shift components are needed for adequate
137 global fitting of the data. Inspection of the actual data shows that most residues are indeed fitted well by
138 only three components (in other words, they fit well to a quadratic equation), but that a small group are
139 not (eg F813 in Figure S3). One should also be able to tell how many components are necessary by
140 looking at the v_i vectors (Arai et al., 2012; Henry and Hofrichter, 1992): essential components should
141 have vectors with smooth shape and high autocorrelation. The first five vectors are shown in Figure 3B,
142 again indicating that the first three components are clearly needed, but not defining clearly whether the
143 fourth is also necessary. The data suggest that no more than four are needed.

144 We have already seen (Figures S3 and S4) that many resonances do not fit well to a quadratic
145 expression, which has three variables, but most fit reasonably well to a cubic equation with four variables.
146 Our proposed chemical shift equation (Eq. 2) has four components. We therefore set all singular values
147 with rank higher than 4 to zero and re-calculated a high-quality 'noise-free' data set D'.

148

149 **Fitting of R3 chemical shift changes to Eq. 2**

150 The column vectors of U' represent the chemical shifts of each species present, but not in a way that
151 normally allows the shifts to be extracted (Ikeda et al., 2011). It is thus common practice to take the data

152 from the SVD analysis, and go on to fit this 'noise-free' data to physically reasonable models in a
153 conventional way (Henry and Hofrichter, 1992). Eq. 2 contains two global variables (ΔG , the difference
154 in free energy between the ground state and the excited state; and ΔV , the difference in volume between
155 these two states) together with four resonance-specific variables (δ_1^0 , $\Delta\delta_1$, δ_2^0 , and $\Delta\delta_2$, discussed in
156 detail below). We first obtained fits for the global variables ΔG and ΔV , using the nuclei most sensitive to
157 pressure-dependent population changes, ie the HN, N and C' nuclei that gave the largest χ^2 values when
158 a quadratic expression was fitted to the 'noise-free' chemical shift vs pressure data (Figure S4).
159 Simultaneous fitting to Eq. 2 for these nuclei resulted in a robust global fit for ΔG and ΔV of -1.6 ± 27 J
160 mol^{-1} and -2.62 ± 0.53 $\text{kJ mol}^{-1} \text{ kbar}^{-1}$ respectively, equivalent to a volume difference of 26.2 ml mol^{-1} . The
161 volume change falls into the middle of the typical range (Kitahara et al., 2013), but the free energy
162 difference is very small, and implies that the ground state (closed conformation) and the excited state
163 (open conformation) have essentially equal populations at 1 bar. This is an unusual and very significant
164 result, because normally the high-pressure excited state is populated to only a few percent at ambient
165 pressure. In this work, we have identified an open conformation for R3, and measured its population.
166 The large population of the excited state provides a good explanation for the unusually curved pressure-
167 dependent chemical shifts.

168 ΔG and ΔV were then fixed, and the data for all nuclei were fitted to obtain the four resonance-
169 specific variables (δ_1^0 , $\Delta\delta_1$, δ_2^0 , and $\Delta\delta_2$, discussed in detail below). This is effectively the same number
170 of variables as used for fitting against a cubic equation. Whereas fitting to a cubic expression gives
171 patterns of residuals that clearly indicate systematic errors (Figure S3), fitting to Eq. 2 gives almost no
172 residual errors. The largest individual deviation was 0.070 ppm, and the overall root-mean-square
173 difference between experimental and calculated data was 0.0026 ± 0.0045 ppm. It is remarkable that
174 such unusual chemical shift patterns can be fitted so well by this simple equation (Figure 4). The fact that
175 the experimental shifts can be fitted so well by Eq. 2 is not conclusive proof that R3 must be following
176 the Boltzmann distribution described by Eq. 2, but does imply that Eq. 2 is a good model for the system.

177 Because Eq. 2 describes the simplest possible model comprising the least number of fitted variables that
178 is compatible with the data, we did not test other more complex models.

179

180 **Structure of R3 open conformation from fitted chemical shift values**

181 For each nucleus, fitting to Eq. 2 returns four variables: δ_1^0 , the chemical shift of the ground state at
182 ambient pressure; $\Delta\delta_1$, the linear pressure-dependent chemical shift change of the ground state; δ_2^0 , the
183 chemical shift of the excited state at ambient pressure; and $\Delta\delta_2$, the linear pressure-dependent chemical
184 shift change of the excited state. This represents a major increase in information content compared to
185 the standard fitting procedure using a quadratic expression, because the δ_1^0 and δ_2^0 fitted chemical shift
186 values provide detailed structural information about the closed and open forms. Significantly, these are
187 the chemical shifts of each form at ambient pressure, and can therefore be interpreted without the need
188 to take into account the effects of pressure on chemical shifts (Wilton et al., 2009). Chemical shifts are
189 powerful structural constraints, as indicated by the success of programs such as CS-ROSETTA (Shen et
190 al., 2008). The δ_1^0 and δ_2^0 chemical shift values for HN, N, C α , C β , and C' nuclei were used as input to
191 TALOS-N (Shen and Bax, 2013), which shows that the closed structure is consistent with the NMR
192 structure (2L7A) (Goult et al., 2013), and comprises a four-helix bundle with random coil termini (Figure
193 5). The open structure has all four α -helices remaining intact with only minor conformational differences
194 within the α -helices compared to the closed structure. There are some differences in the loops between
195 the α -helices, particularly between helices 1 and 2. However, the TALOS-N predictions are least
196 confident in the loops, and it is therefore not possible to calculate a detailed structure for the open
197 conformation using TALOS-N alone.

198 We therefore consider what information can be obtained from the differences in chemical shift
199 between the closed and open forms ($\Delta\delta = \delta_1^0 - \delta_2^0$). Of these, differences for C α and C β are the simplest
200 to interpret because they depend mainly on backbone dihedral angles, and to some extent on sidechain
201 dihedral angles and hydrogen bonding to the backbone (Iwadate et al., 1999), whereas HN, N and C' are

202 also strongly affected by hydrogen bonding to the amide group as a whole. The largest differences in $C\alpha$
203 and $C\beta$ chemical shifts (Figure 6) are mainly found for residues in helix 1, together with those at the N-
204 terminal end of helix 2 and in the turn between them, whilst smaller differences are noted for residues in
205 helices 3 and 4. This distribution of chemical shift differences suggests that in the open form, helix 1 has
206 detached from the rest of the bundle, where its main contact in the closed form is with the N-terminal end
207 of helix 2 (Figure 7). The chemical shifts of the open form do not allow us to calculate the angle between
208 helices 1 and 2. HN and N shifts (Figure S5A, B) support this conclusion, while the C' shifts are similar
209 but less useful (Figure S5C). Chemical shift differences for the random coil region at the N-terminus are
210 very small in all cases, showing that there is no change to the random coil structure, which provides a
211 useful internal control for the quality of the chemical shift analysis.

212 We have also analyzed the differences in pressure-dependent gradients between the closed and
213 open forms ($\Delta\text{gradient} = \Delta\delta_1 - \Delta\delta_2$) (Figures 8 and S6). The gradient reports on how the chemical shift of
214 the nucleus alters with increasing pressure, and is a measure of local compressibility (Kitahara et al.,
215 2013). $\Delta\text{gradient}$ values can therefore highlight regions where the compressibility has changed between
216 the closed and open conformations. Compressibility is determined to a large extent by surface exposure,
217 with large negative $\Delta\text{gradient}$ values indicating regions where the open structure (state 2) is more
218 compressible (which in general means more solvent-exposed). Examination of $\Delta\text{gradient}$ values for $C\alpha$
219 and $C\beta$ (Fig S5 C and D) shows that helices 1 and 2 have the largest changes, becoming generally more
220 exposed in the open state.

221 The chemical shifts of amide protons are strongly affected by hydrogen bonding (Williamson,
222 2013). The $\Delta\text{gradient}$ values therefore provide insights into hydrogen bonding in the open form. To
223 interpret the $\Delta\text{gradient}$ values, it is helpful to consider temperature-dependent shift gradients of amide
224 protons. Although in general non-hydrogen bonded HN have more negative gradients than hydrogen
225 bonded HN (Baxter and Williamson, 1997), strongly hydrogen bonded HN have a more negative gradient
226 than weakly hydrogen bonded HN, because these protons are highly deshielded by the hydrogen bond
227 and are therefore most affected by small changes in bond length (Tomlinson and Williamson, 2012). In

228 the same way, amide protons in strong hydrogen bonds have larger pressure-dependent shift gradients
229 than those in weak hydrogen bonds. Consequently, positive HN Δ gradient values indicate amide protons
230 that have short hydrogen bonds in the closed form, while negative Δ gradient values indicate amide
231 protons with shorter hydrogen bonds in the open form. The Δ gradient values are shown in Figure 7A.
232 Throughout the protein, but particularly in helices 1 and 2, the large negative Δ gradients tend to be on
233 the outer faces of helices while large positive Δ gradients tend to be on the inner faces. The closed NMR
234 structure is composed of four noticeably curved α -helices, and the data therefore indicate that following
235 the twisting out of helix 1 from the bundle, this helix becomes straighter as a consequence of being fully
236 solvated by bulk solvent in the open state. The loss of helix 1 from the bundle then allows the remaining
237 three helices to become somewhat straighter as the protein is solvated differently.

238

239 **Fitting of R3-IVVI chemical shift changes to Eq. 2**

240 Data for R3-IVVI were fitted in a similar way to that described for R3, although in this case using only HN
241 and N shifts, because the protein was ^{15}N labelled. As for R3, fitting the data to a quadratic equation
242 gives a poor fit (Figure S4B). However, the data again fitted well to the four-component model Eq. 2
243 (maximum deviation = 0.018 ppm, overall root-mean-square difference = 0.0011 ± 0.0017 ppm), but with
244 different global parameters: $\Delta G = 0.84 \text{ kJ mol}^{-1}$ and $\Delta V = -3.98 \text{ kJ mol}^{-1} \text{ kbar}^{-1}$ (39.8 ml mol^{-1}). Thus, the
245 free energy difference is larger for R3-IVVI, although still small (for example, compare 4.2 kJ mol^{-1} for
246 ubiquitin (Kitahara et al., 2005) and 11 kJ mol^{-1} for hamster prion (Kuwata et al., 2002)), and implies a
247 population of the excited state of 41% at ambient pressure. The higher energy of the excited state relative
248 to the ground state explains why the chemical shift changes are more typical and less dramatically curved
249 when compared to R3, while the smaller population explains why R3-IVVI binds more weakly to vinculin.
250 The volume change for R3-IVVI is larger than for R3, presumably as a consequence of a larger partial
251 molar volume (a larger volume of packing defects) in the closed state of the mutant R3-IVVI.

