Calcium-controlled conformational choreography in the N-terminal half of adseverin

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Adseverin is a member of the calcium-regulated gelsolin superfamily of actin-binding proteins. Here we report the crystal structure of the calcium-free N-terminal half of adseverin (iA1–A3) and the Ca$^{2+}$-bound structure of A3, which reveal structural similarities and differences with gelsolin. Solution small-angle X-ray scattering combined with ensemble optimization revealed a dynamic Ca$^{2+}$-dependent equilibrium between inactive, intermediate and active conformations. Increasing calcium concentrations progressively shift this equilibrium from a main population of inactive conformation to the active form. Molecular dynamics simulations of iA1–A3 provided insights into Ca$^{2+}$-induced destabilization, implicating a critical role for the A2 type II calcium-binding site and the A2A3 linker in the activation process. Finally, mutations that disrupt the A1/A3 interface increase Ca$^{2+}$-independent F-actin severing by A1–A3, albeit at a lower efficiency than observed for gelsolin domains G1–G3. Together, these data address the calcium dependency of A1–A3 activity in relation to the calcium-independent activity of G1–G3.
Adseverin (also known as scinderin) is a member of the gelsolin superfamily of Ca²⁺-dependent actin-binding proteins, which regulate cytoskeletal dynamics by severing, bundling and capping of actin filaments. The gelsolin superfamily groups a number of modular, evolutionary-related proteins that are composed of 3–6 globular gelsolin homology (GH) domains often fused to non-GH N- and/or C-terminal extensions. In humans, family members include gelsolin, adseverin, villin, supervillin, advillin, villin-like protein, flightless I and CapG (reviewed in ref. 3). Adseverin was first identified in chromaffin cells of bovine adrenal medulla, where it acts as an actin depolymerizing agent that disassembles the cortical network of actin filaments in response to bursts of intracellular calcium, leading to exocytosis of secretory vesicles.

Adseverin has also been shown to be involved in the regulation of osteoclastogenesis and thrombocytectomy, chordyctocytosis and odontoblastic differentiation, as well as in cisplatin resistance in bladder cancer. Recently, several studies revealed a role for adseverin in the proliferation of lung carcinoma and gastric cancer, as well as in cisplatin resistance in bladder cancer. Recently, several studies revealed a role for adseverin in the proliferation of lung carcinoma and gastric cancer, as well as in cisplatin resistance in bladder cancer.

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Here we investigated the structure, dynamics and calcium activation of A1–A3 by using a combination of X-ray crystallography, SAXS, molecular dynamics simulations (MDS) and ensemble optimization. We crystallized A1–A3 in the presence of EGTA, revealing a compact auto-inhibited structure similar to the conformational state observed in inactive full-length gelsolin. Unexpectedly, attempts to crystallize the active form of A1–A3 in the presence of 5 mM calcium led to a structure of Ca²⁺-bound A3 following protein degradation within the crystallization drop. Using SAXS-based atomic ensemble optimization, we were able to draw a comprehensive picture of the conformational landscape of A1–A3 and track Ca²⁺-induced conformational changes. Classical MDS provided insights into the calcium-induced destabilization of inactive A1–A3, and revealed the location of potential low-affinity calcium-binding sites. Finally, actin depolymerization assays showed that mutations that destabilize the inactive A1:A3 interface increase A1–A3 severing activity in calcium-free conditions, demonstrating the importance of this latch.

**Results**

**Calcium-free A1–A3 displays an inactive conformation.** Purified recombinant A1–A3, supplemented with 5 mM EGTA, crystallized in space group C2 and its structure was solved by molecular replacement using the structure of G1–G3 excised from inactive full-length gelsolin as the search model and refined to a resolution of 2.9 Å (Fig. 1a and Table 1). Each domain adopts the classic GH fold, which is organized around a central β-sheet running approximately parallel to the strands, and a shorter α-helix running approximately perpendicular to the strands. The domains adopt the compact arrangement characteristic of inactive gelsolin, with A1 sandwiched between A2 and A3. The structure appears to be relatively rigid, with B-factors indicating that flexibility is limited mainly to the extended A2A3 linker and the short α-helices of A1 and A2 (Supplementary Fig. 1a). In this inactive conformation, the long α-helix of A3 is kinked to enable the formation of the A1:A3 latch, resulting in the masking of the major actin-binding interface on A1. The inactive forms of A1–A3 (iA1–A3) and G1–G3 (iG1–G3) are highly similar with an overall root mean squared deviation (r.m.s.d.) in Cα positions of 1.19 Å over 337 aligned residues, highlighting the structural conservation between gelsolin and adseverin (Fig. 1b). It is noteworthy that contrary to adseverin, iG1–G3 is unstable in the absence of G4–G6, leading to an open conformation of G1–G3 in the presence of EGTA. The iA1–A3 crystal lattice is stabilized by a number of crystal contacts, including a relatively large packing interface of ~1,518 Å² (Supplementary Fig. 1b). Interestingly, comparisons with full-length gelsolin show that this interface largely buries the surfaces in iA1–A3 that bind the C-terminal half of the molecule, suggesting that the crystal environment partially mimics the inactive full-length protein (Supplementary Fig. 1c).
Calcium-bound structure of A3. Attempts to crystallize the active form of A1–A3 (aA1–A3) using a 5-mM CaCl₂ supplemented protein sample led to the appearance of a single crystal after ~ 80 days, from which a 1.8-Å data set was collected. Cell content analysis indicated that the asymmetric unit was too small to accommodate more than a single GH domain, and the structure was solved by molecular replacement using gelsolin domain G3 excised from the structure of full-length gelsolin in calcium-free conditions (PDB 1RGI). This finding was surprising given that homology with gelsolin suggests that in the active form, domains A2 and A3 interact through a Ca²⁺-stabilized interface, while A1 would be separated by the A1A2 linker¹⁸,2⁰, it would thus have been expected that degradation would lead to a structure of isolated A1, or a compact, Ca²⁺-stabilized structure of A2–A3, rather than the Ca²⁺-bound A3 structure obtained here.

