



**ELUCIDATING THE MECHANISMS OF TALIN-MEDIATED CELLULAR
MECHANOTRANSDUCTION**

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ABSTRACT

The ability of a cell to sense their physical environment, including tension and stiffness, has been implicated in a variety of cellular processes from development to disease. One of the key areas of research around this phenomenon concerns proteins whose three-dimensional structure is disrupted by physical forces in such a way as to alter how they behave. This class of 'mechanosensitive' molecules is thought to include a protein, talin, that is vital in the process of cellular attachment to the surrounding environment.

Talin acts as a structural linkage in cells, bridging their internal scaffolding, the cytoskeleton, to the external environment. Here, using an advanced biophysical technique called single-molecule atomic force microscopy (smAFM), it is demonstrated that the majority of the talin molecule can be unfolded by pulling on either end. This unfolding occurs at forces that talin is likely to experience in cells, between 10 and 40 pN (these forces are 10^{12} times smaller than the weight of gravity on 1kg).

Using this smAFM technique, along with computer simulations provided by Vesa Hytönen's group at the University of Tampere, the pattern of how talin unfolds is demonstrated at an atomic level. This pattern is then shown to have similarities with other proteins that share a similar structure to talin. Thus the techniques and results shown here may serve as a template for the study of other structural proteins.

Finally, protein engineering, smAFM and biophysical tools are used to show how the unfolding of talin may be linked to cancer progression. By mutating the talin protein, it was made resistant to unfolding and this increased its binding interaction with Deleted in Liver Cancer 1 (DLC1), a tumour suppressor protein that has been implicated in a variety of cancers. These results demonstrate the crucial role talin unfolding plays in cancer progression and offers a target for future therapeutic endeavours.

DECLARATION OF ORIGINALITY

I hereby declare that the work presented here is my own. Where others have contributed, every effort has been made to accurately and appropriately acknowledge this.

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*As our circle of knowledge expands, so does the circumference
of darkness surrounding it. – Albert Einstein*

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* Denotes joint first authorship

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1 RESEARCH BACKGROUND

This chapter has been adapted from work published as a review article in the FASEB Journal under the title *Talin: a mechanosensitive molecule in health and disease*.¹

1.1 INTRODUCTION

Biochemical signalling in cells is an extremely active area of research; however, our knowledge of how cells manage physical stimuli lags well behind. If we understand that all cells are subjected to forces, it is clear that a holistic approach that integrates biochemical and biomechanical interactions is the only tool that will lead to a full understanding of cell behaviour. Recent advances in the fields of microscopy and biophysical techniques have opened new and exciting possibilities to investigate the influence of mechanical forces in biology, shaping the relatively young and emerging discipline of mechanobiology.

Many hallmark works that established paradigms in mechanobiology have transcended the field and had wide-ranging impacts on other disciplines. For instance, a study that demonstrated that alterations in a cell's physical environment, specifically extracellular matrix (ECM) alone, could encourage a malignant phenotype in cancer cells;² or another, which demonstrated that similar alterations in physical microenvironment could induce stem cells to differentiate into a range of cell types from neurons to osteoblasts.³ These were by no means the first papers to demonstrate the influence of ECM on cell behaviour, but by so effectively tying the microenvironment to such broadly recognized areas as cancer and stem cell research, they did serve to capture the imagination of the scientific community at large.

Cells communicate with their mechanical environment through integrin-mediated focal adhesion complexes,⁴ which connect the cytoskeleton with the ECM. Talin, one of the main molecules in these complexes, was discovered in chicken gizzard smooth muscle and targeted to focal plaques and membrane ruffles.⁵ It was subsequently shown to bind and activate integrin and also bind to F-actin.⁶⁻⁷

Because talin binds to crucial components of the load-bearing apparatus of cells, it has been investigated as a mechanosensitive molecule. This class of molecules has enjoyed popularity recently although what constitutes a mechanosensitive molecule can sometimes be unclear; as such it is worth briefly discussing the definition as used here. All molecules, if exposed to sufficient mechanical force, will have their structure changed similar to how thermal stresses denature proteins. These structural changes will often lead to alterations in a molecule's pattern of interaction with its binding partners, as these interactions are often dependent on the tertiary structure of the proteins that is disrupted by this mechanical (or thermal) stimuli. In order for a protein to be considered mechanosensitive it must be exposed to mechanical forces *in vivo* that are capable of inducing these structural rearrangements.

It is necessary to take stock of our current knowledge of this mechanosensitive molecule and to highlight possible future areas of study given the recent insights into its structural and functional roles and numerous studies outlining its possible role in diseased phenotypes. Given the importance of structure and binding partners, these aspects of talin will be discussed first.

1.2 TALIN STRUCTURE AND BINDING PARTNERS

In vertebrates, talin exists as two closely related isoforms that share 74% amino acid sequence identity. Talin-1 is the only form expressed by most hematopoietic cells and endothelial cells⁸ and talin-2 is most highly expressed in cardiac muscle, skeletal muscle, and brain tissue.⁹ As a result, the effect of talin-2 knockdown/knockout is pronounced in muscle development.¹⁰⁻¹¹ The two isoforms are differentially localized in some cell types as homodimers and may have different functions,¹² and talin-2 can compensate for loss of talin-1 in some conditions.¹³ However, talin-1 appears to be the dominant isoform and most well-studied. Talin will therefore refer specifically to the talin-1 isoform hereafter in this thesis unless stated otherwise.

1.2.1 The talin head

Talin is a 270 kDa, 2541 amino acid membrane-associated protein consisting of two main regions: an N-terminal 'head' and a C-terminal 'tail' (Figure 1.1). The head domain is globular and contains an unusual FERM (4.1, ezrin, radixin, moesin) domain; it contains the three typical FERM subdomains, F1-F3, with an extra insertion in F1. In addition to this insertion it contains an additional subdomain at the beginning, F0.¹⁴ The most prominent function of the talin head is related to the binding and activation of surface-membrane integrins,^{6, 15} along with the binding of membrane phospholipids¹⁶ and the other widely-studied focal adhesion protein, focal adhesion kinase (FAK).¹⁷

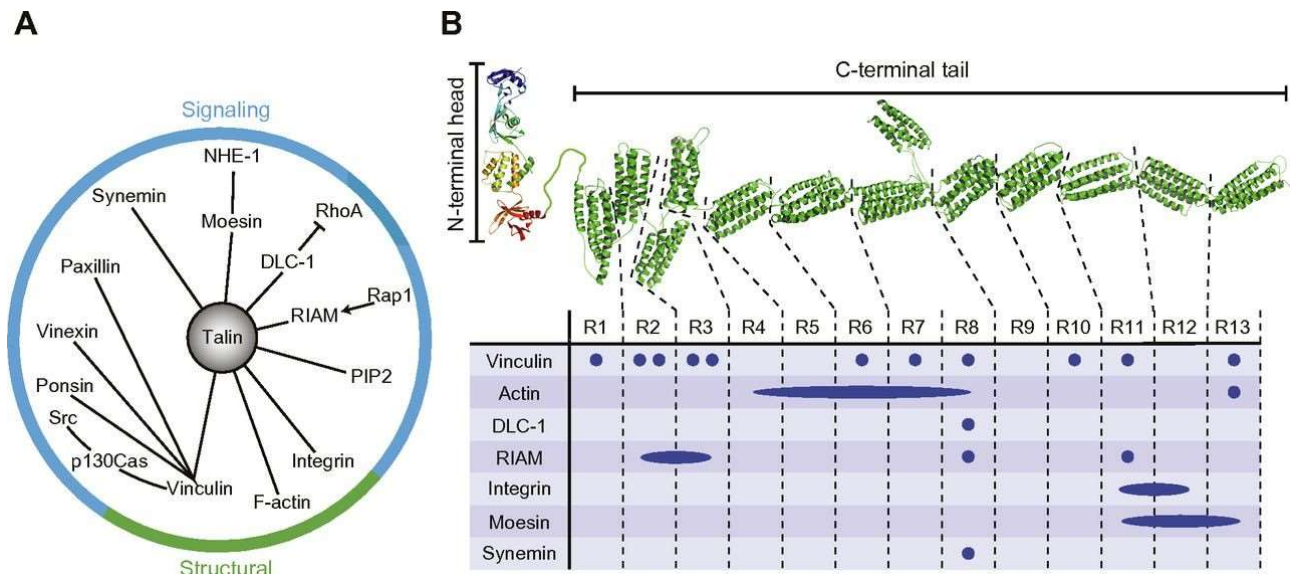


Figure 1.1 Talin structure and binding partners: (A) Relationship between talin and its signalling/structural partners. (B) Structure of talin's integrin binding FERM domain head and mechanosensitive rod domain, along with binding site locations.

Talin is thus a crucial link in the integrin activation process as demonstrated by talin knockdown in various cell types. The F3 unit of the FERM domain binds to the cytoplasmic tail of β integrins at the NPxY motif and results in increased affinity of the integrin to its cognate ligand in a process termed “inside-out” signalling.^{6, 15, 18} Experimental and computational studies have demonstrated that talin binding to β integrin is mediated by favourable membrane electrostatic interactions due to membrane phosphatidylinositol 4,5-bisphosphate (PIP2), resulting in a rotation of the β transmembrane domain, weakening of the integrin inner and outer domain clasps, and separation of the α and β subunits.¹⁹⁻²² A variety of pathways can precipitate inside-out signalling via G-protein coupled receptor (GPCR) signal transduction involving Rho and Rap family small GTPases;²³⁻²⁴ the specific mechanisms vary depending on cell type, GPCR, and integrin subtype. In Jurkat T cells, for example, T-cell receptor stimulation leads to activation of Rap1, which plays a role in cell adhesion and spreading via actin polymerization as well as its interaction with Rap1-GTP-interacting adaptor molecule (RIAM);²⁵ RIAM is required for Rap1-mediated upregulation of LFA-1 ($\alpha_L\beta_2$) affinity and localization of Rap1 to the membrane in these cells.²⁶ RIAM influences integrin activation through binding to talin, and it was further shown that knockdown of

talin or RIAM prevents formation of a RIAM-talin-integrin complex and abrogates Rap1-mediated adhesion through $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$.²⁷⁻²⁸ However, RIAM is not essential for lower affinity $\alpha_4\beta_1$ expressing cells,²⁹⁻³⁰ and talin-1 was not found to be required for GPCR-mediated $\alpha_4\beta_1$ activation, but likely plays a role in subsequent focal adhesion events.³⁰ Recently, *in vivo* knockdown of RIAM in a mouse model unexpectedly did not alter platelet function, perhaps due to compensatory talin recruitment mechanisms, particularly for the $\alpha_{IIb}\beta_3$ integrin.³¹ The contradictory role of talin and its adapters may be due to individual differences among cell and integrin types.

Inside-out signalling and subsequent extension of the integrin ectodomain increases affinity, but only transiently. Without force application that stabilizes the ECM-integrin-talin-actin axis in a process termed “outside in” signalling, the integrin may return to a low affinity state.^{24, 32} (For reviews on inside-out and outside-in signalling in leukocytes see Alon *et al.*³³ and Alon *et al.*³⁴). Anchored ECM ligands induce allosteric changes in integrin conformations³⁵⁻³⁶ leading to strengthening of the high affinity state and propagation as a mechanical signal, which can lead to further downstream signalling.³⁷⁻³⁸ As a crucial bridge between integrins and the cytoskeleton, talin has been implicated in outside-in shear stress-mediated adhesion of VLA-4 and LFA-1.³⁹⁻⁴⁰ A picture of integrin affinity is therefore incomplete without consideration of outside-in signals. This further demonstrates the importance of the physical microenvironment in modulating cell function.