252 A detailed analysis of pressure-dependent chemical shift changes for R3-IVVI using the SVD
253 procedure gave similar results to those obtained for R3 (Figure S7). The chemical shift differences ($\Delta\delta =$

254 $\delta_1^\circ - \delta_2^\circ$) between ground and excited states followed a similar pattern to the R3 data, with the largest
255 changes found in helices 1 and 2 (Figure S8). The differences in pressure-dependent gradients
256 ($\Delta\text{gradient} = \Delta\delta_1 - \Delta\delta_2$) (Figure S9) also show a similar profile (Figure 8), implying an analogous
257 straightening out of helices in the open state of R3-IVVI. We therefore conclude that the primary
258 mechanism resulting in the fast conformational exchange observed at all pressures in solution between
259 the closed and open states for both R3 and R3-IVVI is the twisting out of helix 1 from the four-helix bundle
260 domain, followed by solvation of this helix together with a subtle change in packing of the remaining
261 three-helix bundle.

262

263 **DISCUSSION**

264 A number of publications have demonstrated the use of high pressure NMR spectroscopy to reveal
265 details of higher energy conformers that are in fast exchange with the ground state (Akasaka, 2006;
266 Kalbitzer et al., 2013; Kitahara et al., 2013). Such data are impossible to characterize by NMR
267 approaches under normal conditions since signals are averaged in this exchange regime. In this work,
268 we have introduced two methods that should prove generally useful to such analyses. The first is to make
269 use of singular value decomposition (SVD). Although SVD has been applied to NMR data before (Arai
270 et al., 2012; Jaumot et al., 2004; Matsuura et al., 2004; Sakurai and Goto, 2007), its use has been limited.
271 Two important applications of SVD are to determine the number of components required to fit the data,
272 and then to back-calculate a dataset with greatly reduced noise by zeroing everything apart from the
273 required components, thus providing a much more secure basis for fitting. SVD analysis can be carried
274 out using the widely available Matlab™, which makes it straightforward to do (Supplementary
275 Information).

276 Second, we fitted the data to an equation (Eq. 2), which models the observed pressure-dependent
277 chemical shift using two conformational states whose populations depend on pressure, and for which
278 both states can also undergo a linear pressure-dependent compression. The data fitted remarkably well
279 to this equation, and the fitting yielded parameters that reveal much more about the underlying structural

280 changes than when fitting to a quadratic expression. In particular, we showed that we can accurately
281 quantify the relative free energies of the two states and their populations. Despite the observed chemical
282 shifts being a population-weighted average of the two states, so that neither the pure closed nor open
283 state can be observed directly, the derived chemical shift values for the closed (δ_1°) and open (δ_2°) forms
284 can be used straightforwardly to determine the structures of these two states and chemical shift
285 differences ($\delta_1^\circ - \delta_2^\circ$) highlight where structural differences are located. In addition, the gradient
286 differences ($\Delta\delta_1 - \Delta\delta_2$) indicate the regions where solvent exposure changes on pressure perturbation.
287 This is likely to be a general result. An alternative approach was described recently (Erlach et al., 2014),
288 which provides estimates of the ratio of the difference in compressibility factors and partial molar
289 volumes. For more linear pressure-dependent shifts, that approach may prove more tractable.

290 It is important to emphasize that the high hydrostatic pressure used here is a tool to allow us to
291 characterize the closed and open states. It is not intended as a mimic of the force applied *in vivo*, and
292 hence is a general technique for characterizing any conformational change in proteins. We have
293 described two conformations of R3, that are populated almost equally under our conditions, and which
294 exchange rapidly on the NMR timescale. The application of force also induces a change between two
295 conformations, with a rapid transition. We propose that the two conformations that are seen by pressure
296 and the two conformations seen with force are the same. This proposal is strongly supported by the
297 observation that the conformational change from closed to open is the same under both conditions, as
298 discussed below. Pressure induces opening only of helix 1 (likely followed by the other three), whereas
299 force induces opening of all four helices. Pressure therefore reproduces only the first stage in the
300 unfolding induced by force, and thereby suggests the likely pathway for the force-induced unfolding.

301 This analysis was carried out on wild type and mutant forms of the R3 domain of talin. We showed
302 that wild type R3 is in equilibrium between two forms, each of which is populated 50% at ambient
303 pressure. State 1 (ground state) has higher partial molar volume and is identified as the fully folded form
304 of the four-helix bundle domain, as characterized previously by NMR (Goult et al., 2013). State 2 (excited
305 state) has a smaller volume and is a locally unfolded state. The difference in volume is 26 ml mol⁻¹, which

306 is a typical value for local unfolding but is small for complete unfolding of the entire domain (Royer, 2002).
307 TALOS-N shows that all four helices are still present. The nature of the structural change is shown most
308 clearly by analysis of the difference in C α and C β chemical shifts ($\delta_1^\circ - \delta_2^\circ$) between the two states, which
309 indicates that helix 1 is twisted out from the rest of the protein. Pressure-dependent exchange broadening
310 implies a conformational exchange rate of around 1-2000 s⁻¹, consistent with a hinge motion of this type.
311 Analysis of gradient differences ($\Delta\delta_1 - \Delta\delta_2$) indicates that all helices, but particularly helices 1 and 2,
312 become more linear (less curved) in the open state. R3-IVVI has similar structural changes, but has a
313 larger difference in free energy between closed and open states.

314 Previous studies have shown that RIAM binds to an exposed surface composed from helices 2
315 and 3 of R3 (Figure 9A), requiring very little conformational change in R3 (Goult et al., 2013). In contrast,
316 vinculin binds to the same two helices, but mainly to residues on the opposite face, which are buried in
317 the intact domain (Figure 9B) (Goult et al., 2013). Binding of RIAM and vinculin to R3 is mutually exclusive
318 (Goult et al., 2013). In agreement with this, it has been shown that binding vinculin requires the unfolding
319 of R3 (Goult et al., 2013; Roberts and Critchley, 2009). Indeed, a crystal structure of the vinculin head
320 bound to helix 3 of R3 shows that the single talin helix fits into a groove on the surface of the vinculin
321 head, in a conformation that would require complete separation of helix 3 from the rest of the domain
322 (Fillingham et al., 2005). Moreover, there is evidence that helices 2 and 3 of R3 compose independent
323 binding sites for vinculin, shown by analysis of vinculin binding assays to talin synthetic peptides (Gingras
324 et al., 2005), and by gel filtration showing formation of a 1:2 complex (Goult et al., 2013). The open
325 structure characterized here has the vinculin site on helix 2 exposed, but not well enough to fit neatly into
326 the vinculin groove. Finally, we note that pulling on an R1-R3 construct using magnetic tweezers was
327 interpreted to show unfolding of R3 at a force of about 5 pN (similar to the force expected from a single
328 actomyosin contraction), characterized by an extension of about 18 nm (Yan et al., 2015; Yao et al.,
329 2014). This distance corresponds approximately to the length of R3 when the four helices are opened
330 out completely, whilst retaining their helicity. In summary, a range of experiments suggest that R3 unfolds
331 into the four individual helices when interacting fully with vinculin, yet the results presented here show

332 only the opening out of helix 1, which serves to expose helix 2 yet not render it fully able to bind. We
333 therefore propose that the unfolding of R3 and its interactions with vinculin proceed in a stepwise manner
334 (Figure 9C): RIAM binding to folded R3 is reversible, force pulls helix 1 out from the bundle, which
335 exposes the binding site on helix 2 for vinculin, and thus allows helix 2 to interact with vinculin. Binding
336 of vinculin further opens out the domain, and exposes helix 3. Helix 3 is likely to be a stronger binding
337 site for vinculin than helix 2, as it is one of the three vinculin binding sites on talin identified in the original
338 screen (Bass et al., 1999). Binding of vinculin to helix 3 completely opens out the domain. This stepwise
339 mode of action allows for graduated conversion from RIAM-bound to fully vinculin-bound R3, and
340 provides scope for modulation of the interactions as required by the biological context.

341 This stepwise model agrees well with the energetics determined in this study. The previous
342 observations using magnetic tweezers (Yao et al., 2014) have shown that folded and unfolded forms of
343 R1-R3 are approximately equally populated at a stretching force of 5 pN, whereas the results here
344 suggest that the open and closed forms of R3 are equally populated in the absence of any stretching
345 force. Moreover, it was shown earlier (Goult et al., 2013) that R3-IVVI binds very weakly to vinculin,
346 suggesting a lower population of the open state than observed here. Both these observations are
347 reconciled by noting that our conclusions relate to the initial opening out of helix 1, and not to complete
348 opening of the whole domain, which requires a greater input of free energy.

349 Our data therefore indicate that in the isolated R3 domain (as studied here), the equilibrium
350 between open and closed conformations is delicately poised to allow environmental conditions the
351 maximum opportunity to alter the equilibrium position so as to stimulate closure (and therefore binding
352 to RIAM) or opening (and therefore binding to vinculin). In particular, it is expected that mechanical force
353 on the domain leads to the stepwise opening out of the domain with subsequent binding to vinculin
354 (Figure 9) (Calderwood et al., 2013). This behavior would allow the R3 domain to act as a
355 mechanosensitive switch, altering talin from an initial mode of recruitment by RIAM to the integrin tails,
356 to a functional role of recruiting vinculin to the focal adhesion complexes.

357

358 **Author Contributions**

359 NJB, TZ and MPW did the experiments; NJB, ILB and MPW wrote the manuscript. All authors have given
360 approval to the final version of the manuscript.

361

362 **Acknowledgements**

363 We thank Per Lincoln (Chalmers University, Sweden) for advice on SVD. We thank BBSRC for funding
364 (BB/J014966/1 to NJB).

365

366 **Supplemental Information**

367 Figures S1-S9, Table S1 with Document S1 (a justification of Equation 2).

368 Compressed file Data_S1.tar.zip. Fitted data δ_1° , δ_2° , $\Delta\delta_1$ and $\Delta\delta_2$ for each nucleus, related to Figures 4,
369 6, 8, S3, S4, S5, S6, S8, and S9.

370 This file gives the fitted values together with their associated errors and the overall χ^2 errors. It also
371 contains shift changes, both original data and post-SVD.

372

373 **References**

374 Akasaka, K. (2003). Exploring the entire conformational space of proteins by high-pressure NMR. *Pure*
375 *Applied Chem* 75, 927-936.