The conformation of aA3 is highly similar to aG3, with a r.m.s.d. in Cα positions of 0.82 Å over 93 Cα atoms, and differs from inactive iA3 and iG3 by the position of the second β-strand and the straightening of the long α-helix (Fig. 1c,d). The structure is stabilized by Ca²⁺ coordination at the type II binding site, which involves interactions with Glu280 carbonyl, the carboxylates of Glu281 and Glu305, and three water molecules with bond distances ranging between 2.3 and 2.5 Å (Fig. 1c, inset, and Supplementary Fig. 1d). As previously noted for gelsolin, this Ca²⁺-bound conformation is incompatible with the formation of the A1–A3 latch (Fig. 1d), suggesting that calcium binding at A3 moves A1–A3 away from the inactive conformation through straightening of the A3 long helix resulting in the disruption of the A1:A3 interface¹⁸,²⁷.

Taken together, these results indicate that the Ca²⁺-induced conformational changes in adseverin mirror those observed in gelsolin, although in isolation iA1–A3 retains the inactive conformation that is lost by isolated Ca²⁺-free G1–G3. Furthermore, the structure of aA3 obtained by degradation of A1–A3 in the presence of calcium indicates that A3 may exist in a conformational state where it is distant from A1 and A2.

SAXS experiments. Next, we investigated the structure and calcium activation of A1–A3 using solution SAXS. Scattering profiles for A1–A3 were initially measured in the presence of 2 or 10 mM EGTA, and with increasing concentrations of CaCl₂ (10–500 μM of free Ca²⁺), at protein concentrations of 1, 2, 4 and 8 mg ml⁻¹ (Fig. 2a, curve series 1–4). The samples were aggregation free, as evidenced by the linearity of the Guinier region (Supplementary Fig. 2), with radii of gyration (Rg) ranging between 2.6 and 3.2 nm, and molecular weight estimates ranging from 35 to 42 kDa (Table 2), consistent with the theoretical value of 41.5 kDa. The Ca²⁺-induced changes in the scattering profiles were more pronounced at lower protein concentrations, which is to be expected as the Kds for the high-affinity calcium-binding sites are lower than the protein concentrations used in the experiment (Kd = 0.6 and 3 μM versus c = 24–192 μM). An
Table 1 | Data collection and refinement statistics for iA1–A3 and aA3.

|                  | iA1–A3 (PDB ID: 5A1K) | aA3 (PDB ID: 5A1M) |
|------------------|------------------------|--------------------|
| **Data collection** |                        |                    |
| Space group      | C2                     | I422               |
| Cell dimensions  | a, b, c (Å)            | 135.4, 65.3, 105.2 | 92.5, 92.5, 86.9 |
|                  | x, y, z (°)            | 90, 118.3, 90      | 90, 90, 90       |
| Resolution (Å)   | 59.6–2.9 (3.0–2.9)     | 35.5–1.8 (1.9–1.8) |
| **Refinement**   |                        |                    |
| Resolution (Å)   | 59.6–2.9 (3.0–2.9)     | 35.5–1.8 (1.9–1.8) |
| No. of reflections | 17,745 (2,818)        | 13,831 (2,615)    |
| R<sub>r</sub>/R<sub>free</sub> | 24.0 (30.2)/24.8 | 18.2 (20.4)/18.9 |
| No. of atoms     | Protein                | 5,402              | 815              |
|                  | Ligand/ion             | 0                  | 1                |
|                  | Water                  | 107                | 58               |
| B-factors        | Protein                | 61                 | 20               |
|                  | Ligand/ion             | 0                  | 17               |
|                  | Water                  | 51                 | 28               |
| r.m.s.d.         | Bond lengths (Å)       | 0.008              | 0.008            |
|                  | Bond angles (°)        | 1.120              | 0.980            |

r.m.s.d., root mean squared deviation.

**Figure 2 | SAXS experiments.** (a) SAXS profiles of A1–A3 in the presence of 10 mM EGTA (black spheres), 2 mM EGTA (red spheres) and with increasing calcium concentrations (10, 220, 390 and 490 μM CaCl₂, green, yellow, purple and pink spheres, respectively) measured at protein concentrations of 1, 2, 4 and 8 mg ml⁻¹ (curve series 1, 2, 3 and 4, respectively). Curve series (5) corresponds to a second calcium titration at 4 mg ml⁻¹ of protein using an increased range of calcium concentrations, in order to check for potential changes at very low (10 or 50 nM), as well as higher calcium concentrations (2 or 50 mM; Fig. 2a, curve series 5, and Table 2). These measurements, performed on a different synchrotron visit using a different protein preparation, showed Ca²⁺-induced changes that were similar to those observed in the previously acquired data (Fig. 2a, curve series 3, Supplementary Fig. 3a). All measured R<sub>r</sub> were, however, smaller by 1–2 Å, suggesting mild degradation of the sample (Fig. 2b and Table 2). To gain qualitative insights into the Ca²⁺-induced conformational changes, the data were plotted using the Kratky representation (Fig. 2c). The analysis revealed that A1–A3 is predominantly globular in the absence of calcium, resulting in a bell-shaped curve, and that addition of calcium leads to a more complex profile, suggesting modularity and significant interdomain flexibility<br>