The interaction with and activation of integrins is a very well-studied role of talin and contributes greatly to its observed function within cells, and a recent review by Calderwood *et al.* provides an excellent overview of this phenomenon.⁴¹

1.2.2 The talin rod

The C-terminal talin tail comprises amino acids 487-2541 and as such is the larger of the two sections. The talin tail spans 63 amphipathic α -helices arranged into 4- or 5-helix bundles.⁴² These bundles are

expected to form a linear chain, which leads to this section often being referred to as the talin rod. There are 13 of these bundles (designated R1-R13) in addition to a single helix at the C-terminal end that serves as the dimerization domain.⁴³⁻⁴⁴ The primary interaction of talin with F-actin is believed to occur at the C-terminal end, requiring both this dimerization domain and the final helical bundle (R13).⁴³ The binding of talin to actin at its tail and integrin at its head along the rod provides a connection between the cytoskeleton and the ECM, a connection in which talin serves as a load-bearing mechanical link.

In addition to this structural role, the talin rod also contains a variety of binding sites involved in both the regulation of its own activity and that of other structural and signalling proteins. The most relevant of these are the 11 vinculin binding sites (VBSs) that have been identified. These VBSs are buried within helical bundles and it is currently unclear how many in fact ever bind vinculin *in vivo*.⁴⁵ The talin rod also contains numerous other binding sites including a second actin-binding site,⁴⁶ an auto-inhibition site that interacts with the talin head,⁴⁷ a second integrin binding site,⁴⁴ and binding sites for the intermediate filament protein synemin,⁴⁸ RIAM⁴⁹ and the tumour suppressor protein, deleted in liver cancer 1 (DLC-1).⁵⁰ The presence of a second actin and a second integrin binding site brings more complexity to the simple mechanical bridge of talin binding integrin at its N-terminal and actin at its C-terminal. However, super-resolution microscopy has indicated that the N-terminal end of talin localises to the membrane while the C-terminus is displaced from the membrane, supporting the simpler model of force transmission along the rod.⁵¹⁻⁵² This leaves open the question of what purpose, if any, the secondary binding sites serve, although recent research indicates that talin may exist in alternative configurations depending on the developmental stage and the type of cell studied.⁵³

1.2.3 Talin's mechanosensitivity

The evidence for a configuration in which talin's tail binds to the actin cytoskeleton and its head to integrins, and by extension to the ECM, suggests that talin is a mechanosensitive molecule. The most studied aspect of this mechanosensitivity relates to talin's interaction with vinculin, since the early

structural studies indicated that this interaction requires the unfolding of the bundles containing VBSs.⁵⁴⁻

⁵⁵ Steered molecular dynamics indicated that this unfolding could be precipitated by the application of mechanical force applied at either ends of the bundles⁵⁶ after which vinculin binds to previously buried VBSs (Figure 1.2). The existence of such a mechanical force *in vivo* could occur in one of two ways: either by direct application of force from external sources to the integrins and thus talin; or indeed internally from the contractile nature of the actomyosin network to which talin attaches through its tail.

This hypothesized mechanical activation was demonstrated *in vitro* using atomic force microscopy and magnetic tweezers, where it was shown that the application of force to talin did indeed increase its binding affinity for vinculin (Figure 1.3a-b).⁵⁷ The physiological relevance of this unfolding was further demonstrated when it was shown that it could occur at forces as low as 5 pN, within the range of forces known to be applied to single molecules in focal adhesions.⁵⁸⁻⁵⁹ These molecular studies are supported by cellular studies, in which it was shown that the length of the talin molecule varies in a cell from 60 nm (the length of a structured talin molecule not under the application of force) to as much as 350-400 nm (Figure 1.3c).⁶⁰ Such extension of the molecule would require the unfolding of numerous bundles along the rod as the talin molecule is exposed to force derived from the rearward action of the actin cytoskeleton (the disruption of a single bundle adds 30-50 nm of length to the rod depending on the bundle). Therefore, it seems likely that in focal adhesions talin undergoes cycles of unfolding and that this unfolding is what allows it to bind to vinculin, an interaction that is hindered in its native state for most VBSs. Thus far these molecular studies have focused on the R1-R3 region of the talin rod, although detailed analysis of the structure of focal adhesions indicates that vinculin binds at other locations along the rod; it is likely that other bundles are mechanically activated.⁶¹

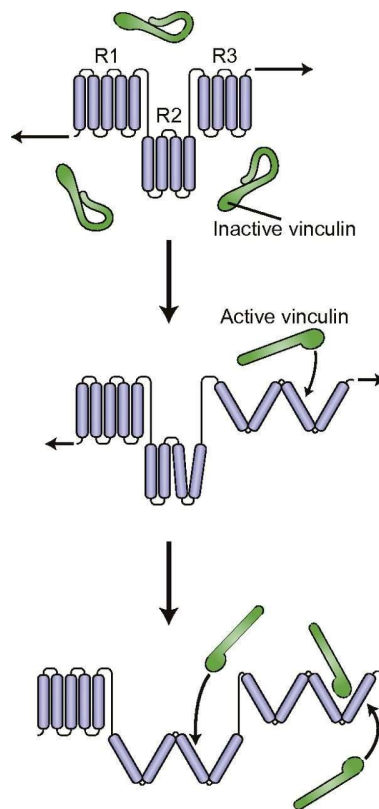


Figure 1.2 Unfolding of talin exposes new vinculin binding sites (VBSs). Without force applied to rod bundles R1-R3, vinculin remains in an auto-inhibited state whereby the tail blocks binding at the vinculin head. When force is applied along the talin rod, the bundles unfold and expose up to 11 new VBSs, which breaks the interaction between the vinculin head and tail, allowing interaction with other proteins such as actin. This process can occur modularly such that the magnitude of force determines the number of exposed VBSs and therefore the number of vinculin binding sites and level of adhesion maturation.

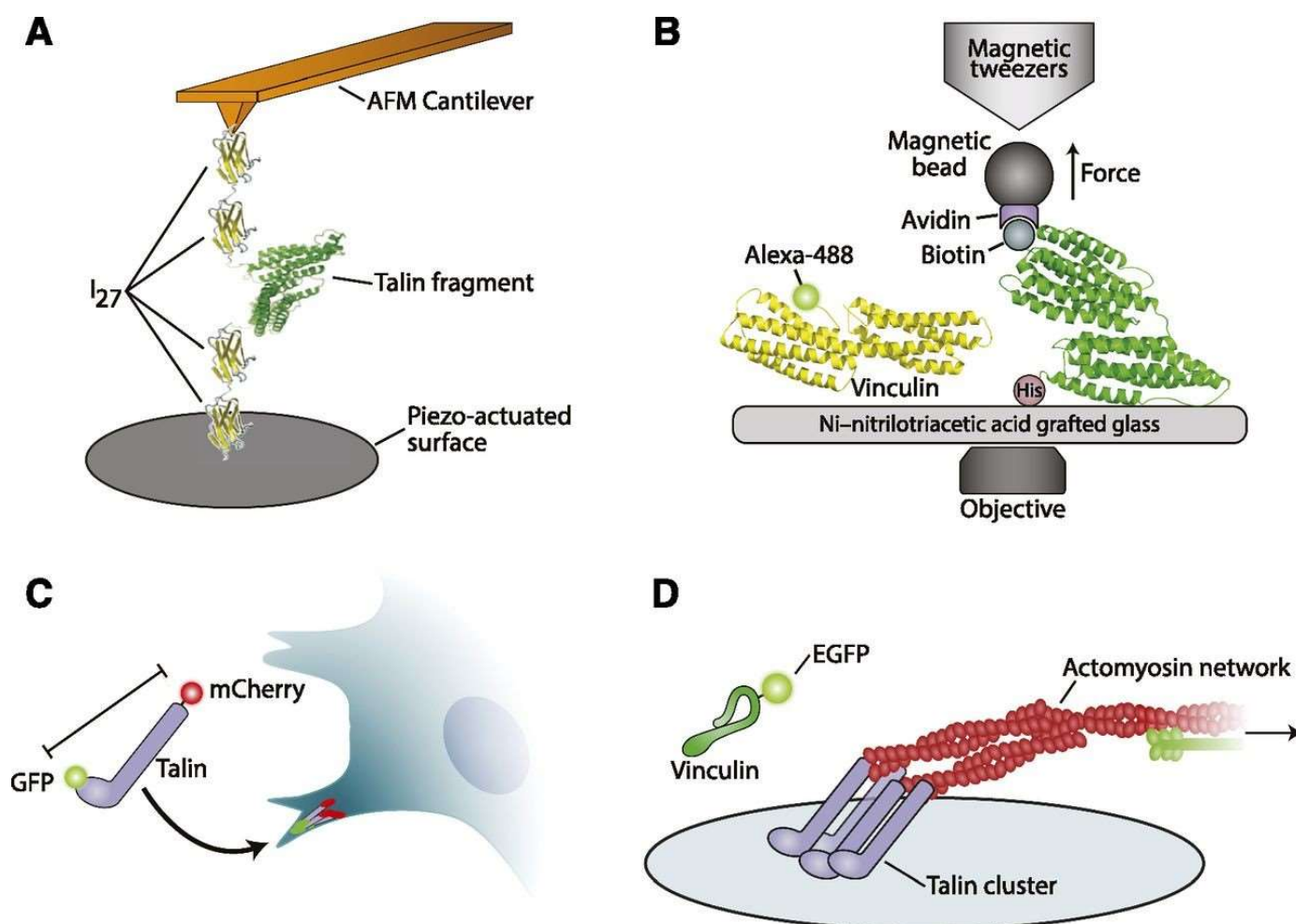


Figure 1.3 Experimental evidence demonstrating the mechanosensitive nature of talin. (A) Single molecule atomic force microscopy (smAFM) was used to reveal the unfolding profiles of the C-terminal rod.⁵⁷ (B) Magnetic tweezers and fluorescent microscopy were combined to examine the interaction of talin with vinculin under force induced unfolding.⁵⁷ (C) In vivo, talin can be stretched up to ~8 times its unbound length as measured by fluorescence microscopy. In contrast to *in vitro* data, cycles of actomyosin contractions alters the force-extension relationship of talin to vinculin.⁶⁰ (D) Pseudo-focal adhesions were created by micro-patterning talin molecules onto a substrate. When a contractile actomyosin network was layered on these adhesions, the talin molecules unfolded and showed an increased affinity for vinculin, as demonstrated by fluorescent microscopy earlier.⁶²

1.2.4 Focal adhesion stabilization

It has been widely accepted in the field of mechanotransduction that the growth of focal adhesions is force dependent; the greater the force applied to a contact, the greater the size and stability of the adhesion.⁶³⁻⁶⁵ However, recent studies have reported a surprising dissociation between the focal

adhesion size and the applied force in mature adhesions, which may imply that mature focal adhesions that anchor stationary cells to their substrates are not mechanosensitive.⁶⁶ Although the reason for this insensitivity has yet to be determined, one possible explanation of this phenomenon is that as the focal adhesion grows and stabilizes, the force experienced by individual mechanosensitive components may be reduced leading to a reduction in force-response.