376 Akasaka, K. (2006). Probing conformational fluctuation of proteins by pressure perturbation. *Chem Rev*
377 106, 1814-1835.

378 Arai, M., Ferreon, J.C., and Wright, P.E. (2012). Quantitative analysis of multisite protein-ligand
379 interactions by NMR: Binding of intrinsically disordered p53 transactivation subdomains with the TAZ2
380 domain of CBP. *J Am Chem Soc* 134, 3792-3803.

381 Atherton, P., Stutchbury, B., Wang, D.-Y., Jethwa, D., Tsang, R., Meiler-Rodriguez, E., Wang, P., Bate,
382 N., Zent, R., Barsukov, I.L., *et al.* (2015). Vinculin controls talin engagement with the actomyosin
383 machinery. *Nature Comms* 6, 10038-10038.

384 Bass, M.D., Smith, B.J., Prigent, S.A., and Critchley, D.R. (1999). Talin contains three similar vinculin-
385 binding sites predicted to form an amphipathic helix. *Biochem J* 341, 257-263.

386 Baxter, N.J., and Williamson, M.P. (1997). Temperature dependence of ¹H chemical shifts in proteins. *J*
387 *Biomol NMR* 9, 359-369.

388 Calderwood, D.A., Campbell, I.D., and Critchley, D.R. (2013). Talins and kindlins: partners in integrin-
389 mediated adhesion. *Nature Rev Mol Cell Biol* 14, 503-517.

390 del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M., and Sheetz, M.P. (2009).
391 Stretching single talin rod molecules activates vinculin binding. *Science* 323, 638-641.

392 Erlach, M., Koehler, J., Moeser, B., Horinek, D., Kremer, W., and Kalbitzer, H.R. (2014). Relationship
393 between nonlinear pressure-induced chemical shift changes and thermodynamic parameters. *J Phys*
394 *Chem B* 118, 5681-5690.

395 Fillingham, I., Gingras, A.R., Papagrigoriou, E., Patel, B., Emsley, J., Critchley, D.R., Roberts, G.C.K.,
396 and Barsukov, I.L. (2005). A vinculin binding domain from the talin rod unfolds to form a complex with
397 the vinculin head. *Structure* 13, 65-74.

398 Gingras, A.R., Ziegler, W.H., Frank, R., Barsukov, I.L., Roberts, G.C.K., Critchley, D.R., and Emsley, J.
399 (2005). Mapping and consensus sequence identification for multiple vinculin binding sites within the
400 talin rod. *J Biol Chem* 280, 37217-37224.

401 Goult, B.T., Bate, N., Anthis, N.J., Wegener, K.L., Gingras, A.R., Patel, B., Barsukov, I.L., Campbell,
402 I.D., Roberts, G.C.K., and Critchley, D.R. (2009). The structure of an interdomain complex that
403 regulates talin activity. *J Biol Chem* *284*, 15097-15106.

404 Goult, B.T., Zacharchenko, T., Bate, N., Tsang, R., Hey, F., Gingras, A.R., Elliott, P.R., Roberts,
405 G.C.K., Ballestrem, C., Critchley, D.R., *et al.* (2013). RIAM and vinculin binding to talin are mutually
406 exclusive and regulate adhesion assembly and turnover. *J Biol Chem* *288*, 8238-8249.

407 Henry, E.R., and Hofrichter, J. (1992). Singular value decomposition: Applications to analysis of
408 experimental data. *Methods Enzymol* *210*, 129-192.

409 Ikeda, K., Yamaguchi, T., Fukunaga, S., Hoshino, M., and Matsuzaki, K. (2011). Mechanism of amyloid
410 β -protein aggregation mediated by GM1 ganglioside clusters. *Biochemistry* *50*, 6433-6440.

411 Iwadate, M., Asakura, T., and Williamson, M.P. (1999). C α and C β carbon-13 chemical shifts in
412 proteins from an empirical database. *J Biomol NMR* *13*, 199-211.

413 Jaumot, J., Marchan, V., Gargallo, R., Grandas, A., and Tauler, R. (2004). Multivariate curve resolution
414 applied to the analysis and resolution of two-dimensional ^1H , ^{15}N NMR reaction spectra. *Anal Chem* *76*,
415 7094-7101.

416 Kalbitzer, H.R., Rosnizeck, I.C., Munte, C.E., Narayanan, S.P., Kropf, V., and Spoerner, M. (2013).
417 Intrinsic allosteric inhibition of signaling proteins by targeting rare interaction states detected by high-
418 pressure NMR spectroscopy. *Angewandte Chemie Int Ed* *52*, 14242-14246.

419 Kitahara, R., Hata, K., Li, H., Williamson, M.P., and Akasaka, K. (2013). Pressure-induced chemical
420 shifts as probes for conformational fluctuations in proteins. *Progr Nucl Magn Reson Spectrosc* *71*, 35-
421 58.

422 Kitahara, R., Yokoyama, S., and Akasaka, K. (2005). NMR snapshots of a fluctuating protein structure:
423 Ubiquitin at 30 bar-3 kbar. *J Mol Biol* 347, 277-285.

424 Kitazawa, S., Kameda, T., Kumo, A., Yagi-Utsumi, M., Baxter, N.J., Kato, K., Williamson, M.P., and
425 Kitahara, R. (2014). Close identity between alternatively folded state N₂ of ubiquitin and the
426 conformation of the protein bound to the ubiquitin-activating enzyme. *Biochemistry* 53, 447-449.

427 Klapholz, B., Herbert, S.L., Wellmann, J., Johnson, R., Parsons, M., and Brown, N.H. (2015).
428 Alternative mechanisms for talin to mediate integrin function. *Current Biol* 25, 847-857.

429 Kuwata, K., Li, H., Yamada, H., Legname, G., Prusiner, S.B., Akasaka, K., and James, T.L. (2002).
430 Locally disordered conformer of the hamster prion protein: A crucial intermediate to PrPSc?
431 *Biochemistry* 41, 12277-12283.

432 Matsuura, H., Shimotakahara, S., Sakuma, C., Tashiro, M., Shindo, H., Mochizuki, K., Yamagishi, A.,
433 Kojima, M., and Takahashi, K. (2004). Thermal unfolding of ribonuclease T1 studied by
434 multidimensional NMR spectroscopy. *Biol Chem* 385, 1157-1164.

435 Noble, B., and Daniel, J.W. (1988). *Applied linear algebra*, 3rd edn (London: Prentice-Hall International).

436 Roberts, G.C.K., and Critchley, D.R. (2009). Structural and biophysical properties of the integrin-
437 associated cytoskeletal protein talin. *Biophys Rev* 1, 61-69.

438 Roche, J., Caro, J.A., Norberto, D.R., Barthe, P., Roumestand, C., Schlessman, J.L., Garcia, A.E.,
439 Garcia-Moreno E, B., and Royer, C.A. (2012). Cavities determine the pressure unfolding of proteins.
440 *Proc Natl Acad Sci USA* 109, 6945-6950.

441 Royer, C.A. (2002). Revisiting volume changes in pressure-induced protein unfolding. *Biochim Biophys*
442 *Acta: Protein Structure Mol Enzymol* 1595, 201-209.

443 Sakurai, K., and Goto, Y. (2007). Principal component analysis of the pH-dependent conformational
444 transitions of bovine beta-lactoglobulin monitored by heteronuclear NMR. *Proc Natl Acad Sci USA* *104*,
445 15346-15351.

446 Shen, Y., and Bax, A. (2013). Protein backbone and sidechain torsion angles predicted from NMR
447 chemical shifts using artificial neural networks. *J Biomol NMR* *56*, 227-241.

448 Shen, Y., Lange, O., Delaglio, F., Rossi, P., Aramini, J.M., Liu, G.H., Eletsky, A., Wu, Y.B., Singarapu,
449 K.K., Lemak, A., *et al.* (2008). Consistent blind protein structure generation from NMR chemical shift
450 data. *Proc Natl Acad Sci USA* *105*, 4685-4690.

451 Tomlinson, J.H., and Williamson, M.P. (2012). Amide temperature coefficients in the protein G B1
452 domain. *J Biomol NMR* *52*, 57-64.

453 Urbauer, J.L., Ehrhardt, M.R., Bieber, R.J., Flynn, P.F., and Wand, A.J. (1996). High-resolution triple-
454 resonance NMR spectroscopy of a novel calmodulin peptide complex at kilobar pressures. *J Am Chem*
455 *Soc* *118*, 11329-11330.

456 Williamson, M.P. (2013). Using chemical shift perturbation to characterise ligand binding. *Progr Nuclear*
457 *Magn Reson Spectrosc* *73*, 1-16.

458 Williamson, M.P. (2015). Pressure-dependent conformation and fluctuation in folded protein molecules.
459 In *High pressure bioscience*, K. Akasaka, and H. Matsuki, eds. (Dordrecht: Springer), pp. 109-127.

460 Wilton, D.J., Kitahara, R., Akasaka, K., and Williamson, M.P. (2009). Pressure-dependent ¹³C chemical
461 shifts in proteins: origins and applications. *J Biomol NMR* *44*, 25-33.