**Ca²⁺-dependent activation of A1–A3.** In order to analyse Ca²⁺-induced changes detected by SAXS, we combined atomistic coarse-grained MDS with ensemble optimization (Supplementary Note 1), demonstrating that A1–A3 transitions from the inactive state (I) to a gelsolin-like active state (A) through two distinct intermediates (Int1 and Int2) characterized by the release of A1/A3 latch and subsequent loss of the A1:A2 interface, respectively (Fig. 3a). All available data could be well fitted using this approach (Fig. 3b), with a goodness of fit $\chi^2 < 2$ for $0.08 < Q < 5$ nm⁻¹ (Table 2), except for data measured in the presence of 50 mM free calcium ($\chi^2 = 2.05$), which was not analysed further. Data measured at 4 mg ml⁻¹ (Fig. 2a,
curve series 5), which showed mild signs of degradation, could, however, only be optimally fitted using ensembles in which the last disordered 15 C-terminal amino acids (residues 350–365) that belong to the A3–A4 linker and are intrinsically disordered could, however, only be optimally fitted using ensembles that include a deletion of the last 15–36 C-terminal amino acids (residues 350–365) that belong to the A3–A4 linker and are intrinsically disordered. This suggests that the transition to the active conformation is highly disfavoured with a population of nearly 20%. Analysis of A2/A3 interdomain distances reveals a small population of Int1 conformers sampling distances very close to the inactive state as the A1/A3 latch opens up (Fig. 3e and Supplementary Fig. 5a). The overall distribution of A2/A3 distances within the optimized ensemble is markedly different from that of the pool (Supplementary Fig. 5f). It is noteworthy that this significant increase in conformational dynamics and shift towards a main population of active conformers is apparent when using an EGTA-to-free calcium ratio of 20:1, the open state (Int2) becomes favoured over the active form (Int1) and Int2 rose to ~15% each, and active conformers increased to approximately 20%. Analysis of A2/A3 interdomain distances reveals a small population of Int1 conformers sampling distances very close to the inactive state as the A1/A3 latch opens up (Fig. 3e and Supplementary Fig. 5a). The overall distribution of A2/A3 distances within the optimized ensemble is markedly different from that of the pool (Supplementary Fig. 5f). It is noteworthy that this significant increase in conformational dynamics and shift towards the active conformation happens at a relatively low free calcium-to-protein ratio (0.1:1), which may be due to binding/unbinding of a single calcium ion to A1–A3 occurring much faster than the conformational changes it induces.

At ~500 μM of free calcium (calcium-to-protein ratio 5:1), which is enough to saturate the three conserved type II calcium-binding sites, the population of active conformers reaches a peak at ~50%, while the inactive form drops to 20%. Int1 and Int2 represent, respectively, 10 and 20% (Fig. 3c,f). This shift towards a main population of active conformers is apparent in the Rg distributions (Supplementary Fig. 4b,c), with the appearance of a broad peak centred at 3 nm. Interestingly, the selected active conformers preferentially sample relatively short distances between A1 and A2 compared with the pool distribution (similar to the G1–G2 distance in gelsolin-bound actin; Fig. 3f and Supplementary Fig. 5c,d,h), which may reflect a conformational optimization of the A1/A2 linker for actin binding. At even higher free calcium concentrations (2 mM, calcium-to-protein ratio 20:1), the open state (Int2) becomes favoured over the active conformation.