The presence of vinculin assists in the process of focal adhesion stabilization, mediated through its interactions with talin and actin.⁶⁷ In *in vitro* systems composed only of substrate-bound talin and actomyosin, the isometric contraction of actin stress fibres exposes VBSs, increases vinculin association, and decreases talin refolding. The actin crosslinker α -actinin can further promote the stability of fibres and decrease vinculin dissociation (Figure 1.3d).⁶² This has also been demonstrated *in vivo* with fluorescence microscopy: actomyosin contraction stretches talin cyclically several times its unbound length, and vinculin binding dampens stretch-contraction cycles.⁶⁰ Additionally when actomyosin contraction is suppressed, vinculin localization at focal adhesions is reduced; but when the substrate under these compromised cells is stretched, vinculin recruitment recovers, highlighting the importance of tension in focal adhesion maturation.⁶⁸ However, recent works have determined that vinculin was not required for proper focal adhesion complex organization,⁵² but may instead be implicated largely in stabilization.⁶⁹

The talin-vinculin interaction provides structural support to the linkage between actin and the ECM; indeed, the magnitude of this mechanical contribution has been investigated using FRET microscopy that measures the tension in the vinculin molecule between its head that binds to talin and its tail, bound to actin. These forces are greatest in nascent adhesions, and then fall as the adhesions mature, consistent with the idea of the growth of adhesions recruiting more vinculin molecules, thus leading to a parallelization of support and reduction in individual tensions.⁷⁰ This is also consistent with the proposed mechanism of mechanotransduction via talin, with high initial forces unfolding numerous binding sites on the talin rod for vinculin to bind, allowing for greater stabilization of the adhesion.

In addition to a mechanical role supporting the bridge between integrins and the cellular cytoskeleton, vinculin also plays an important role in regulating cell behaviour by recruiting other adhesion proteins through its numerous binding domains. Vinculin natively occurs in an auto-inhibited state through an interaction between its head and tail domains and preventing or discouraging its binding affinity for other proteins. The binding of its head to the VBSs on the talin rod is sufficient to break this state, thus releasing the tail and hinge of vinculin for other interactions.⁷¹ One such interaction is that between the hinge region of vinculin and p130Cas, a prominent Crk and Src substrate. Disruption of this interaction reduces a cell's ability to produce traction forces and reduces cell stiffness.⁷² Other proteins thought to be at least partially recruited through their interaction with these regions of vinculin include vinexin, ponsin and paxillin.⁷³⁻⁷⁴ Vinculin may also serve to activate other regions on the talin rod itself, notably the secondary actin binding site located in the middle of rod. Recent work suggests the C-terminal actin binding site engages talin first, leading to an activation through force of the VBSs in the R2-R3 region. The subsequent binding of vinculin to this region induces changes that allow actin to bind to the secondary binding site, providing another mechanism by which the adhesion is stabilized.⁷⁵

1.2.5 Cellular mechanotransduction

These experiments documenting talin's mechanosensitivity with regard to vinculin provide an attractive framework for a possible mechanism of cellular mechanotransduction, the overall process by which cells interpret and respond to physical stimuli. In the first step, the magnitude and duration of any force applied determines how many helical bundles are unfolded. The more helical bundles that are unfolded, the more VBSs become available.⁵⁶⁻⁵⁸ A higher number of VBSs will lead to a greater activation of vinculin by disrupting its auto-inhibition.⁷¹ This will lead to both stabilization of the focal adhesion and an increase in recruitment of proteins shown to be crucial to focal adhesion signalling. This model of focal adhesion strengthening via talin stretching is depicted in Figure 1.4.

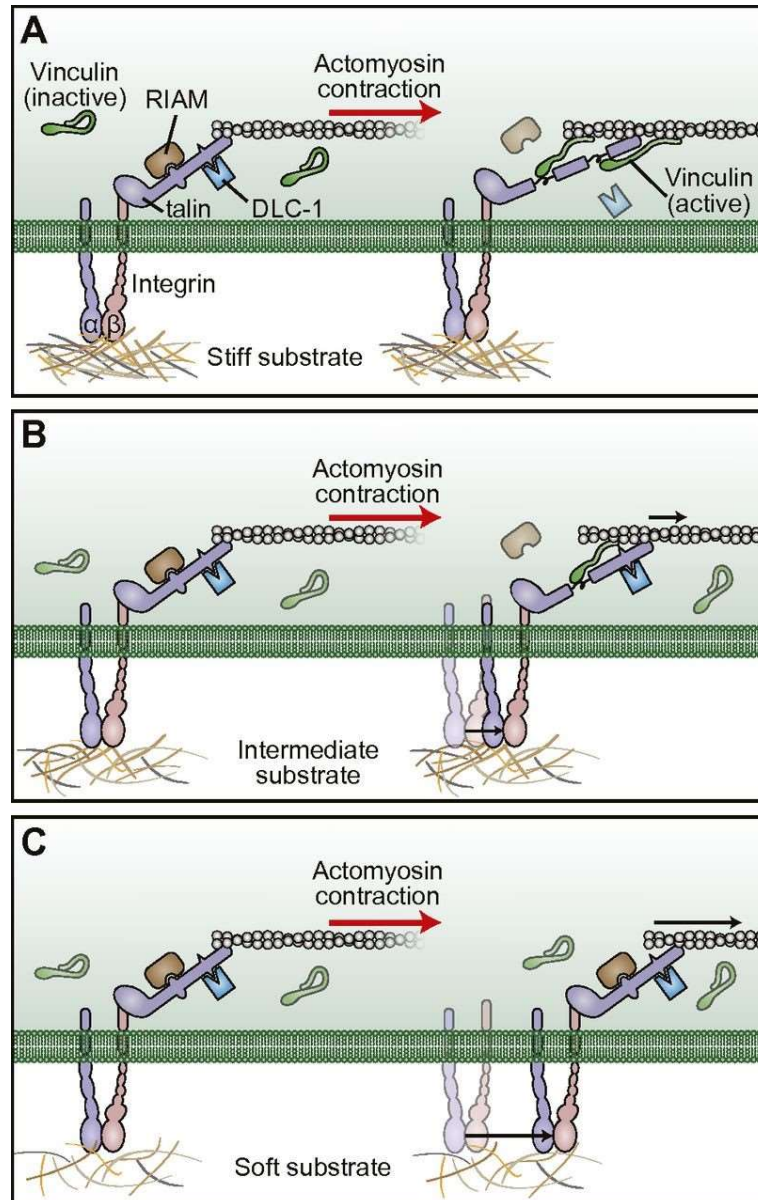


Figure 1.4 Proposed model for the interaction of talin with its binding partners in substrates of different rigidities: (A) stiff, (B) intermediate, and (C) soft ECM substrates. The force applied to talin depends on both the force of actomyosin contraction and the rigidity of the underlying substrate. Very stiff ECM substrates promote firm anchoring of the talin molecule to the cell membrane via integrin. Simultaneous actomyosin contraction concentrates tension along the talin rod, thereby unfolding it. This may decrease the affinity with previously bound adaptor proteins such as RIAM and DLC-1, allowing vinculin binding on newly opened VBSs. Vinculin binding to talin further stabilizes the focal adhesion and leads to focal adhesion maturation. In substrates of intermediate stiffness, fewer VBSs are opened, resulting in a less mature focal adhesion. In very soft substrates, the matrix deformation is such that the force talin is exposed to is reduced leading to an even lower number of exposed VBSs; thus, RIAM and DLC-1 remain bound to the structured rod.

Despite the elegance and promise that this demonstrates as a vital mechanism for cell-ECM mechanotransduction, it remains to be definitively shown that this is indeed how cells 'sense' their mechanical environment. Experimental evidence strongly supports the case for each individual step, but further comprehensive cellular work is required to settle the case for the whole process.

1.2.6 Implications of other binding partners

Part of these comprehensive studies must answer the question that as yet has been unexplored in previous experiments: how do talin's other binding partners relate to its possible role in mechanotransduction? Like vinculin, talin's other binding partners have prominent roles in signalling pathways associated with cell response, development and survival. DLC-1 is a tumour suppressor protein that acts as a GTPase activating protein (GAP) whose loss is frequently associated with a variety of cancerous phenotypes. As a GAP molecule, it down-regulates the activity of members of the Rho family, which are key regulators of the actin cytoskeleton.⁷⁶ In order for its activity to have an effect on cellular behaviour, DLC-1 requires a binding motif that binds to talin and aids in its localisation to focal adhesions.⁵⁰ The binding site for DLC-1 on talin is located in a bundle midway along the talin rod and as such is likely to experience force application within the cell. Whether or not this force will affect the binding of DLC-1 to talin is unknown, although it has been shown that DLC-1 is more commonly found in mature adhesions rather than nascent ones,⁷⁷ a phenomenon that may be explained by mechanotransduction.

The effect of force on talin's interaction with other binding partners may also operate differently than with vinculin, where the unfolding of the bundle increases affinity. It has been suggested, for example, that the same unfolding of the bundles R1-R3 that has been shown to increase the affinity for vinculin could also disrupt the binding of RIAM to the same region, reducing affinity rather than increasing it.⁴² This switch in affinity, potentially force-modulated, from RIAM binding to vinculin is associated with a switch in the nature of the focal adhesions, from sites of actin polymerisation at the leading edge of lamellipodium to stable sites of adhesion that are able to transmit greater forces from the cytoskeleton to the ECM.⁷⁸

1.3 TALIN AND DISEASE

1.3.1 Talin investigation in cancer

Talin's prominent role both in signalling pathways related to cell survival and also in cell adhesion and migration has led to extensive investigation into its possible role in cancer progression. Early studies localized the talin gene to chromosome 9p, and it has been noted that this chromosome frequently contains deletions or mutations in cancerous phenotypes.⁷⁹⁻⁸⁰ Talin has been shown to be significantly upregulated in the metastatic tissue of human prostate specimens as compared with the primary tumours leading to greater adhesion, migration and invasion.⁸¹ In similar fashion, talin is necessary for metastatic breast cancer cells to degrade the ECM to assist in invasion.⁸² Talin is also upregulated in hepatocellular carcinoma, where it is associated with a shorter time to recurrence after resections, in brain tumour glioblastoma multiforme and in ovarian serous carcinoma.⁸³⁻⁸⁵ In oral squamous cell carcinoma, its overexpression has been shown to correlate with overall invasiveness.⁸⁶

1.3.2 Talin regulation in cancers

Because aberrant levels of talin are found in a variety of cancers, it is necessary to determine what is regulating these talin levels and how it affects cancerous phenotypes. One of the factors that influences the levels of protein transcription in cells is microRNA, or miRNA: short, non-coding sections of RNA that regulate gene expression at the post-transcriptional stage.⁸⁷ Due to their effect on protein levels and often aberrant expression in a variety of diseased phenotypes, including cancer, the investigation of their levels has become increasingly popular. For talin, at least four miRNAs have been implicated in its role in cancer: miR-124 and miR-132 are downregulated in prostate cancer, with talin-1 and talin-2 as direct targets, respectively;⁸⁸⁻⁸⁹ miR-9, which targets talin-1, is downregulated in ovarian serous carcinoma;⁸³ and finally Trastuzumab, a monoclonal antibody that has been used as an effective treatment for breast cancer,⁹⁰ has been shown to upregulate miR-194, which in turn reduced the levels of talin-2.⁹¹ Although

these miRNAs seem to specifically target one isoform or the other, it is currently unclear as to whether it is the relative expression levels of the two isoforms or the absolute levels of one that is relevant to cancer progression. Recent work has suggested that the two isoforms might be tuned differently for mechanosensing, with talin-1 requiring engagement of the actomyosin machinery to activate its vinculin binding sites whereas talin-2 appears to be able to partly bind vinculin without the application of force.⁹² As the distinct roles of the two isoforms in mechanotransduction becomes clearer, it may provide indications as to why the specific targeting of one or the other is important.