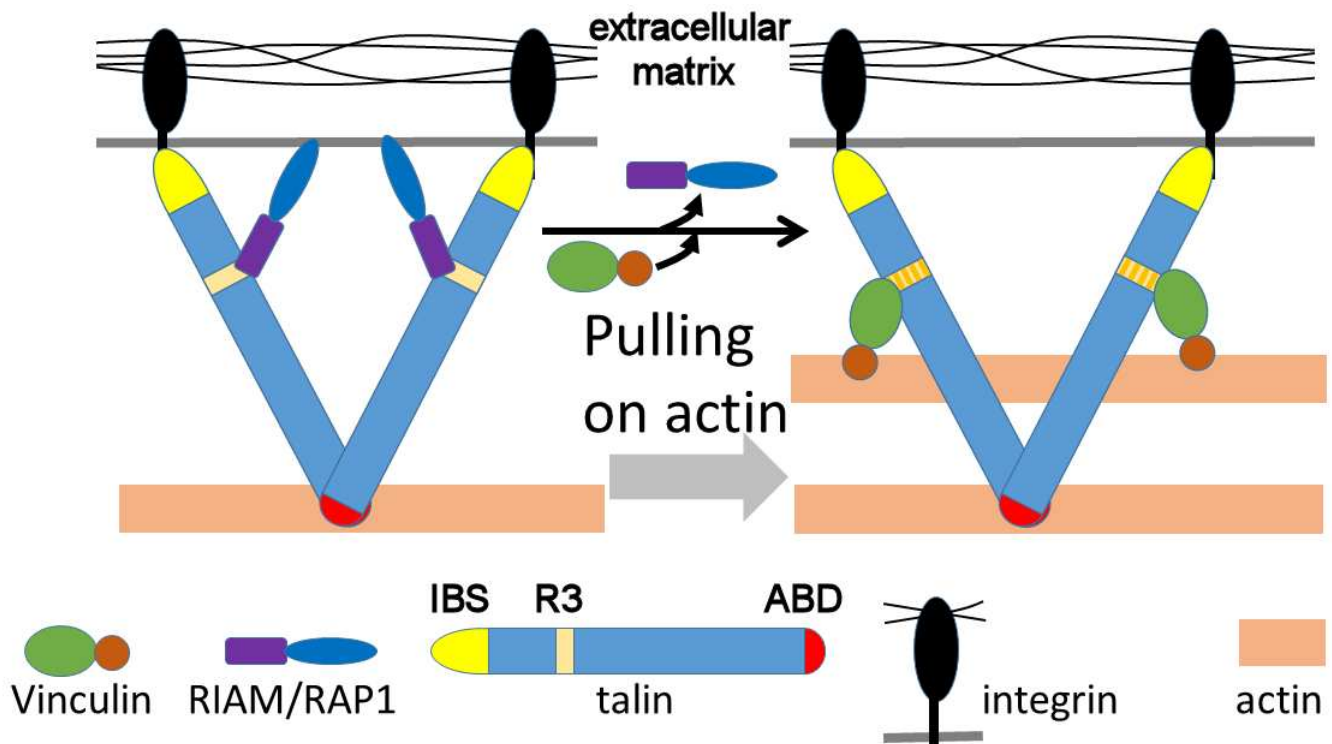
462 Yan, J., Yao, M., Goult, B.T., and Sheetz, M.P. (2015). Talin dependent mechanosensitivity of cell focal
463 adhesions. *Cell Mol Bioeng* *8*, 151-159.

464 Yao, M., Goult, B.T., Chen, H., Cong, P., Sheetz, M.P., and Yan, J. (2014). Mechanical activation of
465 vinculin binding to talin locks talin in an unfolded conformation. *Scientific Reports* *4*, 4610.

466 Yao, M., Goult, B.T., Klapholz, B., Hu, X., Toseland, C.P., Guo, Y., Cong, P., Sheetz, M.P., and Yan, J.
467 (2016). The mechanical response of talin. Nature Comms 7, 11966.

468

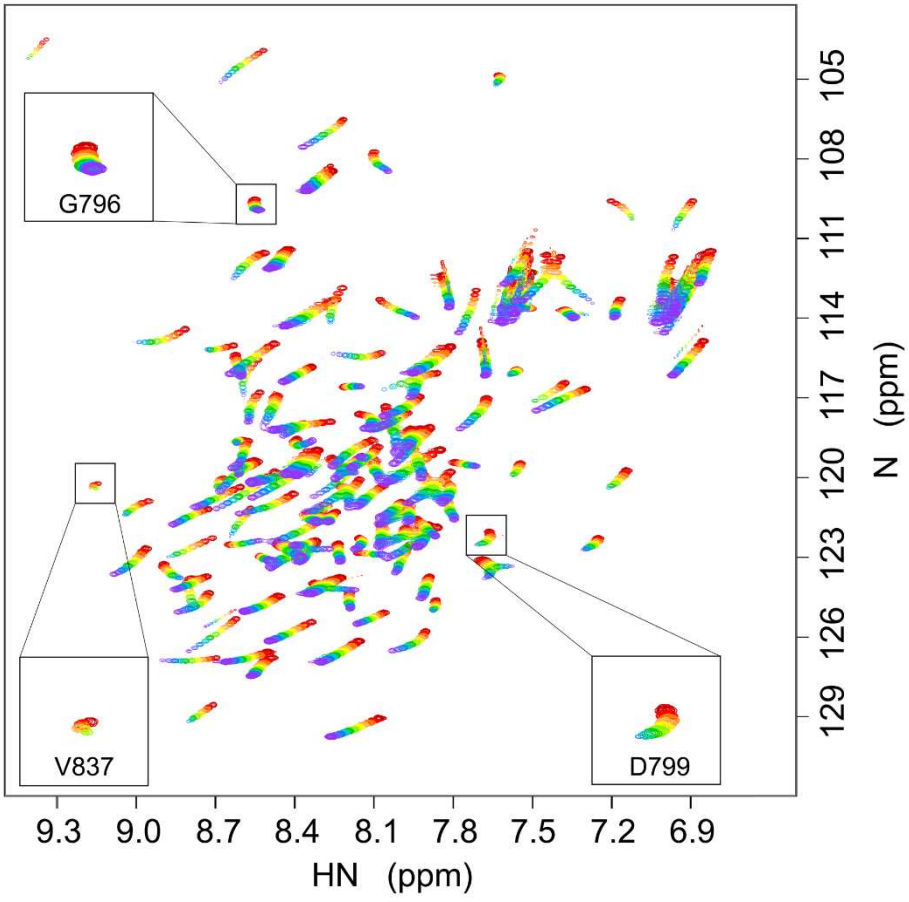
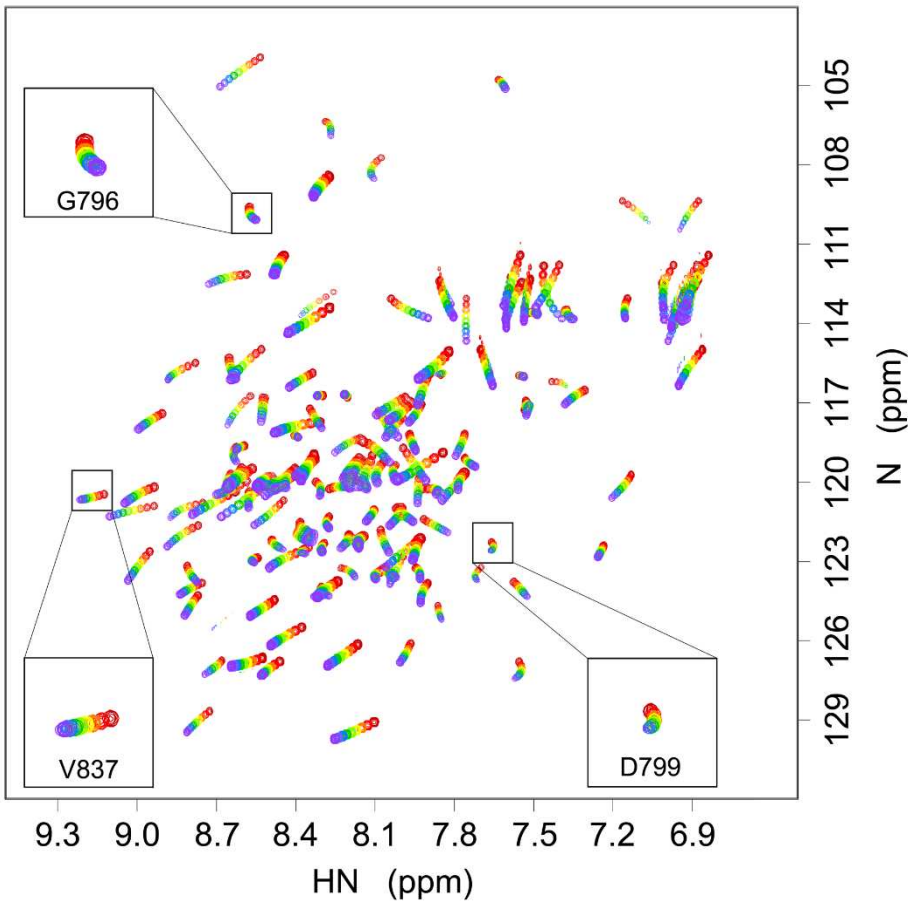
469 **Figures**



470

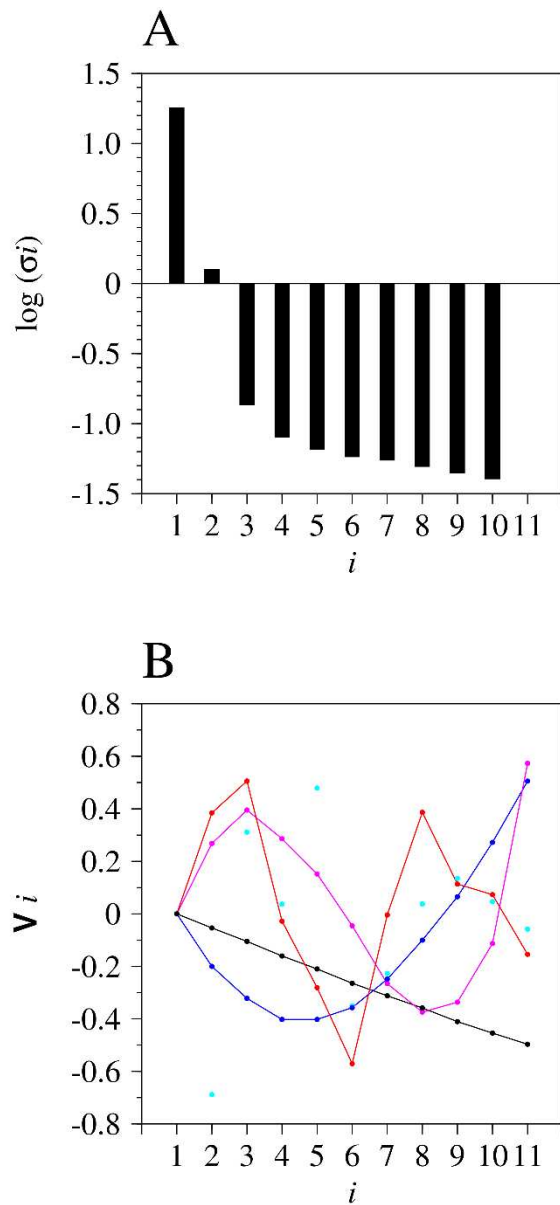
471 **Figure 1. Model for the role of talin in the formation of focal adhesion complexes.**

472 Talin is a long rod-like protein. One end contains an integrin binding site (IBS) while the other contains
473 an actin binding domain (ABD). At rest, RIAM binds to the closed R3 domain of talin and anchors it to
474 the cell membrane. When actin filaments are pulled, the R3 domain undergoes a conformational change
475 which causes RIAM to dissociate and vinculin to bind. Vinculin attaches talin to the actin cytoskeleton
476 and thereby stabilizes the focal adhesion complex. Figure adapted from Klapholz et al. (2015).