### Table 2 | SANS-derived parameters.

| EGTA/free calcium concentration | c (mg ml⁻¹) | MW (kDa) | Rg (nm) | Dmax (nm) | χexp |
|---------------------------------|------------|----------|---------|-----------|------|
| 10 mM EGTA                      | 1.00       | 40       | 2.64    | 9.0       | 0.915|
| 10 mM EGTA                      | 2.00       | 42       | 2.62    | 9.1       | 1.123|
| 10 mM EGTA                      | 4.00       | 39       | 2.65    | 9.1       | 1.524|
| 10 mM EGTA                      | 8.00       | 40       | 2.67    | 9.3       | 1.674|
| 2 mM EGTA                       | 1.00       | 39       | 2.61    | 9.1       | 0.833|
| 2 mM EGTA                       | 2.00       | 39       | 2.61    | 9.3       | 1.098|
| 2 mM EGTA                       | 4.00       | 39       | 2.69    | 9.1       | 1.198|
| 2 mM EGTA                       | 8.00       | 40       | 2.70    | 9.2       | 1.461|
| 10 mM Ca²⁺                     | 1.00       | 36       | 3.05    | 10.0      | 0.803|
| 10 mM Ca²⁺                     | 2.00       | 39       | 3.05    | 10.6      | 0.865|
| 10 mM Ca²⁺                     | 4.00       | 38       | 2.95    | 10.3      | 0.939|
| 10 mM Ca²⁺                     | 8.00       | 39       | 2.90    | 10.1      | 1.291|
| 220 μM Ca²⁺                    | 1.00       | 33       | 3.07    | 10.4      | 0.786|
| 220 μM Ca²⁺                    | 2.00       | 37       | 3.02    | 10.4      | 0.862|
| 220 μM Ca²⁺                    | 4.00       | 38       | 2.96    | 10.3      | 0.947|
| 220 μM Ca²⁺                    | 8.00       | 40       | 2.98    | 10.4      | 1.361|
| 380 μM Ca²⁺                    | 1.00       | 36       | 3.03    | 10.5      | 0.827|
| 380 μM Ca²⁺                    | 2.00       | 36       | 3.03    | 11.5      | 0.862|
| 380 μM Ca²⁺                    | 4.00       | 38       | 3.02    | 10.0      | 1.162|
| 380 μM Ca²⁺                    | 8.00       | 40       | 2.97    | 9.9       | 1.281|
| 490 μM Ca²⁺                    | 1.00       | 35       | 3.10    | 10.5      | 0.830|
| 490 μM Ca²⁺                    | 2.00       | 36       | 3.10    | 10.4      | 0.874|
| 490 μM Ca²⁺                    | 4.00       | 39       | 3.19    | 10.2      | 0.914|
| 490 μM Ca²⁺                    | 8.00       | 40       | 3.19    | 10.5      | 1.131|
| 19 mM EGTA                      | 4.00       | 38       | 2.56    | 8.8       | 1.386*|
| 49 mM Ca²⁺                      | —          | 38       | 2.57    | 8.9       | 1.323*|
| 175 μM Ca²⁺                    | —          | 38       | 2.60    | 8.7       | 1.343*|
| 550 μM Ca²⁺                    | —          | 39       | 2.91    | 9.7       | 1.006*|
| 2.3 mM Ca²⁺                     | —          | 43       | 3.16    | 10.9      | 0.947*|
| 50 mM Ca²⁺                      | —          | 43       | 3.61    | 12.3      | 1.262*|
| —                               | —          | 43       | 3.67    | 13.5      | 2.053*|

SANS, small-angle X-ray scattering.

*Optimal fitting of the data was obtained using ensembles that include a deletion of the last 15 C-terminal amino acids (residues 350–365) that belong to the A3–A4 linker and are intrinsically disordered in solution.
conformation (55 versus 5%), while the inactive conformation is reduced to 5% (Fig. 3c and Supplementary Fig. 4c). These observations are in line with the increase in the measured $R_g$ (3.6 nm; Table 2), and the predominance of the open state may explain the degradation of A1–A3 that led to crystallization of A3 (3.6 nm; Table 2), and the predominance of the open state may enable the detection of regions that display conformational sensitivity to calcium on fast timescales, as well as potential low-affinity sites, within reasonable simulation times (for example, ref. 33). For each system, we ran two independent simulations of 1 μs and 0.1 μs in the presence and absence of calcium, respectively. The protein structure was stable over the course of the simulations, with the r.m.s.d. from the starting structure stabilizing ~3–4 Å after an initial rise during the first 20 ns (Fig. 4a,b). Analysis of the root mean square fluctuations

MDS of calcium binding to inactive A1–A3. Ensemble optimization demonstrated a complex calcium-induced remodelling of A1–A3 conformational landscape. To gain insights into the initial events that shift the equilibrium towards the active state, we performed explicit solvent MDS of iA1–A3 in the presence of 50 mM CaCl$_2$ or 50 mM NaCl (Fig. 4). Although well above the physiological range, such high calcium concentrations enable the detection of regions that display conformational sensitivity to calcium on fast timescales, as well as potential low-affinity sites, within reasonable simulation times (for example, ref. 33). For each system, we ran two independent simulations of 1 μs and 0.1 μs in the presence and absence of calcium, respectively. The protein structure was stable over the course of the simulations, with the r.m.s.d. from the starting structure stabilizing ~3–4 Å after an initial rise during the first 20 ns (Fig. 4a,b). Analysis of the root mean square fluctuations

Figure 3 | Proposed mechanism of calcium activation of A1–A3 in solution. (a) Model for A1–A3 transition between the inactive and active (actin-binding competent) states. The release of the A1/A3 latch from the inactive form (I) leads to a first intermediate (Int1), followed by the loss of the A1A2 interface (Int2). Finally, the calcium-stabilized interface between A2 and A3 is formed, resulting in the active conformation (A). (b) Fitted SAXS profiles. For clarity, only a subset of the data shown in Fig. 2a is represented. Experimental data are shown as black spheres and theoretical SAXS profiles for optimized ensembles of 50 models are drawn as red lines. (1) 490 μM CaCl$_2$, 1 mg ml$^{-1}$ of A1–A3; (2) 490 μM CaCl$_2$, 2 mg ml$^{-1}$ of A1–A3; (3) 490 μM CaCl$_2$, 4 mg ml$^{-1}$ of A1–A3; (4) 49 nM, 175 and 550 μM, and 2.3 mM CaCl$_2$, 4 mg ml$^{-1}$ of A1–A3; (5) 490 μM CaCl$_2$, 8 mg ml$^{-1}$ of A1–A3; (6) 2 mM EGTA, 8 mg ml$^{-1}$ of A1–A3 mg ml$^{-1}$. (c) Relative populations of inactive (I, black), intermediate (Int, red and Int2, green) and active (A, blue) conformations of A1–A3, plotted as a function of free calcium concentration ($x$ axis, top). All data measured at 4 mg ml$^{-1}$ were used (Fig. 2a, curve series 3 and 5). Error bars represent the s.d. of ensemble optimization results obtained from 10 independent ensembles of 6,000 models (see Methods section). (d–f) Two-dimensional (2D) histogram representations of the distribution of A1–A2 and A2–A3 interdomain distances (taken between their centres of mass) within the optimized ensembles, using data measured in the presence of 2 mM EGTA (d), 10 μM (e) and 550 μM free Ca$^{2+}$ (f). The 2D histogram was calculated using a binning of 15 × 15 and coloured from red to blue (0–50 models).
indicated flexibility mainly in the linker regions, which was enhanced by calcium (Fig. 4c).