As well as the modulation of talin's overall expression levels, it is also important to take note of the regulation of the protein itself and its activity as well. Firstly, there is cathepsin H, a protease for which talin is a substrate. This protease is upregulated in prostate cancer cell lines and, owing to its action on talin, cathepsin H inhibition increases integrin affinity and reduces migration of the cancer cells on fibronectin.⁹³ The apparent cleavage target of cathepsin H, the F0 subdomain of the talin head, does not bind directly to the integrin tails but is thought to interact, along with F1, with membrane phospholipids to enhance integrin activation.¹⁴ Equally, an important part of focal adhesion dynamics involves the action of calpain, another protease that acts on talin, cleaving it at the linker region between the talin head and tail regions, which leads to the disassembly and turnover of focal adhesions.⁹⁴ Calpain activity has been linked to the presence of common oncoproteins and has a subsequent effect on focal adhesion mechanics and cell migration, possibly through its cleavage of talin, and therefore its regulation of adhesion turnover.⁹⁵

1.3.3 Talin and cancer treatment

The importance of talin within pathways relevant to cancer and also in the cancerous phenotypes themselves suggests that talin could be an attractive avenue of cancer treatment. Indeed, as discussed previously with Trastuzumab, it seems that cancer treatments already in use may act through a mechanism related to talin function. This, however, is not the only example of such an investigation. Talin

expression in cells has been shown to affect the chemoresistance of those cells to cancer treatments, such as cisplatin, a common chemotherapy drug, whereby knockdown of talin confers chemoresistance to cisplatin treatment through NF- κ B activity.⁹⁶⁻⁹⁷ Conversely, a lower expression level of talin-1 in a variety of breast cancer cell lines has been shown to increase their chemosensitivity to Docetaxel, a well-established chemotherapy drug used in metastatic breast cancer treatment.⁹⁸ Taking the idea of talin's potential role in chemotherapy pathways further, it has been shown that, similar to Trastuzumab, some established and putative cancer therapies act directly on talin levels in cells. These include Reseveratrol, which has been studied extensively in prostate and colon cancers, and DZ-50, a novel Doxazosin derivative, both of which have been shown to reduce levels of talin in cancer cells.⁹⁹⁻¹⁰⁰

1.3.4 Possible mechanisms by which talin modulates cancer

Although the previous studies provide good evidence for talin's importance in cancerous phenotypes, they do little to explain it. Given its multitude of binding interactions, there are a number of potential avenues that could provide an explanation. Its activation of integrins, and thus its control over adhesion, could be vital for migration and thus metastasis. Stable adhesions help to protect the cancer cells from anoikis (cell-death caused by insufficient cell-ECM interaction) and also assist in migration by providing the anchorage needed for cellular traction; conversely stable adhesions can also restrict cell migration by holding the cell in place, which is why the upregulation of talin proteases, and adhesion turnover, as discussed earlier could be an important facet of cancer progression. Alongside its role in migration, talin's downstream signalling partners have been shown to be important in cell cycle regulation. Thus, talin could influence cancer via these downstream pathways. Numerous studies, that will be discussed in this chapter, seem to indicate that it is in fact a combination of these factors.

Several studies have indicated that talin-1 is overexpressed in cancer and particularly in metastatic cells.^{83, 86, 88, 101-102} The effect of talin-1 overexpression in prostate cancer cells appears to activate β 1 integrins, which increases subsequent adhesion, resistance to anoikis, and metastatic potential.¹⁰³ Thus,

talín-1 inactivation reduces cell adhesion and motility, also likely through its relationship with integrins.⁸⁴ One study found that active $\beta 1$ integrin in prostate cancer depends on the phosphorylation of talin-1 at residue S425 and can be blocked with a non-phosphorylatable mutant talin-1 or silencing of p53-activated Cdk5, which is responsible for S425 phosphorylation. Without phosphorylation or association with binding partners, talin is known to exist in an auto-inhibited form and does not bind to integrins.¹⁰⁴ Active (phosphorylated) talin at S425 is also required for activation of downstream effectors FAK, AKT, and Cas, which are involved in cell survival and migration.¹⁰⁵ Similarly, aberrant expression of talin binding partners that assist in recruitment of talin or the conformational change required for binding to the cytoplasmic tail of integrins could potentially lead to a more cancerous phenotype through modulation of cell adhesion and migration. Talin-1 shRNA silencing in $\beta 3$ expressing prostate cancer cells also reduced levels of Src and integrin-linked kinase-1 (ILK-1).⁸¹ *In vivo*, shRNA knockdown of talin-1 or expression of non-phosphorylatable talin-1 in metastatic prostate cells inhibited colonization to bone.¹⁰⁵ Talin-1 level of expression has also been shown to scale with disease progression.^{81, 88} A summary of these interactions is shown in Figure 1.5.

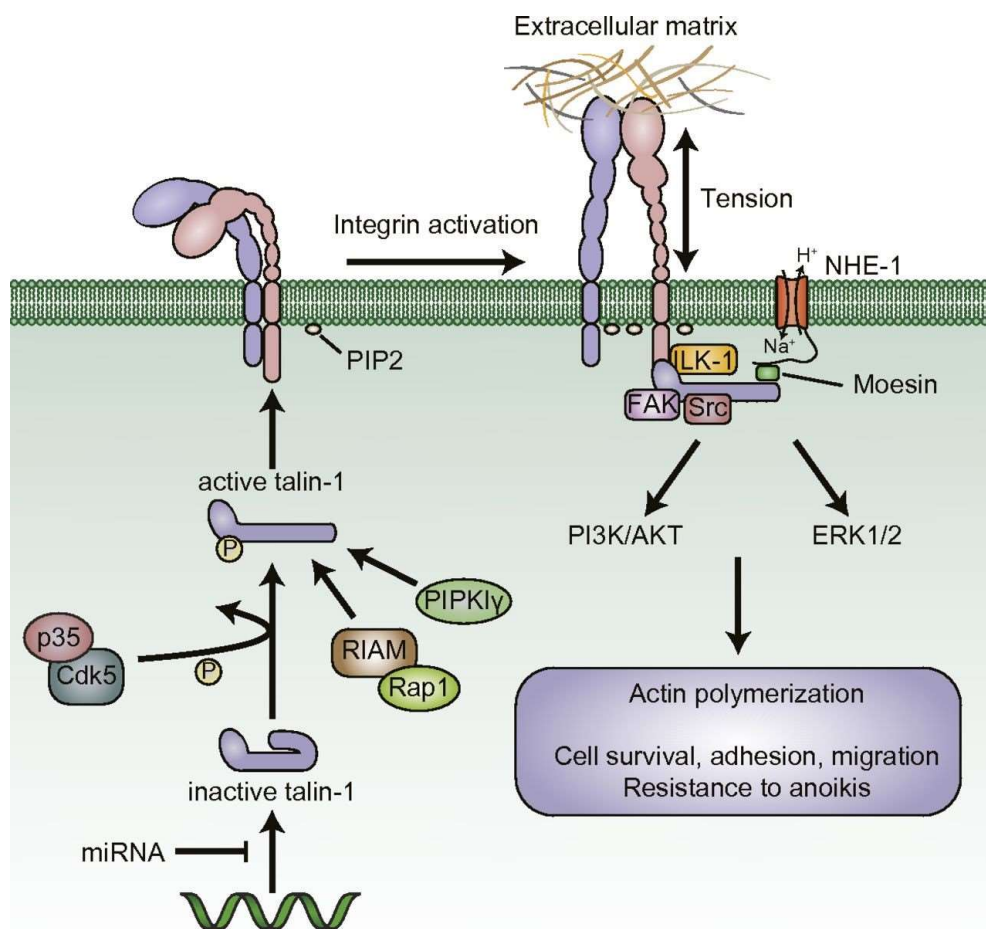


Figure 1.5 Role of talin in malignant phenotypes. Talin is activated by a conformational switch mediated by phosphorylation at residue S425 by Cdk5 as well as recruitment factors such as Rap1-activated RIAM. Upregulation of talin activating proteins is often related to neoplastic transformation. Once at the membrane, PIP2 (generated by PIPK1 γ) further assists in conformational changes in both talin and the integrin cytoplasmic tail separation needed for integrin extension and activation. MicroRNAs may target talin expression levels to influence downstream focal adhesion formation. In the absence of such inhibitors, intermediate or high affinity integrins, generated from talin head binding to the β cytoplasmic tail, ligate with extracellular matrix (ECM) components. If talin is dually anchored by rigid integrin-ECM and cortical actin, the focal adhesion complex can undergo force-induced maturation, leading to enhanced downstream signalling associated with a cancerous phenotype. Likewise, structural biology and steered molecular dynamics have shown that force application activates integrins.³⁶ Tumours generate stiffer ECM, and cells are submitted to greater forces that may trigger the activation of integrin and downstream partners including talin. Membrane-localized talin also recruits the moesin/NHE-1 complex at lamellipodia, which drives a pH gradient needed for ECM remodelling and actin polymerization. Adapted from ^{81-82, 105}. This figure was created by Tyler Lieberthal.

Another important aspect of migration, alongside adhesion, is the formation and stabilization of invadopodia, which are involved in degradation of the ECM and metastasis. ECM degradation is

mediated by the sodium/hydrogen exchanger 1 (NHE-1), which acidifies the peri-invadopodial microenvironment and promotes protease activity;¹⁰⁶⁻¹⁰⁸ NHE-1 has therefore been implicated in metastasis of melanoma, breast cancer, and pancreatic ductal adenocarcinoma as a result of stromal remodelling and often in partnership with other membrane proteins.¹⁰⁹⁻¹¹³ NHE-1 inhibition has also been shown to promote chemotherapy-induced apoptosis *in vitro*.¹¹⁴⁻¹¹⁵ Beaty *et al* determined that talin is required for invadopodia maturation and the recruitment of NHE-1 with the shared binding partner, moesin, to focal adhesions.⁸² Surprisingly, the interaction between the talin head domain and β integrin is not required for talin localization to invadopodia. Nevertheless, silencing of talin prevents intracellular alkalinisation at invadopodia, which is required for actin polymerization, motility, and metastasis in mammary adenocarcinoma cells.⁸² Inhibition of NHE-1 and cytosolic alkalinisation may also affect downstream pathways related to cell proliferation and survival (e.g. RhoA, MAPK).¹¹⁶ Thus, talin anchoring of NHE-1 at invadopodia promotes neoplastic transformation both intracellularly and extracellularly.

Talin's function in cancerous phenotypes is not limited just to its influence on adhesion and motility but rather also depends on its downstream signalling, as emphasized by its overexpression in non-adherent cells. In suspension, cells do not receive mechanical stimulation required for anchorage-dependent survival pathways. However, overexpression of talin-1 confers anoikis resistance via adhesion-independent phosphorylation of FAK, AKT, Src, and ILK-1 effector GSK3 β .⁸¹ This multiplicity of mechanisms demonstrates that talin plays a prominent and varied role in cancerous phenotypes.

The combination of these demonstrates a number of ways in which the expression levels of talin may influence cancer progression. Higher levels of talin lead directly to greater activation of integrins. This integrin activation is vital for the formation of adhesions that are necessary for migration, a critical aspect in cancer metastases. Equally important for metastasis is the formation of invadopodia, and given talin's role in recruiting NHE-1 complexes to these, it is likely that higher talin levels result in more active invadopodia formation and activity. Finally, independent of migration, higher talin expression levels will

boost the phosphorylation of downstream partners that in turn confer resistance to anoikis, another crucial aspect of cancer progression.

Aside from talin's expression levels, it remains to be seen how talin's role as a key component of cellular mechanotransduction might impact its role in cancer progression. The effect of substrate stiffness on cancer metastasis is well-documented,² as is the fact that fibrotic environments (e.g. cirrhosis) are frequently associated with an increase in cancer development and progression.¹¹⁷ Similarly, other mechanical stimuli arising from interstitial or osmotic pressure of solid tumours, which may be a result of uncontrolled cell proliferation and vascular collapse, can also activate genes implicated in neoplastic transformation.¹¹⁸ Because the forces generated from ECM rigidity are transmitted through integrins,¹¹⁹ and by extension talin, the mechanosensitivity of talin could mediate cancerous phenotypes. If it is shown that talin is the key component in a cell's ability to sense substrate rigidity, via a method such as is outlined in Figure 1.4, then that would go a long way to explaining the many links between talin activity and cancer that have been outlined above. Already it has been shown that the increase in malignant progression on stiff ECM is reliant on vinculin recruitment to focal adhesions in a force-dependent mechanism, highlighting the importance of this line of inquiry.¹²⁰ In addition, the role of DLC-1 and its interaction with talin remains to be fully investigated with regards to cancer. Because DLC-1 has been shown to be a potent tumour suppressor and its loss can contribute to the development of cancers,¹²¹ then any alteration to its activity by its association with talin must be considered and investigated as a potential mechanism by which talin controls cancer progression. Therefore, a thorough understanding of all talin's binding interactions and how they are modulated must be fully developed in order to construct a complete picture of its role as a metastatic mediator and potential as a therapeutic target.