A**B**

478 **Figure 2.** ^{15}N -HSQC spectra of R3 and R3-IVVI acquired at pressures from 1 bar (red) to 2.5 kbar
479 (violet).

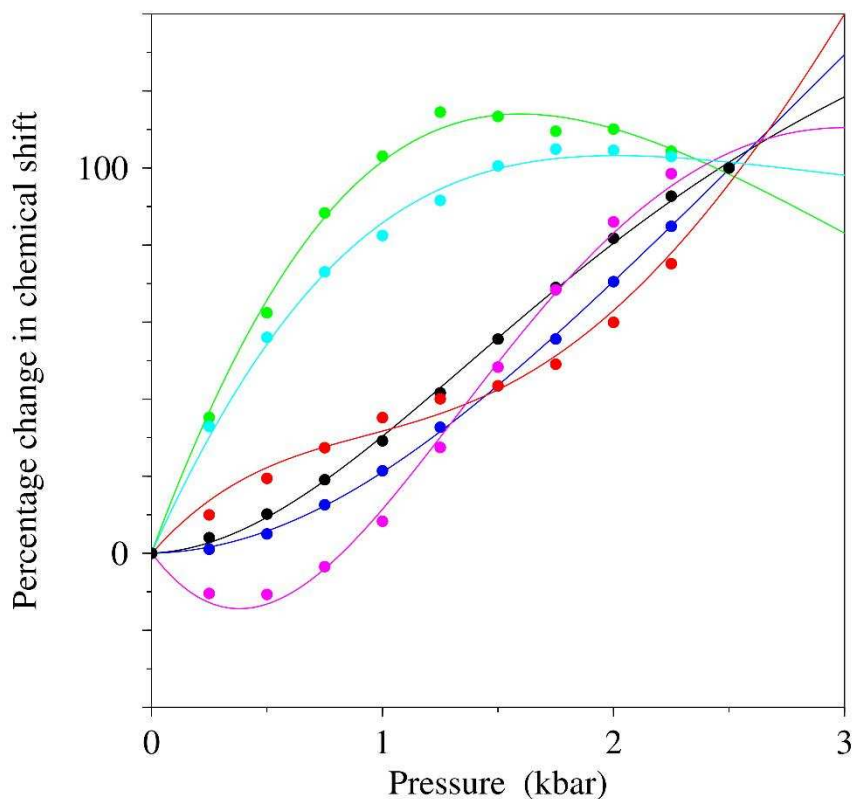
480 (A) R3 and (B) R3-IVVI. The insets show the pressure-induced changes in backbone amide peak position
481 for G796 and D799, positioned at the N-terminal end of helix 1, and V837 which is located at the center
482 of helix 2. These residues together with others show dramatic curvature for R3, whereas their behavior
483 is less curved for R3-IVVI. For a direct comparison of spectra of R3 and R3-IVVI, see Figure S1. For
484 chemical shift differences and their locations on the structure, see Figure S2.



485

486 **Figure 3. Analysis of SVD fitting.**

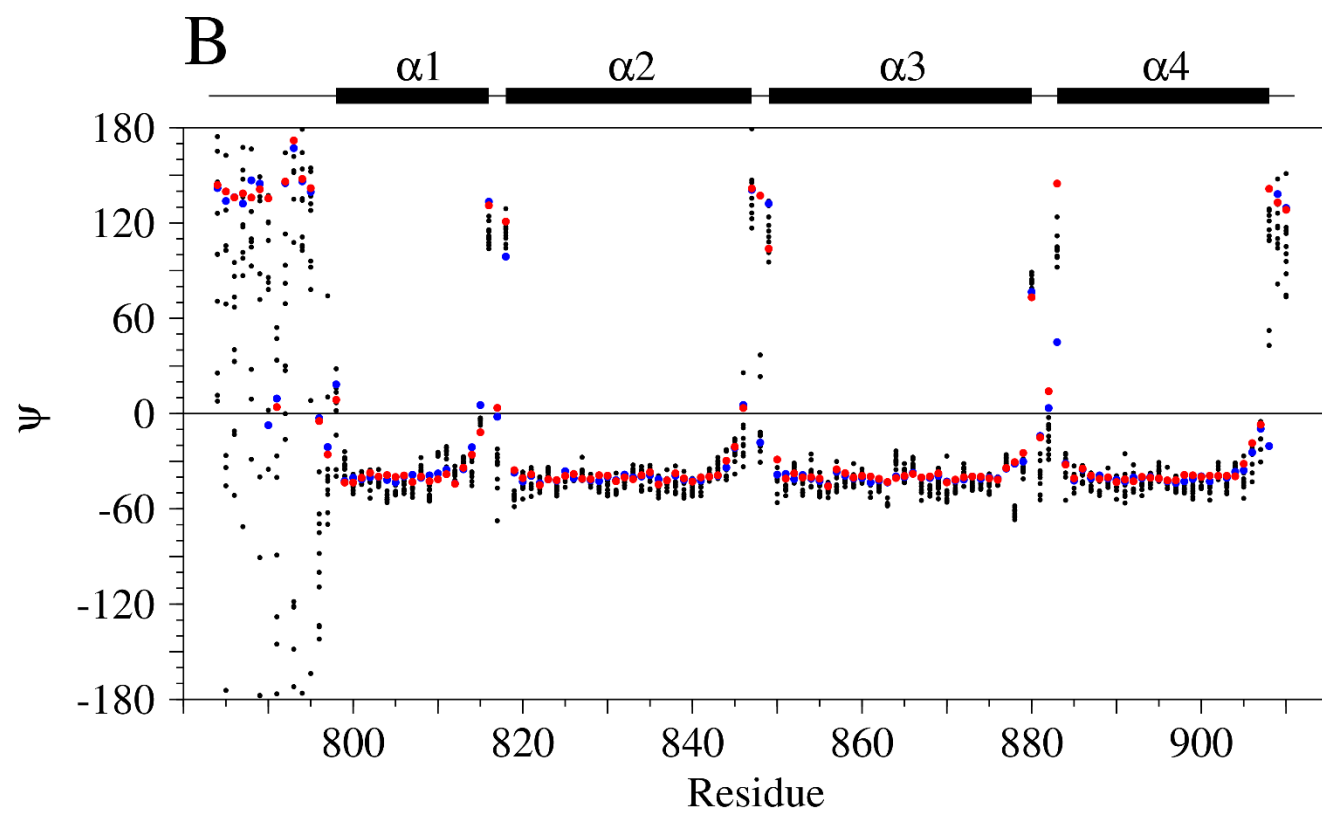
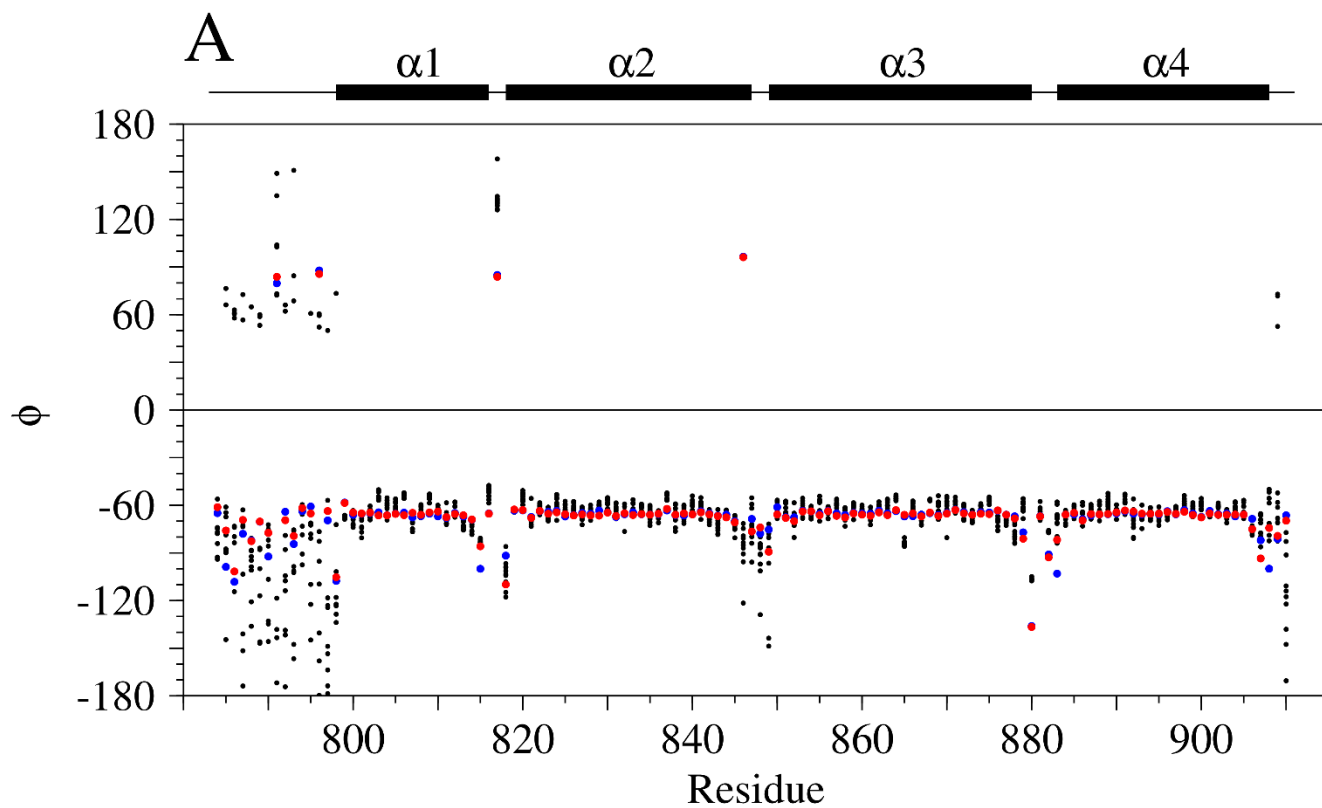
487 (A) Plot of $\log(\sigma_i)$ vs. i for the SVD combined analysis of backbone amide HN and N observed chemical
488 shift vs. pressure data for R3. The value of σ_{11} is 0. (B) Plot of the first 5 of the eleven column vectors
489 of \mathbf{V} . Vectors 1 to 4 are indicated by circles and lines colored black, blue, magenta and red, respectively
490 and vector 5 is shown as cyan circles. For fitting to more simple quadratic and cubic equations, and
491 locations of poorly fitting residues, see Figures S3 and S4. For the equivalent analysis of SVD fitting for
492 R3-IVVI see Figure S7.