We identified bound calcium by analysing time-averaged distances between Ca$^{2+}$ and protein atoms (Fig. 4d). Ca$^{2+}$ clusters mainly around the conserved type II binding sites (Fig. 4d, inset 1, 2 and 5), as well as the A2A3 linker and the C-terminal disordered extension belonging to the A3A4 linker, in a manner that is consistent with the distribution of negative charges on the protein surface (Supplementary Fig. 7). Additional Ca$^{2+}$ is observed between the short $\alpha$-helices of A1 and A2, and their second and fourth $\beta$-strands, respectively (Fig. 4d, inset 3 and 4). These sites involve calcium coordination by Glu9 and Glu28 carboxylates, and the backbone oxygen of Leu29 in A1, and Glu219 and Glu173 carboxylates and the hydroxyl of Tyr175 in A2. These interactions create additional contacts between the short $\alpha$-helices and $\beta$-strands of A1 and A2, and...
may represent low-affinity-binding sites that stabilize the GH domains at high calcium concentrations.

Examination of Ca\(^{2+}\) dynamics at the high-affinity sites indicates that only the type II binding site at A2 can bind Ca\(^{2+}\) in the inactive conformation (Fig. 4d, inset 5), showing stable coordination of Ca\(^{2+}\) by Asp164 and Glu186 side chains, as well as Gly163 carbonyl oxygen. In contrast, binding at A1 is hindered by the interaction of Asp44 with Arg23 side chain and Val122 backbone nitrogen, resulting from the interaction with A2 (Fig. 4d, inset 1). Similarly, formation of the type II binding site in A3 is prevented by the kink of the long \(\alpha\)-helix that interacts with A2, resulting in distances between Glu305, Asp256 and Glu281 that are too large to coordinate calcium tightly (Fig. 4d, inset 2).

Next, we analysed the effect of calcium on the dynamics of the A2A3 linker, which is located close to the type II binding site at A2 (Fig. 5), and in actin-bound G1–G3 contacts calcium through Asp259 side chain (Asp238 in adseverin), thus providing additional stability to the active state\(^{20}\). In the absence of calcium, the extended A2A3 linker samples the conformational space in the vicinity of the crystal structure (Fig. 5a). In contrast, calcium perturbs the linker dynamics, leading to an initial increase in flexibility within the first 100-ns of simulation (Fig. 5b) followed by sampling of calcium-induced, non-native conformational states of the linker associated with calcium binding to the type II binding site in A2 and to the acidic patch of the A2A3 linker (Fig. 5a,d). The observation of calcium-induced changes in A2A3 linker dynamics suggests that this linker acts as a calcium-sensitive conformational switch that modulates the stability of the inactive, intermediate and active states.

**Impact of A1/A3 interface mutations on F-actin severing.** The detailed picture of the Ca\(^{2+}\)-dependent activation mechanism of A1–A3 brought by the combination of X-ray crystallography, SAXS and MDS explains its reported Ca\(^{2+}\)-dependent severing activity, which contrasts with the open conformation and Ca\(^{2+}\)-independent severing of actin filaments described for the equivalent fragment of gelsolin (G1–G3; refs 30,31). These differences prompted us to investigate the contribution of the stability of the A1/A3 latch to severing activity. To this end, two mutants of A1–A3 (Met310Asp and Glu314Ser) were designed that were aimed at disrupting hydrophobic interactions between Met310 and Phe64, and a salt bridge involving Glu314 and Arg97 at the A1/A3 interface (Fig. 6a). Interestingly, structural comparisons show that in gelsolin, the equivalent residue to Glu314 is a serine, resulting in loss of the salt bridge, while the Met310/Phe64 pair is replaced by Leu332/Tyr87, suggesting a potentially less stable G1:G3 interface (Fig. 6b).

F-actin depolymerization by A1–A3 and its mutants, as well as by G1–G3 was monitored by the loss of fluorescence of pyrene-labelled F-actin in the presence of 1 mM EGTA (Fig. 6c) or 500 \(\mu\)M CaCl\(_2\) (Fig. 6d). In the presence of Ca\(^{2+}\), all proteins showed a similar actin filament depolymerization pattern, with rapid loss of fluorescence. In contrast, in the presence of EGTA, A1–A3 and the M310D and E314S mutants showed reduced severing activity, while only G1–G3 severing activity remained robust, as expected from previous reports\(^{29,30}\). Interestingly, A1–A3 severing of F-actin was roughly 10 times slower than in the presence of Ca\(^{2+}\) (Fig. 6c,d). Although the M310D and E314S mutants showed increased activity compared with wild-type A1–A3, they remained much less active than G1–G3 in Ca\(^{2+}\)-free conditions, suggesting that the destabilization of the A1/A3 latch in the mutants may only partially shift the equilibrium towards the active state. In addition, M310D was slightly more active than E314S, indicating a larger contribution to destabilizing the interface, as may be expected based on the nature of the mutation (hydrophobic to charged versus charged to hydrophilic).