1.3.5 Haematological disorders

Due to talin's extensive list of binding partners, there are a number of potential pathologies in which it could be implicated. One of its most critical roles is the activation of integrin mediated by its head domain.

This process is vital to the ability of cells to be able to adhere to substrates, and this is crucial to platelet and neutrophil activation. These cells, usually suspended in the fluid, either in the cardiovascular or lymphatic systems, must adhere to the endothelium and transmigrate once they arrive at the site of injury where they are required to act. This initial firm adhesion relies on the activation and engagement of integrins, which bind leukocytes to solid substrates under shear forces; this activation in turn relies on talin. Talin is required to induce the slow-rolling of neutrophils along substrates that precedes their arrest mediated by the integrin lymphocyte function-associated antigen-1 (LFA-1).¹²² Similarly, in platelets, the loss of talin reduces the activation of integrin affinity.¹²³ Further, knockdown of talin in platelets abrogates adhesion and spreading, which indicates impaired $\alpha_{IIb}\beta_3$ function and the β_1 integrins that cooperate with β_3 integrins, as well as impaired aggregation and thrombus formation.¹²⁴ These mechanistic studies then support the proteome analysis of patients with myelodysplastic syndromes, whose sufferers often experience bleeding complications, that present lower levels of talin, which is likely to have a subsequent effect on integrin activation.¹²⁵ It is clear from many studies that talin is required for integrin activation in hematopoietic cells and talin loss produces leukocyte adhesion deficiency. Kindlin, which cooperates with talin to increase integrin valency, is also associated with leukocyte adhesion deficiency-III (for a review on talin and kindlin interactions see ¹²⁶). Yet, although the presence of talin appears indispensable in most cases, recent evidence suggests that talin recruitment mechanisms are more nuanced in these cells: RIAM is a more potent talin recruitment factor for β_2 integrins and this appears to account for the normal platelet phenotype in RIAM^{-/-} mice, whereas β_2 -expressing neutrophils and T-cells exhibit defects in adhesion and trafficking with loss of RIAM.^{29, 31, 127} Further work is required to determine the alternative pathways by which talin is recruited to β_1 and β_3 integrins to determine how platelets are protected from RIAM deletion.

Talin's mechanosensitivity is evident, perhaps indirectly, by distinct roles it plays in platelet function. Talin^{-/-} platelets exhibit defective clot retraction even in the presence of integrin-activating exogenous manganese. However, clot retraction in a talin mutant (L325R) in which talin retains binding partners

without activating integrins can be rescued with Mn. This rescue can be blocked either by inhibiting actin polymerization or with extracellular integrin antagonists, indicating that clot retraction depends both on talin's ability to activate integrins and to bridge ligand-bound integrins with actomyosin contractile machinery.¹²³ Similarly, talin can upregulate a subset of high affinity conformation $\alpha_4\beta_1$ in Jurkat T-cells in shear stress-free conditions and also contribute to adhesion and shear resistance, whereas paxillin is only involved in the latter process. Talin further mediates CXCL12-stimulated adhesion under shear, yet this chemokine does not alter integrin affinity in the presence of soluble ligand (shear free conditions).⁴⁰ These studies demonstrate that the presence of talin is insufficient for proper cell function; talin must be subjected to force whether by cell contraction or application of shear stress.

Exactly how haematological pathologies link to talin-1 levels is unclear, although clues are provided by a study on von Willebrand disease, another haematological disorder. Type 2B of this disease involves gain-of-function mutations of the von Willebrand factor which impairs the activity of Rap1.¹²⁸ This GTPase is integral to talin's recruitment to focal adhesions via its association with RIAM.⁴⁹ As in the discussion with cancer earlier, some have used these results and attempted to utilise these pathways in designing therapies. For example, existing therapies that seek to decrease platelet aggregation and thus ease atherothrombotic complications involve inhibition of the adenosine diphosphate receptor P2Y₁₂ which has been shown to cause the activation Rap1.¹²⁹ Indeed, putative therapies have been based on the targeting of alternative receptors that also induce the activation of Rap1 but with a different time-dependent profile, such as calcium and diacylglycerol-regulated guanine nucleotide exchange factor 1 (CalDAG-GEFI).¹³⁰ Given this evidence on both sides—on the one hand that talin's activation of vinculin is vital for platelet aggregation and on the other that diseases and therapies affect one of talin's primary regulators, Rap1—it is impossible to escape, once again, the importance of talin's binding interactions to the progression or treatment of diseased phenotypes.

1.4 CONCLUSIONS

Talin, as can be seen in this review, has a prominent role in a number of cellular processes and signalling pathways that are vital to a cell's functionality from its regulation of integrin activation to its apparent influence on Src/ERK pathways. It is also apparent that force restructures talin: its rod domains are able to be unfolded at forces <30 pN and this structural change has an impact on its binding to vinculin.⁵⁷⁻⁵⁸ There remains, however, two avenues of investigation that must be addressed in order to fully cement talin's role as a mechanosensitive molecule. One track must be to establish that this modulation of binding affinity under force occurs natively in cells and not just in the carefully controlled setting of single-molecule setups by investigating talin's mechanosensitivity at the cellular level. Although some studies, such as demonstrating talin's change in length,⁶⁰ have been conducted in cells, there is still a lack of definitive evidence that this extension is important for modulating binding affinities in the same manner as has been shown at the single-molecule level. There are already indications that this would be the case: first by a study that showed that when force is applied by layered actin cytoskeleton to clusters of talin molecules rather than by a piezo cube or magnetic bead, this force does indeed increase the affinity of vinculin binding to this pseudo-focal adhesions.⁶² Secondly, cellular studies have shown that the force on a single integrin molecule peaks at 40 pN during initial adhesion¹³¹ and that individual talin molecules experience average forces in the 7-10 pN range with a proportion experiencing higher forces.⁹² These levels of mechanical tension would be sufficient to unfold the R1-R3 region.⁵⁸ The second track will be to establish whether this unfolding affects the binding of other molecules to talin and their subsequent interactions. The emerging importance of the DLC-1 protein and its localisation to focal adhesions,⁵⁰ as well as the interactions with RIAM⁴² and vinculin's downstream interactions^{72, 74} necessitate studies into the effect of force on all of talin's adapter proteins and the phenomena that they govern.

The importance of these future studies is highlighted by the disease states that seem to be affected by talin's expression and function. Its role in both cancer and haematological disorders is well documented but not necessarily explained, and the inconsistencies remain to be reconciled. It is likely that, given its

wide range influence, these are not the only disease pathologies to which talin contributes, nor are they the only situations where possible therapies might be designed around its role. Recently talin's activation of integrin has been shown to be critical to the function of podocytes in the kidney and that an increase in the processing of talin by calpain-cleavage may aggravate nephrotic syndrome in patients.¹³² There are sure to be more examples, given the well-appreciated importance of the cell-ECM interaction in a variety of areas from cell motility to survival, from cancer to stem cell differentiation. Thus, it is important for us to gain a full understanding of the molecule that seems to be at the centre of it all, in order to fully understand its position and then harness its full potential.

1.5 RESEARCH OBJECTIVES

The conclusions drawn above on the nature of the talin molecule and its role in focal adhesion, and thus cellular, mechanotransduction lead to two further avenues of investigation for this research endeavour. The first line of research is to determine the mechanical properties of the full talin rod. Prior research has focused on the first three bundles of the talin rod, R1-R3.⁵⁷⁻⁵⁸ Given, however, the research mentioned earlier concerning the extension length of the talin rod⁶⁰ and the detailed architectural studies⁶¹ on the talin rod in cells, it is highly likely that the remaining 10 bundles of the rod are involved in talin's mechanosensitivity. Thus, it is necessary to understand how these remaining bundles respond to force and the nature of their unfolding dynamics.

Secondly, there is a need to study the mechanosensitivity of talin in cell models. This is needed both to confirm that the molecular studies are correct in suggesting talin's role in cellular mechanotransduction and also to help elucidate how its mechanosensitivity might influence the role of its important binding partners. Given the role that its binding partners such as DLC1 have in cancer progression,¹³³⁻¹³⁵ this line of investigation may give some hints as to the reason for talin's seeming importance in disease phenotypes.

This leads to two clear research objectives:

1. Investigate the unfolding dynamics of all bundles in the talin rod
2. Conduct cellular experiments to investigate talin's mechanosensitivity and the impact that this may have on key binding partners

The first of these research objectives is approached in Chapters 3 and 4. The second research objective forms the motive behind Chapter 5.

2 SINGLE-MOLECULE ATOMIC FORCE MICROSCOPY

2.1 ATOMIC FORCE MICROSCOPY

α -helical structures within proteins, like those found in the talin rod, are extremely sensitive to mechanical tension. They have been shown to unfold in the range of tens of piconewtons, much weaker than the structures that can be formed by β -sheets.¹³⁶⁻¹³⁷ As such their unfolding dynamics are difficult to study, requiring a spectroscopic device that is capable of resolving piconewton-scale forces and nanometre-scale extensions: an atomic force microscope (AFM).

The AFM was devised by Binnig and Quate in the 1980's as an extension to scanning tunnelling microscopy (STM), extend the ability to profile surfaces at an atomic scale.¹³⁸ STM functioned by measuring the tunnelling current between the tip of a stylus and the surface to be studied, as the stylus was moved over the surface. The stylus' position (or the surface's) in the z-axis was carefully controlled, typically by a piezoelectric actuator, so as to maintain a constant tunnelling current.¹³⁹ By recording the spatial adjustments necessary to maintain this current, a 'picture' of the surface's topography could be constructed. The drawback of this technique was that it required a conductive surface. AFM was designed to remove this restriction by introducing a second stylus, or probe. This conductive AFM probe was brought into physical contact with the non-conductive surface to be investigated. The resultant deflections in the AFM probe were then measured by an STM probe.

Although this early AFM setup was successful at characterising non-conductive surfaces it was complicated by the use of STM as detection device. In 1988, Meyer and Amer adapted AFM using a laser detection device instead of STM. In this method, a laser was reflected off the back of the AFM probe at an angle and onto a detector, which was given a reflective coating. As the AFM probe was deflected, the resultant shift in laser position on the detector was measured (Figure 2.1). The laser displacement can

be calibrated to AFM probe deflection by moving the surface through a set distance and measuring the resultant displacement.¹⁴⁰

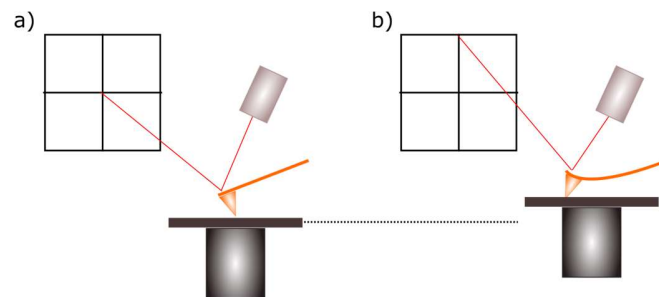


Figure 2.1 Deflection of AFM probe as measured by laser displacement. (a) A laser is reflected off the back of the AFM probe onto a detector. (b) As the surface deflects the AFM probe, the laser position on the detector is displaced. This displacement is measured and used to determine the surface topography.