493

494 **Figure 4. Examples of ‘noise-free’ chemical shift vs. pressure data for R3.**

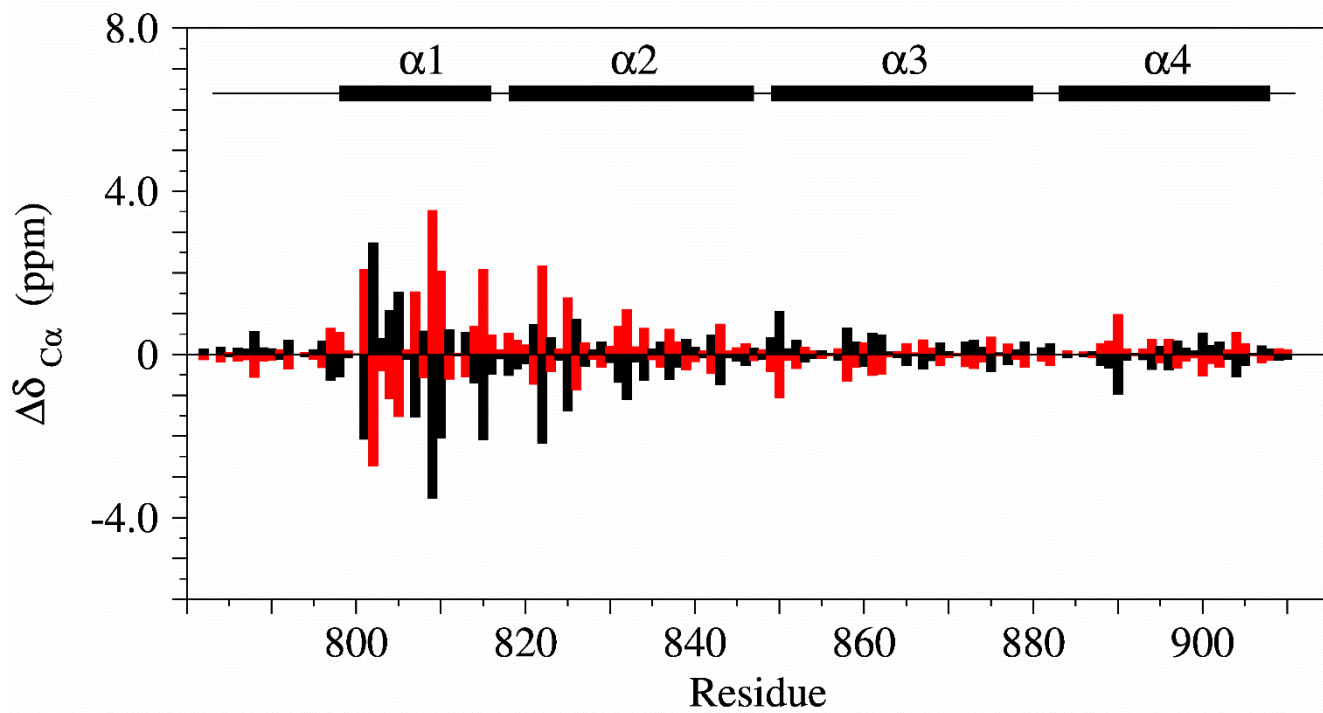
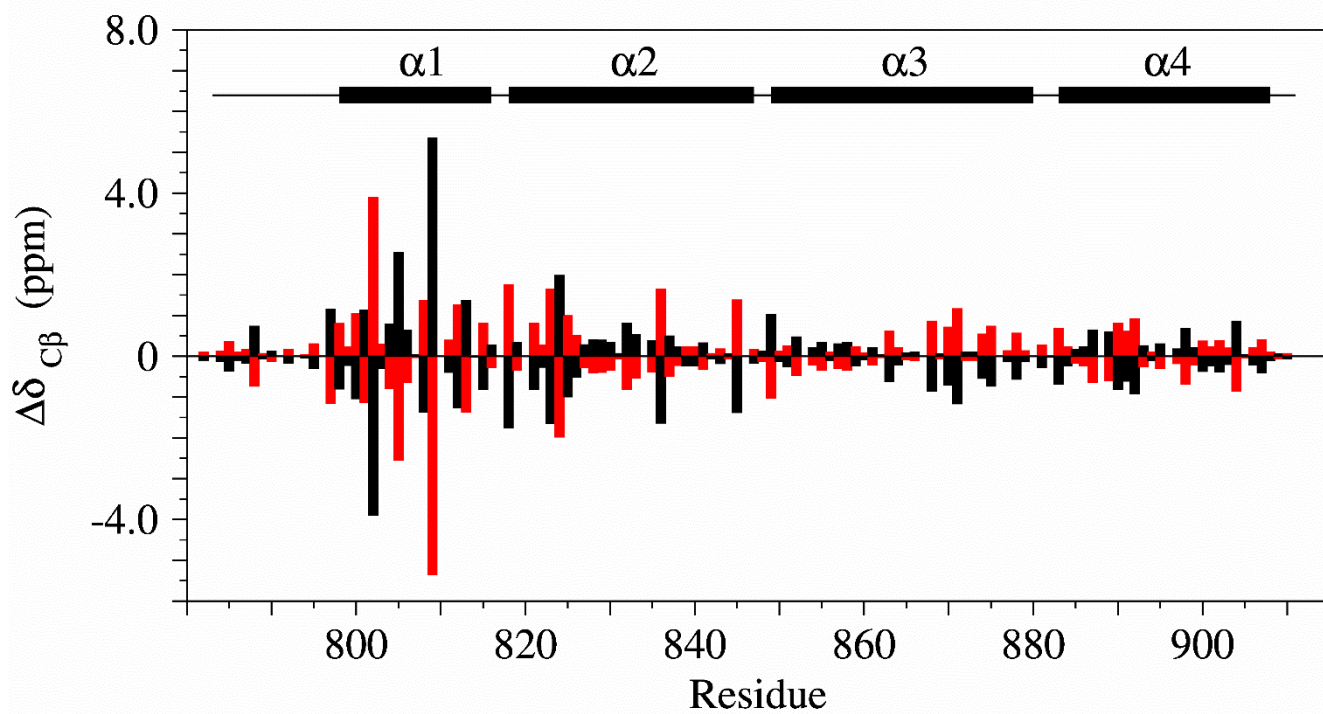
495 All curves are rescaled to a maximum chemical shift change of 100%, to illustrate the variable response
496 of specific nuclei to increasing pressure. Experimental data are indicated by circles, and the fits to Eq. 2
497 (fitted with a global ΔG and ΔV and resonance-specific δ_1° , δ_2° , $\Delta\delta_1$ and $\Delta\delta_2$ parameters) are shown by
498 lines: F813 HN (black), V823 N (blue), I828 N (green), R797 C' (red), T809 C' (magenta) and A877 C'
499 (cyan).



500

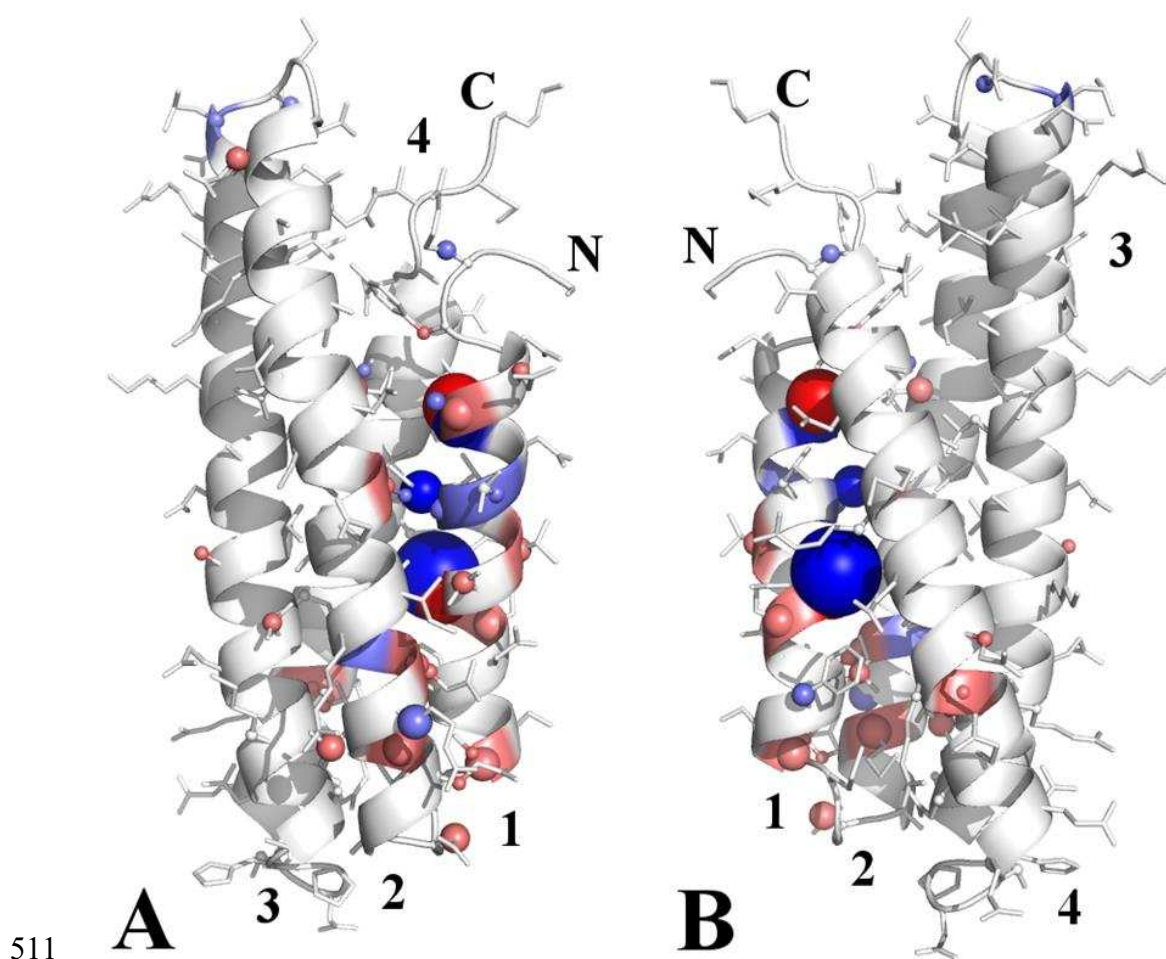
501 **Figure 5. TALOS-N predictions for backbone dihedral angles.**

502 (A) ϕ and (B) ψ dihedral angles of the ground state (closed conformation: blue circles) and the excited
503 state (open conformation: red circles), compared to the 10 lowest energy members of the 2L7A NMR
504 structural ensemble (black circles). The four α -helices are indicated.