**Figure 5 | Effect of calcium on A2A3 linker dynamics as observed in MDS.** In each panel, the crystal structure of iA1–A3 is shown in grey cartoons for comparison with the A2A3 linker represented using a larger cartoon loop radius. (a) Linker dynamics in the absence of calcium. Ten overlaid snapshots of the protein taken at 10 ns of interval (10–100 ns) are shown in cartoon representation with the protein backbone coloured from blue to red (N- to C-terminus). (b-d) Linker dynamics in the presence of calcium, illustrating the increased flexibility and deviation from the crystal structure. Ten overlaid snapshots of the protein taken at 10 ns of interval were extracted from one of the two 1 \(\mu\)s MDS of iA1–A3 in the presence of calcium and are shown as rainbow-coloured cartoons (0–90, 400–490 and 900–990 ns in b-d respectively). In each case, bound calcium ions are represented in magenta spheres for one of the snapshots, with coordinating side chains as sticks (100 ns in b, 500 ns in c and 1,000 ns in d).

**Discussion**
The structural data presented here have revealed that isolated A1–A3 can adopt, in the crystal and in solution, an inactive conformational landscape of A1–A3 that is characterized by the formation of the A1/A3 latch as observed in calcium-free full-length gelsolin. Using SAXS and ensemble optimization, we have demonstrated that the conformational landscape of A1–A3 is composed of a...
heterogeneous mixture of dynamically exchanging conformers, where the inactive conformation coexists with activation intermediates and gelsolin-like active conformers in a 9:1 ratio in the absence of calcium. The addition of moderate (10 μM)-to-high (500 μM) calcium concentrations increased conformational exchange, resulting in a larger proportion of intermediate forms (30%), and progressively shifted the equilibrium towards the active form, up to a maximum of ~55%. These calcium concentrations correlate well with the ~50 μM peaks in subplasmalemmal calcium observed in adrenal chromaffin cells, which enable adseverin to depolymerize the cortical network of actin filaments. Interestingly, we found that higher calcium concentrations (2 mM, close to blood plasma calcium concentration of 1.0–1.5 mM) induced further expansion leading to a main population of open conformers (Int2). Because of its intrinsic disorder, this open conformation may result in faster actin binding and severing of gelsolin-like active conformers in a 9:1 ratio in the presence of 1 mM EGTA (c) or 0.5 mM CaCl2 buffer (d).

Figure 6 | Calcium-independent actin-severing activity of A1–A3 is increased by mutations that disrupt the A1/A3 interface. (a) Details of the A1/A3 interface. The mutated residues Met310 and Glu314 are shown as grey sticks. (b) Similar view of the G1/G3 interface. (c,d) Actin depolymerization assays. A total of 6 μM of each protein (actin control, black; A1–A3wt, red; E314S mutant, green; M310D mutant, yellow; and G1–G3, purple) was added to 6 μM of F-actin in the presence of 1 mM EGTA (c) or 0.5 mM CaCl2 buffer (d).

This crystal structure revealed the presence of a single calcium ion (Fig. 4). This bound calcium likely plays a major role in shifting the equilibrium towards the active state at low calcium concentrations by perturbing the conformation of the A2A3 linker (Fig. 5), which undergoes significant refolding during the inactive-to-active transition (Fig. 7a). However, an additional, perhaps more important, mechanism by which calcium binding at A2 may shift the conformational landscape is through stabilization of the active A2:A3 interface (Fig. 7b), likely resulting in an increased lifetime of the active conformation. The inaccessibility of the A1 and A3 type II calcium-binding sites in the inactive conformation implies that the increase in the population of active conformers observed at calcium-to-protein ratios > 1 is a consequence of calcium binding at A1 and A3 in the Int1, Int2 and A conformers, which, in combination with the straightening of the long α-helix of A3, prevents the molecule from transitioning back to the inactive form. Finally, we show that very high calcium concentrations destabilize the active A2:A3 interface, leading to a majority of open conformers. Based on the MDS results, we propose that additional calcium binding in the A2A3 linker region, and in particular at the highly acidic stretch 232-DGGDDDD-238, may cause the observed destabilization. Interestingly, potential additional low-affinity sites that might play a role in stabilizing the A1 and A2 domain structure were also identified in MDS (Fig. 4), although they have not been verified experimentally. The A2A3 linker appears to be optimized for calcium-induced conformational change through allosteric regulation by association with calcium (Fig. 5). Furthermore, 232_DGGDDDD-238 in the A2A3 linker may offer sequential aspartic acids to the occupied type II calcium-binding site on A2. Thus, as A3 approaches A2, a pathway of calcium/aspartic acid interactions may lead to the docking of the A2:A3 interface, which is stabilized by the final aspartic acid in the
sequence (Asp238) coordinating the type II calcium bound to A2 (Fig. 7). Such functionality in linker regions is beginning to be recognized across a range of multidomain proteins.

Actin depolymerization assays have shown that A1–A3 is able to slowly sever actin filaments in EGTA conditions, although this activity was roughly 10% of the severing rate observed in the presence of 500 μM calcium. Interestingly, this correlates well with the proportion of active conformers detected by SAXS in similar conditions, which are, respectively, 5 and 50% in the presence of 2 mM EGTA and 500 μM calcium. This suggests that F-actin binding by A1–A3 is dominated by conformational selection of the active form, and that the inactive-to-active transition may be relatively slow compared to F-actin binding and severing, preventing population shift from occurring. Alternatively, the large concentration of G-actin present in the assay might hinder population shift through sequestering of G-actin by A1–A3 following an initial binding event to the inactive conformation.