2.2 FORCE SPECTROSCOPY USING AFM

Although AFM was designed as an instrument to measure surface topography, its sub-nanometre resolution was found to be useful in other scenarios, most notable force spectroscopy. In this mode the deflection of the cantilever is translated into a force, using the calibrated cantilever spring constant of the probe.¹⁴¹ This allows the AFM to probe the mechanical properties of a surface, as well as its topography. This can be used to measure the properties of a compliant surface by indenting it with the probe and measuring the resultant force,¹⁴¹ to measure ligand-receptor unbinding,¹⁴² or to measure the unfolding of modular proteins (Figure 2.2).¹⁴³

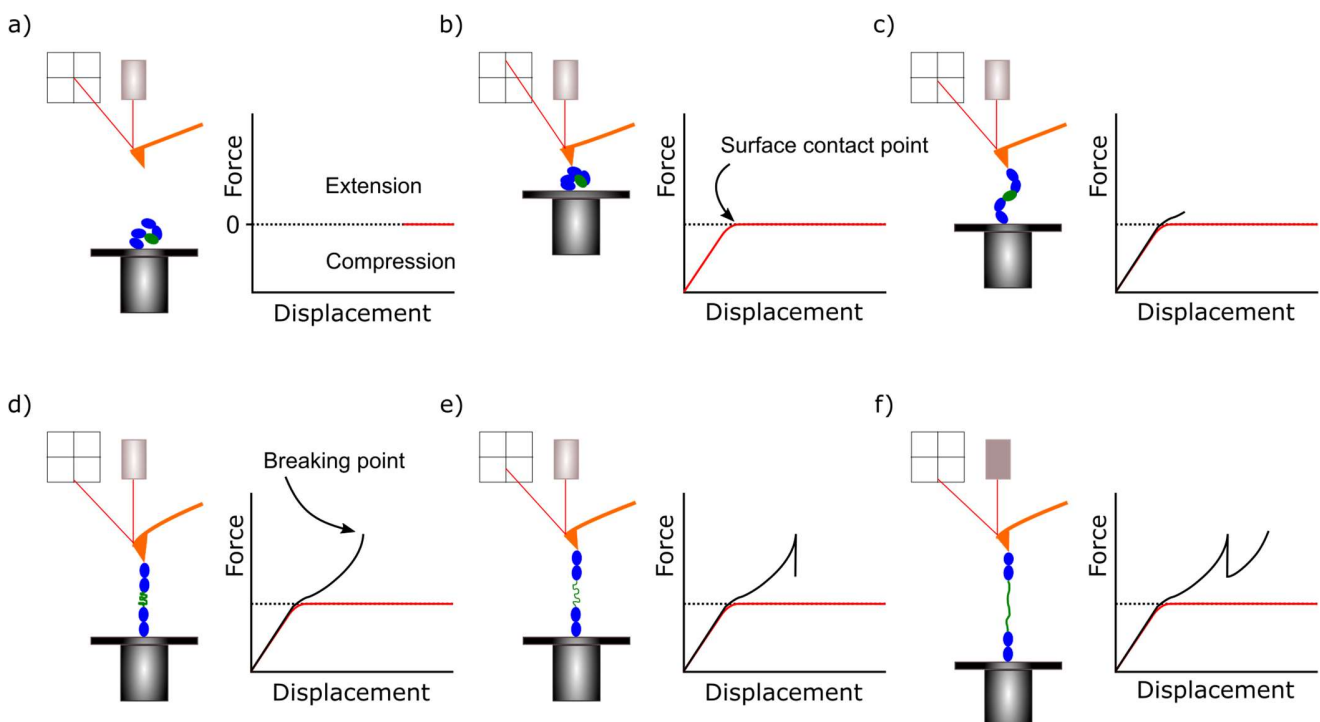


Figure 2.2 Use of AFM for single molecule force spectroscopy. (a) As the AFM probe approaches the surface-mounted protein (blue and green ovals), there is no force registered before contact. (b) The probe is pressed into the surface to attach to the protein, registering a negative (indentation/compression) force. (c) As the probe is retracted and the protein is extended, tension builds in the protein, deflecting the probe and resulting in a positive (retraction/extension) force. (d) At a threshold force the weakest subdomain (green) reaches a breaking point and its tertiary protein structure is disrupted. (e) The disruption of the tertiary structure releases tension and allows the probe to retract, reducing the measured force. (f) As the disrupted protein is stretched, the tension builds in the protein again, increasing the deflection of the cantilever and thus the measured force.

2.2.1 Calibration of AFM probe spring constant

In order to use an AFM in force spectroscopy mode it is necessary to calibrate the spring constant of the AFM probe so that its measured deflection can be converted into a force. One of the most commonly used methods was developed by Hutter and Bechoefer.¹⁴⁴ By modelling the cantilever as a weight on a spring, and invoking the equipartition theorem, it is possible to relate the spring constant to the magnitude of oscillations that the cantilever undergoes in response to thermal noise:

$$k = \frac{k_b T}{\langle q \rangle^2} \quad (1)$$

where k is the probe spring constant, k_b is Boltzmann's constant, T is the temperature measure in Kelvin, and $\langle q \rangle^2$ is the mean-square spring displacement. If the thermal fluctuations are measured in the frequency domain then the mean-square spring displacement is equal to the integral of the power spectrum. To fully calibrate the probe the sensitivity, or relationship between the change in laser displacement and cantilever deflection must also be measured. This is most commonly achieved as described earlier: the probe is brought into contact with a hard surface, it is then moved by a set distance. In this way the exact cantilever deflection is known, and the laser displacement can thus be measured to determine the sensitivity.

This Hutter method was shown by Butt and Jaschke¹⁴⁵ to need adjustment if the sensitivity is measured as described above. This is because the shape of a cantilever undergoing thermal fluctuations is different to a cantilever that is being loaded at its point. Since the laser reflection measures the inclination of the cantilever rather than its deflection, it is necessary to take this discrepancy into account. This leads to a correction factor, $\beta^* = 0.817$, that must be applied to the spring constant to Equation 1 when using rectangular probes.¹⁴⁵ This correction factor was used throughout this research project, as only rectangular probes were used.

3 CHARACTERIZATION OF THE FULL TALIN ROD

This chapter has been adapted from work published in ACS Nano, under the title *All subdomains of the talin rod are mechanically vulnerable and may contribute to cellular mechanosensing*. It was a joint work undertaken with Vesa Hytonen's group at the University of Tampere. They are responsible for the SMD results shown. Results were interpreted, and conclusions drawn collaboratively.

3.1 INTRODUCTION

As discussed in Chapter 1, talin plays a vital role as the connection between trans-membrane integrins and actin cytoskeleton. Alongside this structural role, it also acts as a regulatory hub for numerous important binding partners. Previous research has demonstrated a possible link between talin's structural and regulatory functions: the tension that it is exposed to when performing its structural role has the potential to disrupt its tertiary protein structure and affect its binding to partners.⁵⁶⁻⁵⁷

These previous studies on talin's mechanosensitivity have been focused around a small, N-terminal sub-region of the rod, bundles R1-R3. The compact nature of this region and the abundance of VBSs leads to the possibility that this region alone is responsible for talin's mechanosensing while the remainder of the rod, proposed to be linear in structure, is responsible for force propagation.⁴² Up until now, however, the response of this linear region to force has never been investigated.

The fact that talin is the most prominent candidate for consideration as a cellular mechanosensor means that the elucidation of the biomechanical properties of the full rod in response to force is indispensable to the understanding of its function. The presence of numerous relevant binding sites in the remainder of the rod (R4-R13), including six VBSs and binding sites for other proteins including RIAM, synemin and DLC1, means that the response of this region to force could have a profound effect on the modulation of talin's interactions.^{48-49, 123}

There are, however, difficulties associated with studying the full-length talin rod using single-molecule techniques. Firstly, it is a large, amphipathic domain (~2000 aa) and as a result can be difficult to handle. Secondly the length of the fully-stretched talin rod domain is longer than the maximum travel distance (<1 μm) allowed by high-resolution single-molecule atomic force microscopy (smAFM) setups. Thirdly studies conducted on the complete rod would lose information about how the observed unfolding relates to individual bundle structures.

In this chapter, these restrictions are circumvented by separating the rod into smaller subdomains. These subdomains are then analysed using a state of the art smAFM setup, combined with recent advances in single-molecule stretching techniques. The subdomains are inserted into polyprotein constructs; these constructs contain flanking, fingerprinting molecules that allow for correct trace determination through a characteristic unfolding pattern (Figure 3.1b,c).⁵⁷ The constructs also contain an N-terminal HaloTag enzyme that, when coupled to ligand-prepared surfaces, ensures that the talin subdomains are extended in the desired orientation.¹⁴⁶ This unique combination of smAFM techniques allows us to establish an outline of the pattern of unfolding of the talin rod. All-atom steered molecular dynamics (SMD) simulations are then performed on each of the individual bundles, to provide a profile for how this applied tension is rearranging these bundles at an atomic level. These complementary datasets allow us to compare the relative stabilities of the bundles for the full rod and thus propose a sequence for how talin might unravel under force application *in vivo*.