A**B**

506 **Figure 6. Differences in chemical shift ($\Delta\delta = \delta_1^\circ - \delta_2^\circ$) between the ground state structure (closed**
507 **conformation) and the excited state structure (open conformation) of R3.**

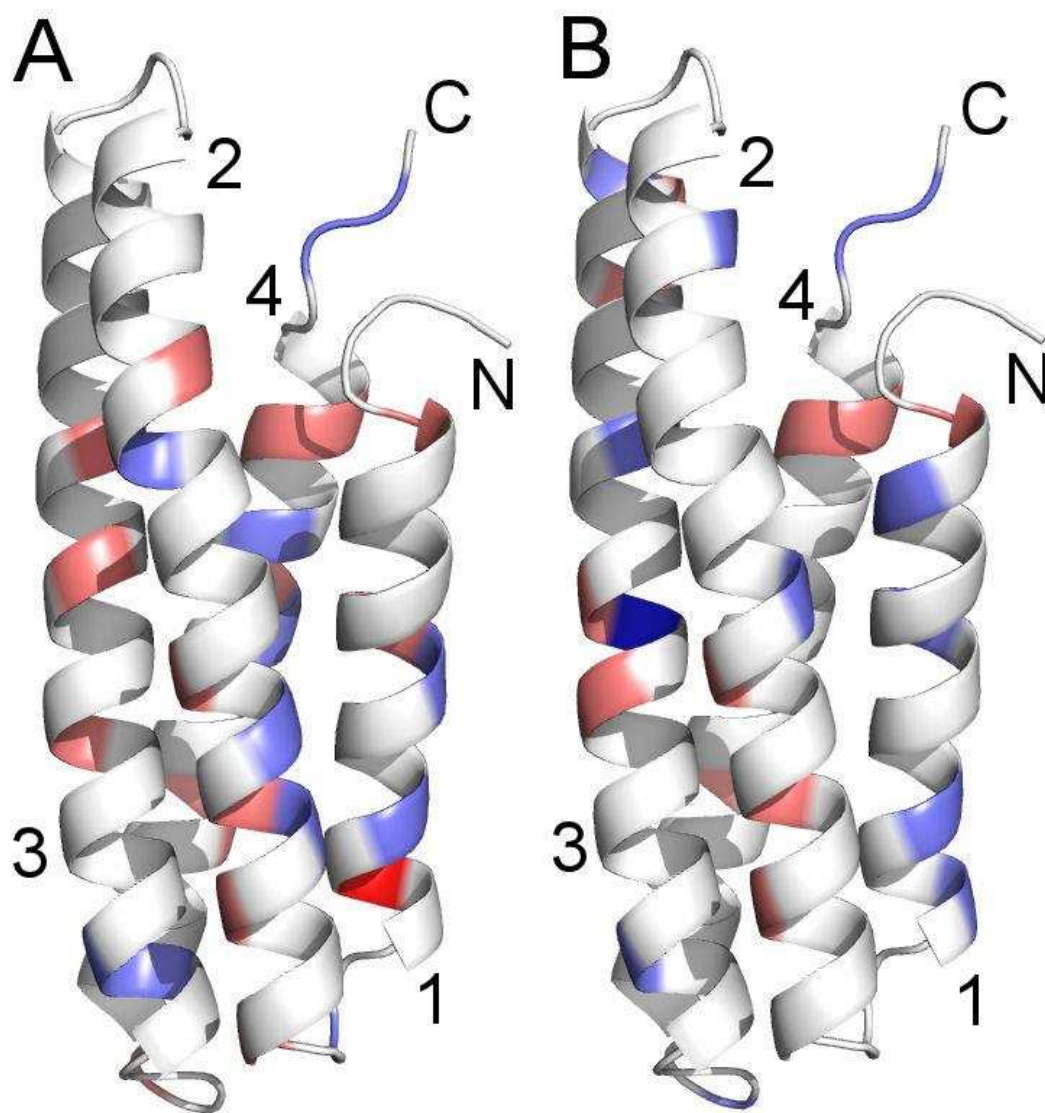
508 (A) $C\alpha$ nuclei and (B) $C\beta$ nuclei. The bars indicate relative chemical shift changes from δ_{obs} for δ_1° (ground
509 state: black) and δ_2° (excited state: red). The four α -helices are indicated. For corresponding differences
510 in HN, N and C', see Figure S5, and for the differences for R3-IVVI, see Figure S8.



512 **Figure 7. Differences in chemical shift ($\Delta\delta = \delta_1^\circ - \delta_2^\circ$) between the ground state (closed**
513 **conformation) and the excited state (open conformation) of R3.**

514 Differences are shown on the lowest energy NMR structure. The disordered N-terminus is not shown
515 and the cartoon depictions comprise residues A795 to K911. The N- and C-termini are indicated and
516 helices are numbered. (A) $\Delta\delta$ values for $C\alpha$ nuclei (colored backbone and spheres) and $C\beta$ nuclei

517 (spheres on sidechain sticks) with positive values in blue and negative values in red. Only differences >
518 1 standard deviation are indicated, with large differences (> 3 standard deviations) in deeper colors and
519 larger spheres. (B) Same as panel (A) except that R3 is rotated 180° about a vertical axis.

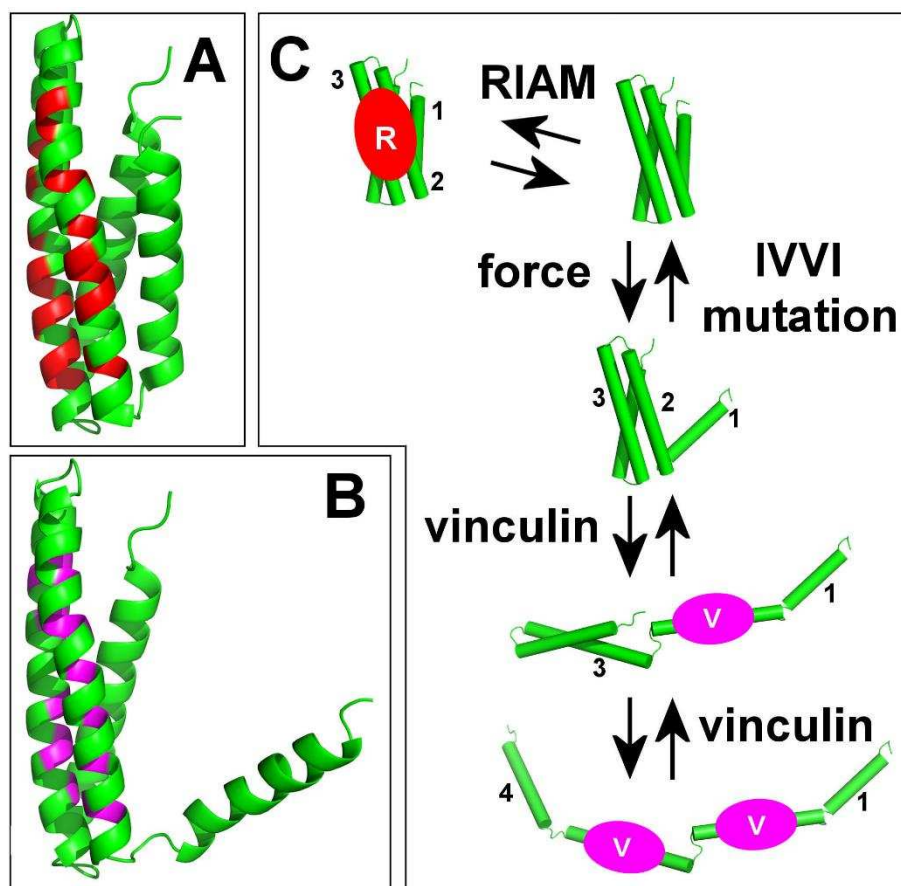


520

521 **Figure 8. Differences in pressure-dependent gradients ($\Delta_{\text{gradient}} = \Delta\delta_1 - \Delta\delta_2$) for HN nuclei**
522 **between the ground state (closed conformation) and the excited state (open conformation).**

523 (A) R3 and (B) R3-IVVI, shown on the lowest energy NMR structure. The disordered N-terminus is not
524 shown and the cartoon depictions comprise residues A795 to K911. The N- and C-termini are indicated
525 and helices are numbered. Δ_{gradient} values > 1 standard deviation are shown with positive values in

526 blue and negative values in red, with large differences (> 3 standard deviations) indicated as deeper
527 colors. For a graphical view of the gradient values for R3 and R3-IVVI respectively, see Figures S6 and
528 S9.



529

530 **Figure 9. Proposed mode of action of talin domain R3.**

531 (A) Binding site on R3 for RIAM in the closed state. (B) Binding site on R3 for vinculin in the open state.
532 (C) In the unactivated full-length protein, R3 is closed, and helices 2 and 3 form a binding site for RIAM,
533 which is able to bind reversibly to the closed domain. Force, provided by movement of the actin
534 cytoskeleton relative to talin, pulls helix 1 out from the bundle, exposing the binding site on helix 2 for
535 vinculin. This enables vinculin to bind, further opening out the bundle, which then exposes helix 3, forming
536 a second vinculin binding site and leading to complete opening of all four helices. The IVVI mutation
537 stabilizes the closed state and disfavors vinculin binding.