The M310D and E314S mutants of A1–A3 showed increased severing activity in the absence of calcium when compared with wild-type A1–A3, most probably due to a partial shift towards the active conformation, which highlighted the importance of the A1/A3 contacts in stabilizing the inactive state. However, the activity of the mutants in calcium-free conditions remained much lower than for wild-type A1–A3 in the presence of calcium, or G1–G3 that displays fast depolymerization activity regardless of the presence of calcium. The fact that these mutations were unable to completely turn A1–A3 into a Ca$^{2+}$-independent severer suggests that the high level of activity of G1–G3 does not primarily result from the instability of the inactive state G1–G3. In support of this, comparison of predicted A4G for the interfaces between domains 1–2 and 1–3 in iA1–A3 and iG1–G3 using the Pisa Web server revealed no major difference in stability, and a ~150-ns MDS of iG1–G3 suggested that the G1/G3 interface is stable on such timescales (Supplementary Fig. 8), also indicating that the domain rearrangements required for activation are slow.

A second structural difference between the two proteins may reside in the stability of the active form, which is likely influenced by the sequence of the A2A3 linker. Indeed, the concentration of negative charges in the highly acidic stretch of A1–A3, as well as the loss of ionic interactions between Lys166 and Glu263, may lead to an active structure that is less stable than aG1–G3 (Fig. 7b,c). Consistent with this, SAXS studies of G1–G3 and fragments G1–G2 and G2–G3 showed that G1–G3 mainly adopts an active/open conformation in solution regardless of calcium concentration, explaining its calcium-independent activity. Although ensemble analysis was not performed on this data, the construct used (gelsolin residues 1–371) has the same length as A1–A3 (residues 1–365 + 5 residues remaining from the cleaved purification tag), which enables direct comparisons between measured $R_g$. Interestingly, a mild compaction of G1–G3 from 3.4 to 3.2 nm was observed upon addition of 1 mM calcium. This $R_g$ of 3.2 nm fits well with the $R_g$ of A1–A3 in the presence of 500 μM calcium, which is dominated by active conformers. It is thus tempting to speculate that this slight calcium-induced compaction of G1–G3 may result from a shift between open (Int2) and active conformations through stabilization of the G2-G3 interface by calcium binding at the type II site of G2. In addition, the same study showed that mutations in the G2G3 linker could induce Ca$^{2+}$-dependent severing activity in G1–G3. The mutants were found to adopt a collapsed state in the absence of calcium, which enabled direct comparisons between measured $R_g$. In conclusion, we have drawn a quantitative picture of the conformational landscape of A1–A3 and provided structural insights into how calcium binding induces large conformational changes by affecting the relative stability of the accessible conformational states of A1–A3. Our biophysical characterization of A1–A3 in solution helps in explaining the apparent structural similarity yet different calcium-regulated dynamics that characterize the N-terminal halves of adseverin and gelsolin. The origin of these differences is best understood in the context of the full-length proteins. In the absence of a gelsolin C-terminal α-helix latch, adseverin requires tighter interactions than gelsolin in the A1/A3 latch to maintain calcium control of activation.

Methods
Cloning, expression and purification. The cloning and Escherichia coli expression of the N-terminal halves of adseverin (A1–A3, residues 1–365) and gelsolin (G1–G3, residues 25–372) have been described previously in refs 22,20. Briefly, the constructs were engineered into a modified pET-21d (+) (Novagen) plasmid, pSY5, to encode an 8-histidine tag, followed by a PreScission protease cleavage site,
The N terminus of the proteins. The A1–A3 M310D and E143S mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene) and the following oligonucleotide pairs: 5′-GGAAGGGAAGGCCTGACAG TAGAACGACCTAGAATTATTC-3′ for M310D and 5′-GGCTGCAATAGAGACA GCCGTAATCTTACAGAAAGT-3′. All crystals were grown in liquid nitrogen after being soaked in a mother liquor solution containing 100 mM Bis-Tris propane (pH 7.5) or 30% polyethylene glycol 6,000, 100 mM EGTA, 20 mM Tris-HCl (pH 7.2), and 150 mM NaCl). Proteins were concentrated to 10 mg ml⁻¹ by Ultra-15 10 kDa, Amicon Ultra centrifugal filter units.

Crystallization and data collection. Crystallization was carried out by vapour diffusion using a Cartesian Technologies pipetting system. Solutions containing 10 mg ml⁻¹ of A1–A3 in 50 mM NaCl and 10 mM Tris-HCl (pH 7.5) supplemented with 5 mM of either EDTA or CaCl₂ crystallized in 20% polyethylene glycol 3,350, 100 mM Bis-Tris propane (pH 7.5) or 30% polyethylene glycol 6,000, 100 mM HEPES (pH 7.0), respectively, which led to the crystal structures of iA1–A3 and aA3. Crystal data and data collection parameters are listed in Table 1. The crystals were used for data collection for 45 min in an SS-34 rotor in a RC 5C Plus centrifuge (Sorvall) at 4°C. Solvent content and crystallographic data were obtained from 30 randomly selected images using the JADE program (Fuller). The crystallographic data were processed with the XDS program (Kabsch) and the crystal structure was solved using the SHELX-2014 software (Sheldrick).