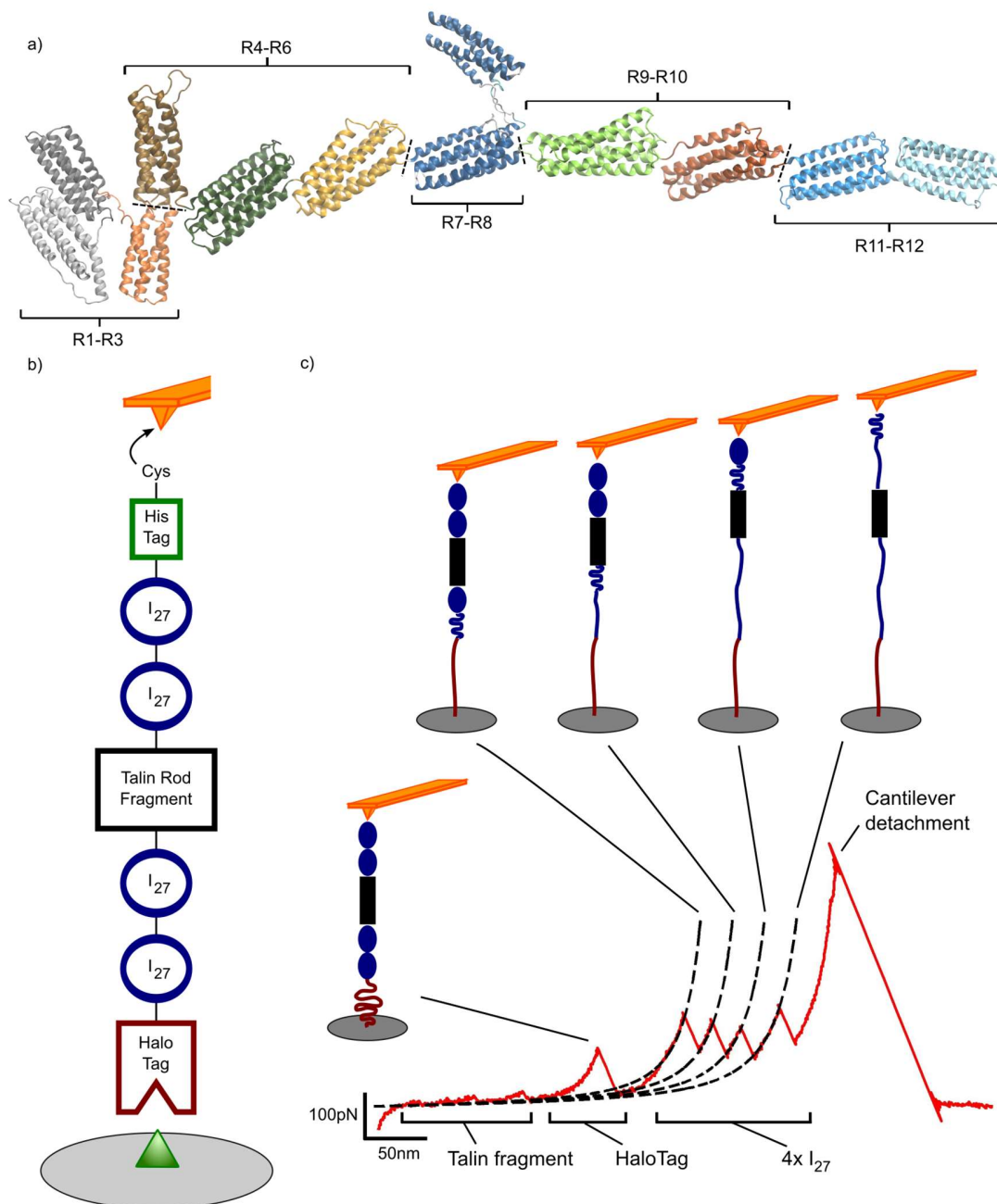


Figure 3.1 Talin rod poly-protein constructs for smAFM experiments. (a) Crystal structure of talin rod, showing the α -helical bundles R1-R12. The rod was separated into different fragments as indicated for the AFM experiments. (b) Schematic of the polyprotein constructs containing: the talin fragment to be investigated; four flanking I_{27} domains for fingerprinting; the HaloTag enzyme that binds to its associated ligand on the surfaces; a HisTag for protein purification; and a terminal cysteine for cantilever tip attachment. (c) Example of fingerprint used for identification. After the unfolding of the talin rod fragment, the first peak corresponds to the unfolding of the HaloTag enzyme. This is followed by the unfolding events for the four I_{27} domains, creating the characteristic 'sawtooth' pattern. The final peak represents the detachment of the polyprotein from either the surface or the cantilever. The dashed lines indicate a worm-like chain model fit applied to the I_{27} unfolding events. (b, c) Not to scale.

3.2 METHODS

3.2.1 Generation of polyprotein constructs

The constructs and experimental procedure for the smAFM were similar to those described before.¹⁴⁷ The talin fragment polyprotein constructs, including flanking I27, were synthesized and cloned in to pFN18a (Appendix 1). The polyproteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RILP competent cells, using the T7 promoter present in the plasmid. Protein expression was induced with IPTG when the culture reached an OD_{600 nm} of 0.6. Cells were lysed by applying 0.2 mg/ml lysozyme for 30 minutes at 25 °C, followed by sonication with a sonifier cell disruptor model SLPe (Branson Ultrasonics Corporation, USA) and clarification of the lysate using centrifugation. The clarified lysate was subjected to Ni-NTA affinity chromatography beads in a batch process. The proteins eluted with imidazole were analysed for purity with SDS-PAGE and used at a final concentration between 1-10 µg/mL.

3.2.2 Preparation of ligand-functionalized surfaces

Glass coverslips were functionalized with the chloroalkane ligand to HaloTag as previously described.¹⁴⁶ The glass coverslips were first cleaned using Helmanex III (1% in water), acetone and ethanol washes. The surfaces were then prepped with O₂ plasma cleaning for 15 mins. Surfaces were then silanized using (3-aminopropyl)trimethoxysilane, diluted to 1% in ethanol. Surfaces were then washed with ethanol and then dried with N₂. These amine-functionalized surfaces were then incubated with 10 mM succinimidyl-[(Nmaleimidopropionamido)tetracosaethyleneglycol] ester (SMPEG24 – Thermo) diluted in 100 mM borax buffer (pH 8.5) for 1 h. The final step involved incubating the surfaces overnight with 10 mM HaloTag Thiol O4 ligand in the same buffer. The surfaces were quenched 50 mM 2-mercaptoethanol in water.

3.2.3 AFM experiments and analysis

We used a commercial AFS-1 from Luigs & Neumann, GmbH, based on a device developed at the Fernandez Lab, Columbia University.¹⁴⁸ The cantilevers used were gold-coated OBL-10 levers from Bruker. The spring constants varied between 4-10 pN/nm as measured by equipartition theorem with the appropriate adjustments for cantilever geometry.¹⁴⁴⁻¹⁴⁵ Around 20 μ l of protein solution was incubated on functionalized coverslips for 30 mins prior to the experiments to allow for HaloTag binding. The cantilever was pressed into the surface with a force of $\sim$ 300 pN to bind the cantilever to the polyprotein. Force extension experiments were conducted at 400 nm/s retraction rate. Data analysis was carried out using Igor Pro (Wavemetrics) using custom analysis scripts (Appendix 2) where the worm-like chain model was applied.

3.2.4 Steered Molecular Dynamics (SMD) simulation

SMD is a technique whereby the effect of force on proteins is computer simulated on an atom-by-atom basis. It provides much greater insight into how a protein unfolds beyond the forces involved. It has been used previously to demonstrate that vinculin binding to talin requires the unfolding of bundles.⁵⁶ The SMD in this thesis was performed by Vesa Hytönen's group at the University of Tampere.

In SMD all tested talin rod domains were analysed for mechanical stability separately with the exception of R7. Bundle R7 was modelled together with inseparable R8 domain. The following structures from RCSB Protein Data Bank (RCSB PDB) were used for the models; R3 (id 2L7A residues 796-909), R4 (id 2LQG), R5 (id 2L7N), R6 (id 2L10), R7-R8 (id 2X0C), R9 (id 2KBB), R10 (id 2KVP), R11 (id 3DYJ residues 1975-2145), R12 (id 3DYJ residues 2131-2291). Anchors and extra sequence parts used for structure crystallization were removed from all analysed models in PyMOL ver.1.4.1.

SMD simulations were performed using Gromacs ver4.6.7. at Sisu supercomputer, CSC, Finland.¹⁴⁹⁻¹⁵⁰

For each model CHARMM27 force field and explicit TIP3P water model in 0.15 M KCL neutral solution

was used. Pulling vector was set between C α of first and last residue of appropriate domain. Consequently, each structure was oriented according to the pulling vector in z direction of the simulation box. Each model was minimized to 100,000 steps and equilibrated for 1 ns. The system equilibration was monitored by following the temperature, pressure and density parameters over time. The structure equilibration was assessed by plotting the backbone RMSD value for each bundle subjected to pulling throughout the equilibration, *i.e.* R8 was omitted from the analysis. For all bundles the RMSD reached plateau within 0.8 ns of 1 ns equilibration. All equilibration parameters were stabilized within 1ns for all modelled structures. Simulations were performed with constant velocity pulling at 0.1 nm/ns ($n=1$) and 2 nm/ns ($n=3$). All simulations were run at NPT conditions with Berendsen thermostat and Berendsen barostat. Temperature control was set to 310 K and time constant 0.1 ps and pressure control to 1.0 bar and compressibility 4.5×10^{-5} bar. Temperature and pressure control were applied separately for protein and solution parts. Furthermore, equilibration was performed at isotropic conditions while pulling at semi-isotropic conditions where pressure control was turned off in the pulling direction (z-axis). The conditions used were validated by using an extensive set of simulations. Spring constant was set to 1,000 KJ/mol nm.²

Force of the spring was calculated according to $F = k_{spring}(x_{ideal\ t} + x_0 - x_t)$, where k is the spring constant, $x_{ideal\ t}$ is the ideal elongation with velocity at time point, x_0 is the pulling vector length at time=0, and x_t is the pulling vector length at time point. Resulting trajectories were analysed with VMD ver1.9.1 at 100 ps time window.¹⁵¹ Average of 10 force values was presented to minimize fluctuation and eliminate noise caused by low time step (2 fs) and sampling (1000) setting for data collection and additional 5 window stride load setting in VMD analysis.

3.3 RESULTS

3.3.1 Generation of talin rod constructs

The talin rod is comprised solely of alpha-helical bundles and the structures of these individual bundles have been resolved using structural biology methods. In this thesis, the current accepted structural model of the full-length talin rod was used.¹ The talin rod consists of 63 α -helices, which then form into bundles (R1-R13), each containing four or five helices. The talin rod was first divided into five separate fragments in order to characterise their unfolding properties under force application by smAFM. The fragments were based on the best available knowledge of the organization of the bundles for the whole rod (Figure 3.1a): the boundaries between the fragments were placed similarly to those used to fragment the rod for the determination of the crystal structures.⁴² In the case of two of the fragments, the boundaries were dictated by their structures: bundle R8 is inserted between the third and fourth helices of R7 and thus they were expressed together; and R11 and R12 share a common, elongated helix between them. The fragment R1-R3 has previously been analysed and thus was included as a reference to those earlier works.² The remaining bundles, R4-R6 and R9-R10, were clustered into two fragments. R13 was excluded as talin's primary actin binding site occurs in this bundle and thus it is unlikely to be extended from its N and C termini.

In order to improve the efficiency of data capture and analysis, these fragments were engineered into polyprotein constructs (Figure 3.1b). All constructs were produced in *E. coli* and purified to homogeneity according to previous protocols.^{57, 148} These constructs ensure that the talin fragments were extended in the correct orientation, *i.e.* from their N and C termini. The use of an N-terminal HaloTag protein and C-terminal cysteine residue has been shown previously to provide specific, stable attachment of polyproteins to the Halo-ligand-functionalized smAFM surface and gold-coated cantilever tip, respectively.³ The immunoglobulin domain, I₂₇, from titin has been used to provide a molecular 'fingerprint' to determine valid force extension traces.^{57, 152} Only traces which displayed the unfolding of the HaloTag and the characteristic 'sawtooth' pattern of at least three I₂₇ domains were accepted for

analysis (Figure 3.1c and Figure 3.2). It is possible that the inclusion of flanking I_{27} domains may affect the unfolding properties of the talin sub-domains through non-specific protein interactions. This is expected to have a minimal effect on the results as the non-specificity of these interactions is likely to be weaker than the tertiary structures of the sub-domains themselves. Once the polyprotein has undergone initial stretching the I_{27} domains will have separated and thus any potential effect will be abrogated. The use of I_{27} domains in this way is well established in the literature.^{57, 153}

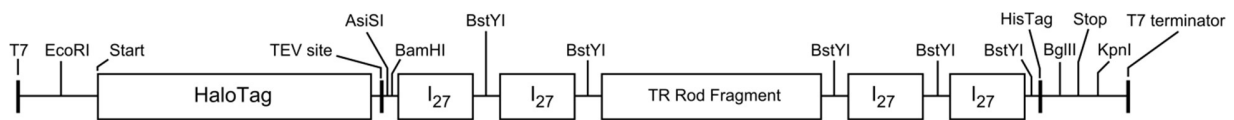


Figure 3.2 Partial vector map for polyprotein plasmids. The main features and restriction sites are shown for the T7-promoted coding region.

3.3.2 Unfolding pattern of talin rod fragments

The talin rod constructs were extended at a constant velocity (400 nm/s) in order to investigate how the individual fragments unfold. The molecular fingerprint, first used to filter for valid traces as discussed above, was then used to match traces to one another by aligning the sawtooth patterns of the I_{27} domains. These aggregated overlaid traces display that the talin rod fragments unfold in a consistent manner with defined unfolding events (Figure 3.3a-e). For each trace, these unfolding events were then fitted to a Worm-Like Chain model and the unfolding length of each event was measured, with the HaloTag peak as a reference point. Histograms of these unfolding lengths demonstrate the number of discrete unfolding events and the regular intervals at which they occur (Figure 3.3f-j).