538

539

540 **STAR★ METHODS**

541 Detailed methods are provided in the online version of this paper and include the following:

542 Key resources table

543 Contact for reagent and resource sharing

544 Methods details

545 Expression and purification of protein

546 High-pressure NMR

547 Data analysis

548 Data and software availability

549

550 **ABBREVIATIONS**

551 R3, domain R3 of talin; R3-IVVI, T809I/T833V/T867V/T901I mutation of R3; RIAM, Rap1-GTP-interacting

552 adaptor molecule; SVD, singular value decomposition

553

554

STAR★ METHODS

555 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>E. coli</i> BL21 STAR (DE3)	Thermo Fisher	Cat #C601003
Chemicals, Peptides, and Recombinant Proteins		
¹⁵ NH ₄ Cl	Sigma Aldrich	Cat # 299251
¹³ C ₆ -glucose	Sigma Aldrich	Cat # 389374
TEV protease	Recombinant-In house	N/A
Hi-Trap QFF	GE-healthcare	Cat #17-5053-01
TCEP	Sigma Aldrich	Cat # C4706
TSP	Sigma Aldrich	Cat # 269913
Deposited Data		

R3-IVVI assignments	BioMagResBank	26880
Experimental Models: Recombinant DNA		
pET151/D-TOPO plasmid	Goult et al. 2013	
Software and Algorithms		
SVD	Matlab	
TALOS-N	Shen & Bax, 2013	https://spin.niddk.nih.gov/bax/software/TALOS-N/

556

557 **Method details**

558 **Expression and purification of protein**

559 Wild-type mouse talin fragment R3 (residues 787-911) and R3-IVVI mutant (T809I/T833V/T867V/T901I)
560 were previously cloned into pET151/D-TOPO expression vector (Invitrogen), encoding an N-terminal
561 hexa-histidine tag (Goult et al., 2013). Recombinant proteins were expressed and purified as described
562 previously (Goult et al., 2009). Briefly, protein was produced in *E. coli* strain BL21 STAR (DE3) cultured
563 in LB or 2xM9 minimal medium containing 1 g/L ¹⁵NH₄Cl and 4 g/L glucose or 2 g/L ¹³C₆-glucose. Cells
564 were grown at 37°C to an OD₆₀₀ of 0.6, cooled to 18°C and induced using 0.5 mM IPTG for 16 hours.
565 His-tagged protein was purified by nickel-affinity chromatography following standard protocol and the tag
566 was removed by cleavage with TEV protease, followed by the reverse purification. Protein was further
567 purified using anion exchange chromatography using a 5 ml Hi-Trap QFF column (GE Healthcare).
568 Protein concentrations were determined using absorbance at 280 nm and calculated extinction
569 coefficients.

570

571 **High-pressure NMR**

572 Samples (300 μl) contained 1 mM R3 or R3-IVVI and were dissolved in 20 mM sodium phosphate pH
573 6.5, 150 mM NaCl, 0.5 mM TCEP in 10% ²H₂O/90% H₂O and placed in a ceramic tube connected to a
574 high-pressure pump (Daedalus Innovations) (Urbauer et al., 1996). Spectra were obtained at 298 K on
575 an 800 MHz Bruker Avance I spectrometer equipped with a room-temperature probe (R3) or on a 600
576 MHz Bruker DRX spectrometer fitted with a cryoprobe (R3-IVVI), at regularly spaced pressures up to 2.5

577 kbar. For R3, 2D ^{15}N -HSQC, 2D HN(CO)CACB and 2D HNCOSY spectra were acquired every 250 bar from
578 1 bar to 2.5 kbar, while for R3-IVVI, 2D ^{15}N -HSQC spectra were acquired every 310 bar from 1 bar to
579 2.48 kbar. Proton chemical shifts were referenced to 3-trimethylsilyl-2,2,3,3-($^2\text{H}_4$) propionate (TSP, Sigma
580 Aldrich) at 0.0 ppm. ^{15}N and ^{13}C chemical shifts were calculated relative to TSP by use of the
581 gyromagnetic ratios of ^{15}N , ^{13}C and ^1H nuclei ($\gamma(^{15}\text{N})/\gamma(^1\text{H}) = 0.101329118$ and $\gamma(^{13}\text{C})/\gamma(^1\text{H}) =$
582 0.251449530). Peaks were picked using Felix (Felix NMR Inc., San Diego, CA) and exported to text files.
583 R3 backbone assignments at ambient pressure were taken from Goult et al., 2013 and were confirmed
584 using 3D HNCACB, 3D HNCOSY and 3D HN(CA)COSY spectra at ambient pressure. Backbone assignments
585 for R3-IVVI at ambient pressure were obtained using standard triple-resonance methods and have been
586 deposited in BioMagResBank (<http://www.bmrb.wisc.edu/>) under the BMRB accession code 26880.

587

588 **Data analysis**

589 SVD was carried out using MatlabTM. SVD is a well-established technique for factorizing matrices, related
590 to principal component analysis, and is used extensively in signal processing and statistics (Henry and
591 Hofrichter, 1992; Noble and Daniel, 1988). It is reported to be the least biased way of extracting the
592 meaningful data from an original overdetermined set containing experimental noise (Henry and
593 Hofrichter, 1992). Any real $p \times q$ matrix D can be factorized as

$$594 \quad D = UWV^T$$

595 where U is a $p \times p$ unitary matrix, V is a $q \times q$ unitary matrix and V^T is its transpose, and W is a $p \times q$
596 diagonal matrix, whose diagonal elements σ_i are real and non-negative. The σ_i are called the *singular*
597 *values* of D , and are normally presented in descending numerical value. The number n of non-zero σ_i
598 defines the rank of the matrix, ie the number of independent components (here, the number of
599 independent molecular species whose chemical shifts are required in order to fit the data). In practice,
600 only a few of the σ_i have large values, with the remainder having values which are small but not exactly
601 zero, since they arise from uncorrelated noise in the experimental data. It is therefore possible to set all

602 the randomly near-zero σ_i to zero, leaving a reduced W' as a $n \times n$ diagonal matrix, and at the same time
603 reduce U and V to only n columns. The resulting D' matrix, calculated from $D' = U'W'V'^T$ is thus a 'noise-
604 free' version of D , in which only correlated noise remains. SVD is therefore very useful, for (a) deriving
605 the rank of D , ie the minimum number of independent components required to generate the chemical
606 shift changes observed, and (b) removing most of the noise from the data, therefore improving
607 subsequent fitting of the data.

608 The experimental data set was factorized to yield the U , W and V matrices. This analysis was
609 carried out separately for amide proton and amide nitrogen chemical shifts, and also for both sets
610 combined. The fitting was of similar quality for all three sets, as were the results, and therefore we report
611 the use of combined HN and N shifts, because a global analysis of all chemical shift values together
612 should provide the most robust fitting (Arai et al., 2012). In particular, the noise on each individual point
613 is reduced in an ideal case by $pq^{-1/2}$, which here is approximately a factor of 50 when combining ^1H and
614 ^{15}N shifts together (Henry and Hofrichter, 1992). The raw data D consisted of separate lists of backbone
615 ^1HN , ^{15}N , $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $^{13}\text{C}'$ chemical shift changes for R3, and backbone ^1HN and ^{15}N chemical shift
616 changes for R3-IVVI at each pressure. Analysis of the $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$ and $^{13}\text{C}'$ shifts was carried out
617 independently, because the number of residues with usable non-overlapped signals was different for
618 each group. Rather than using absolute chemical shift values, the experimental data were all input as
619 changes in chemical shift from the initial chemical shift value. SVD fitting of absolute shifts generates
620 one very large singular value (reflecting the starting chemical shift values) together with other much
621 smaller ones, whereas fitting of chemical shift differences generates singular values of a more similar
622 size. Subsequent least-squares fitting is then more robust (Henry and Hofrichter, 1992).

623 Peaks that showed evidence of intermediate exchange broadening in the 2D HN(CO)CACB
624 spectra ($\text{C}\alpha$ preceding T802, D803, I805, V808, E810, F813 and S814, and $\text{C}\beta$ preceding L806, V808,
625 T809, E810 and F813), together with a small number of peaks with very small chemical shift changes,
626 were removed from the analysis. Following the SVD, all components of U , W and V above rank 4 were
627 set to zero and used to calculate the 'noise-free' dataset D' . These chemical shift changes were rescaled

628 to have the same maximum shift change, to avoid biasing the fitting by a few resonances with very large
629 chemical shift changes. The rescaled shifts were fitted to Eq. 2 using a Levenberg-Marquardt non-linear
630 least-squares algorithm. Global ΔG and ΔV values (together with resonance-specific δ_1^0 , δ_2^0 , $\Delta\delta_1$ and $\Delta\delta_2$
631 parameters) were first obtained by fitting to Equation 2 using a subset of resonances which had the most
632 curved pressure-dependent chemical shift changes, based on the value of χ^2 when fitting the data to a
633 quadratic expression. The error in the fitted ΔG and ΔV values was estimated by using different residues
634 and nuclei for the fitting, and also by carrying out a Monte Carlo-type search, varying ΔG and ΔV and
635 calculating the goodness of fit of the data. Once reliable values of ΔG and ΔV had been obtained for this
636 subset of resonances, the values were fixed, and resonance-specific δ_1^0 , δ_2^0 , $\Delta\delta_1$ and $\Delta\delta_2$ were calculated
637 for all other resonances, after which the data were rescaled to the original values for subsequent analysis.
638 During the fitting for R3, it was observed that the values for δ_1^0 and δ_2^0 were in every case almost exactly
639 of the same magnitude but of opposite sign. This is a consequence of fitting the chemical shifts as
640 differences from the starting value, and of the very small absolute value of ΔG for R3 (ie close to 50%
641 populations of the two states at ambient pressure). In order to obtain more robust fits, δ_1^0 and δ_2^0 were
642 therefore restrained to be equal in magnitude but of opposite sign (for R3 only), thereby reducing the
643 number of fitted parameters by one. Backbone dihedral angles were calculated for the ground and
644 excited states using TALOS-N (Shen and Bax, 2013), using chemical shift values for HN, N, C α , C β , and
645 C' nuclei obtained from δ_1^0 and δ_2^0 , respectively.

646

647 A typical Matlab™ script for carrying out SVD analysis is as follows:

648 <arrange the data into a matrix n rows by m columns, where n is the number of peaks and m is the number of
649 titration points, and import it. For ¹⁵N-HSQC data, the list contains both H and N shifts>

650 d=data;

651 [u,s,v]=svd(d,0);

652 vsmall=v(:,1:4);

```
653 ssmall=s(1:4,1:4);
654 usmall=u(:,1:4);
655 temp=ssmall*vsmall';
656 noiseless=usmall*temp;
657 e=noiseless';
```

658 **Contact for reagent and resource sharing**

659 Further information and requests for resources and reagents should be directed to and will be fulfilled by
660 the Lead Contact, Mike Williamson (m.williamson@sheffield.ac.uk).

661

662 **SUPPLEMENTAL INFORMATION**

663 Figures S1-S9, Table S1 with Document S1 (a justification of Equation 2).

664 Compressed file Data_S1.tar.zip. Fitted data δ_1° , δ_2° , $\Delta\delta_1$ and $\Delta\delta_2$ for each nucleus, related to Figures 4,
665 6, 8, S3, S4, S5, S6, S8, and S9.

666 This file gives the fitted values together with their associated errors and the overall χ^2 errors. It also
667 contains shift changes, both original data and post-SVD.

668