SAXS experiments. Protein samples (10 mg ml⁻¹ in 0.2 mM EGTA, 20 mM Tris-HCl (pH 7.2) and 150 mM NaCl) were mixed with 10 × calcium or EGTA stock solutions to achieve the required free calcium concentrations, which were calculated using the Ca-EGTA Calculator v1.3 webserver (http://maxchelator.stanford.edu/CaEGTA-TS.htm). SAXS data were collected at the European Synchrotron Radiation Facility, Grenoble, France. Crystals were frozen in liquid nitrogen after being soaked in a mother liquor solution containing 100 mM Bis-Tris propane (pH 7.5). After washing the beads, proteins were eluted by raising the imidazole concentration to 250 mM. The His-tag was cleaved by addition of human rhinovirus 3C protease followed by overnight dialysis against 130 mM NaCl and 20 mM Tris-HCl (pH 7.5). Proteins were further purified by size-exclusion chromatography on a Hitrap S/10 Superdex 200 prep grade column (GE Healthcare), first equilibrated with 125 ml of 100 mM EGTA, 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, re-equilibrated with 250 ml gel-filtration buffer (0.2 mM EGTA, 20 mM Tris-HCl (pH 7.2) and 150 mM NaCl). Proteins were concentrated to 10 mg ml⁻¹ by Ultra-15 10 kDa, Amicon Ultra centrifugal filter units.

Starting for the inactive state (I) were obtained from the crystal structure of calcium-free N-terminal half of adseverin or from a homology model based on PDB 1RGI for the active state (A)14. Complete models including the missing disordered termini were generated in Modeller53. An additional homology model based on the structure of the gain-of-function mutant of the actin capping protein CapG (PDB 2J7Z; ref. 54), which differs from the active structure by having a different interface between domains 2 and 3, was also generated and used as a qualitative control of the accuracy of the ensemble optimization approach. Starting conformers for intermediates 1 and 2 (Int1 and Int2) were obtained by dissolving domain 3 and then domain 1 from the iA1–A3 model. This was performed by defining and altering backbone torsions within the interdomain linker regions in VEGA3. An additional model for Int2 was generated by dissolving domain 3 from domain 2 in the active structure, leading to an alternative conformer of Int2 where the alpha helix in the A2A3 linker is folded. Models of A1–A3 (I, Int1, Int2, A and the CapG-derived model) were simulated in GROMACS55 using an atomistic coarse-grained structure-based model56,57. A timestep of 0.0005 time units was used and the simulation was coupled to a temperature bath via Langevin dynamics. 10,000 Snapshots were extracted from each simulation, and a 1,000 models from each simulation were randomly dispatched into 10 independent ensembles of 6,000 models.

Ensemble optimization. For each model from each ensemble, theoretical SAXS patterns were calculated with the programme CRYOSOL58, and ensemble optimization fitting was performed for each of the 10 independent ensembles with GAJOE uses a genetic algorithm to select from a large pool of conformers optimized sub-ensembles that minimize the discrepancy between the experimental and calculated curves $\chi^2_{exp}$ according to the following equation:

$$\chi^2_{exp} = \frac{1}{K} \sum_{j=1}^{K} \left[ \frac{d(Q_j) - \langle d(Q_j) \rangle}{\sigma(Q_j)} \right]^2$$

where $K$ is the number of points in the experimental curve, $\sigma$ is the s.d. and $\mu$ is a scaling factor. The ensemble size was varied from 1 to 50 models and variations in the quality of the fit to the experimental data $\chi^2_{exp}$ were monitored in order to analyse protein flexibility (Supplementary Fig. 6). Additional validation of the ensemble calculations was also performed by fitting the experimental data to pool ensembles including only I, A or I + A models, which yielded fits of poorer quality than those that included the intermediate states (I + Int1 + Int2 + A), mainly through the elongation of the interdomain linker.

Actin depolymerization assays. Polymerization buffer (final concentration, 50 mM KCl, 0.2 mM ATP, 2 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EGTA and 50 mM HEPES, pH 7.5) was added to 10% pyrene-labelled G-actin (6 μM) in 96-well, flat-bottomed plates (Corning), and was incubated for 30 min to allow the formation of F-actin. Calcium was then added to obtain the required free calcium concentration. Reactions were equilibrated for 1 h before 6 μM of the respective proteins (A1–A3, A1–A3-M310D, A1–A3-E3145 or G1–G3) were added. The final volume in each well was 100 μl. Fluorescence intensity was measured kinetically at excitation and emission wavelengths of 365 and 407 nm, respectively, using a Safire2 fluorimeter (Tecan).
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**Acknowledgements**

We thank the European Synchrotron Radiation Facility (Grenoble, France) for beamtime, and the staff of beamlines ID29, ID14-2 and ID14-3 for assistance with crystallography and SAXS data collection.

**Author contributions**

S.C., R.C.R., J.M.G. and C.L. designed the research; S.C., J.M.G. and C.L. performed experiments; S.C., R.C.R., J.M.G. and C.L. analysed data; and S.C., R.C.R., J.M.G. and C.L. wrote the paper.

**Additional information**

**Accession codes.** The final refined coordinates and structure factors have been deposited in the PDB with accession codes 5A1K and 5A1M.

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Chumnarnsilpa, S. *et al.* Calcium-controlled conformational choreography in the N-terminal half of adseverin. *Nat. Commun.* **6**:8254 doi: 10.1038/ncomms9254 (2015).