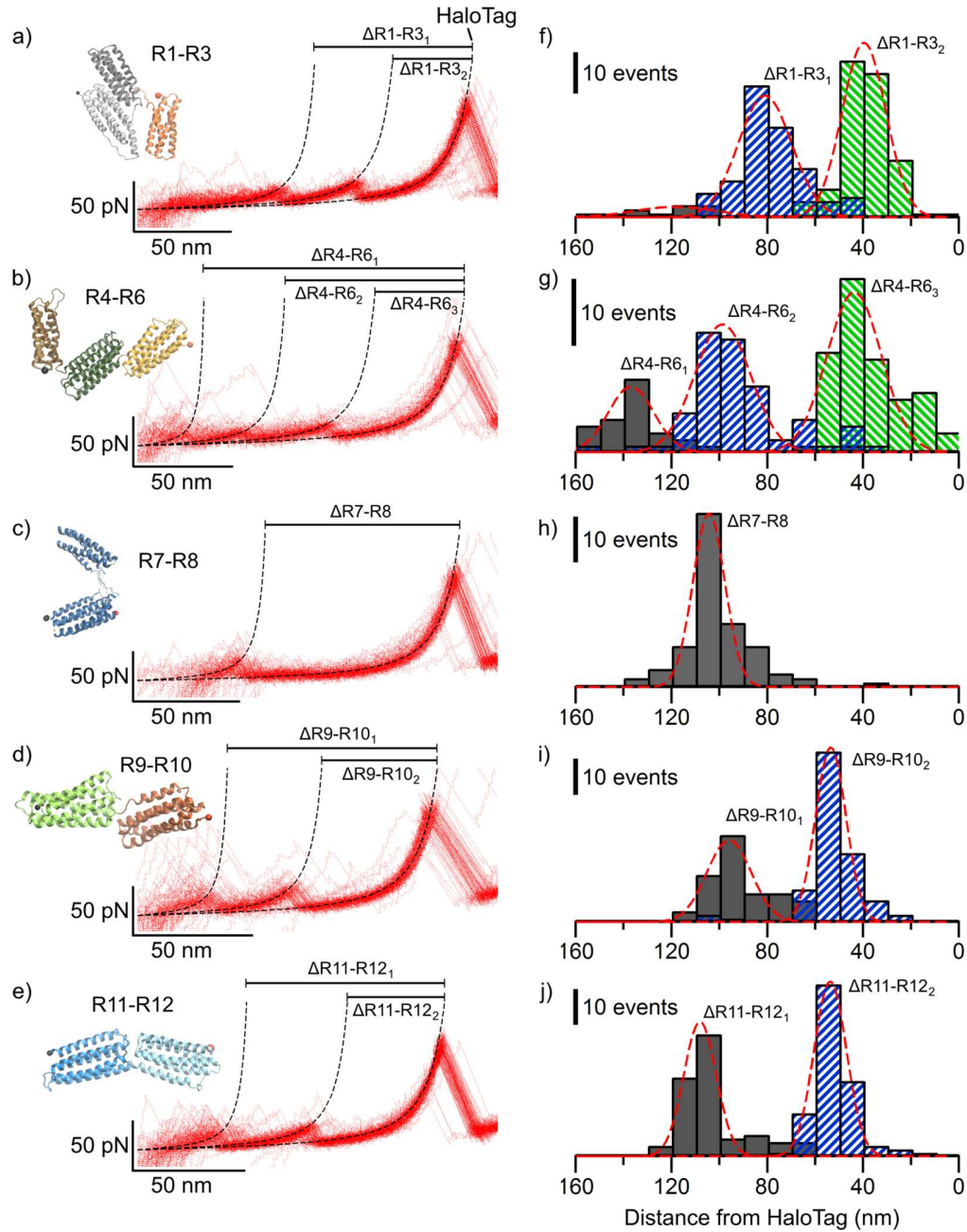


Figure 3.3 Unfolding pattern of talin rod fragments. (a-e) Aggregated force extension traces reveal the unfolding pattern of each fragment. The fingerprints of each trace were used for alignment, revealing the number of unfolding events and the extension associated with each. Inset: crystal structures for each fragment. Each event is fitted to the worm-like chain model (dashed lines). The method for measuring the extension of each event is also shown. (f-j) Histograms of the extensions associated with each unfolding event. The extension associated with each unfolding event was measured, using HaloTag as a reference point. The x-axes have been reversed so that the order of the unfolding peaks in the histogram matches the order of unfolding in the traces, i.e. the furthest peak from HaloTag is the first to unfold. Red dashed lines show Gaussian fits applied to the histograms. Extension lengths are summarized in Table 3.1. (a, f) R1-R3, number of traces analysed, $n = 101$. (b, g) R4-R6, $n = 76$. (c, h) R7-R8, $n = 82$. (d, i) R9-R10, $n = 91$. (e, j) R11-R12, $n = 103$.

For R4-R6, R9-R10 and R11-R12 the number of unfolding events is the same as the number of bundles in the fragment. This indicates that each bundle has a single dissociation event that allows it to unravel. The distances between the peaks of the histograms can be analysed to determine the length of unfolding that occurs for each event. These lengths tend to be 10-30% shorter than the theoretical fully-extended length of the amino acid chain for each bundle (Table 3.1).¹⁵⁴ This indicates that either the bundles partially unravel before their main dissociation event or that, after they dissociate, they still retain some of their α -helical structure.

For R1-R3, a three-bundle construct, only two events were usually observed, although in several traces the anticipated three events were discernible. This apparent difference is explained by previous work on this fragment that has shown that the R3 bundle unfolds at forces as low as 5 pN which is below the resolution of this smAFM apparatus.⁵⁸ As with the other fragments, the length of unfolding is less than the theoretical maximum length of the amino acid chains (Table 3.1).

The R7-R8 fragment is different to the others both in structure and in the unfolding pattern that it generates. In all traces only a single unfolding event occurs, despite the presence of two bundles. Additionally, the unfolding length of 104 ± 7 nm is much greater than the potential unfolded length of either individual bundle (Table 3.1). This indicates that a single dissociation event results in both bundles unravelling. This notion is supported by the structural work on this fragment which demonstrated that the R8 bundle is much weaker than the R7, and stabilised by its insertion between the third and fourth helices of R7.⁵ It can therefore be surmised that once the R7 bundle is ruptured and begins to unravel, the instability of the R8 bundle allows it to be unravelled without a second dissociation event.

Table 3.1. Summary of smAFM unfolding events

		Force (pN)	Position (nm)	Position Delta (nm)	Theoretical Maximum Extension (nm)	Observed Relative Extension (%)	Number of Events
R1-R3	Peak 1	9 ± 1	118 ± 17	37 ± 28	161	71	11
	Peak 2	19 ± 3	81 ± 11	41 ± 20			90
	Peak 3	25 ± 2	40 ± 9	40 ± 9			100
R4-R6	Peak 1	11 ± 3	137 ± 10	38 ± 21	162	85	26
	Peak 2	14 ± 3	99 ± 12	55 ± 23			65
	Peak 3	21 ± 2	44 ± 11	44 ± 11			74
R7-R8	Peak 1	20 ± 3	104 ± 7	104 ± 7	115	90	82
R9-R10	Peak 1	17 ± 3	96 ± 9	42 ± 16	116	83	62
	Peak 2	26 ± 4	54 ± 6	54 ± 6			86
R11-R12	Peak 1	18 ± 4	108 ± 7	54 ± 14	121	89	78
	Peak 2	22 ± 4	54 ± 7	54 ± 7			88

Forces and positions are the mean $\pm$ standard deviation of the Gaussian fits shown in Figures 2 and 3. Positions are measured relative to HaloTag peak. Theoretical maximum extensions are calculated using the known number of amino acids and a fully-extended length of 0.4 nm per amino acid.¹⁵⁴

3.3.3 Talin rod bundles dissociate over a range of forces

As well as illuminating the pattern of fragment unfolding, the force extension data can be analysed to determine the force associated with each unfolding event. These forces were combined into histograms that demonstrate the range of forces involved (Figure 3.4a-e). The unfolding of all talin rod fragments seems to occur over a range of forces between 10-40 pN. Within that range, individual unfolding events seem to occur at specific forces, indicating that different bundles may exhibit different levels of mechanical stability (Table 3.1). This indicates that talin may consist of mechanical elements with a range of mechanical stabilities, such as has been proposed previously for fibronectin.¹⁵⁵ Further analysis was performed to dissect the mechanical characteristics of the talin rod components.

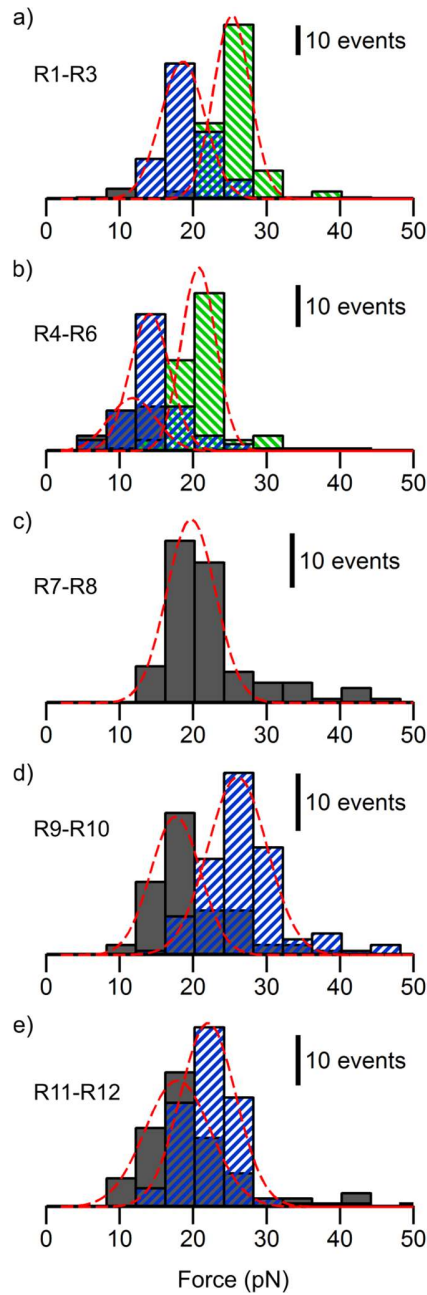


Figure 3.4 Unfolding force of talin rod fragments using smAFM. (a-e) Histograms of the unfolding force for each event. Grey bars represent the unfolding event furthest from HaloTag and thus the first to occur. Blue bars, where applicable, show the next event further from HaloTag. Finally, green bars show the last unfolding event, and closest to HaloTag, for those traces exhibiting three events. Red dashed lines show Gaussian fits applied to the histograms. Forces are summarized in Table 3.1. (a) R1-R3. (b) R4-R6. (c) R7-R8. (d) R9-R10. (e) R11-R12.

3.3.4 SMD and AFM analysis reveal hierarchy of bundle stability

To demonstrate the unfolding pattern of talin, SMD data generated by Vesa Hytönen's group has been included here to complete the picture. Constant velocity end-to-end pulling simulations at 0.1 nm/ns and 2 nm/ns revealed that all tested talin rod domains unfold over a range of forces (Figure 3.5). 2 nm/ns constant velocity simulation allowed us to obtain a comprehensive picture of the unfolding pathway within 20 ns simulation. Three simulations were performed for each of the bundles to ensure the reproducibility of the traces obtained. Simulations at 0.1 nm/ns focused on the initial breaking of the domains over approx. 40 ns. This lower pulling velocity was used to test the method sensitivity and the impact of the velocity on the final force magnitude, rather than for a comparison of trajectories. Overall, similar patterns of unfolding force magnitude were observed for both tested velocities. The pulling rates used for these simulations were much greater than physiological, with the expectation that pulling at speeds closer to those expected *in vivo* would result in dramatically lower forces as revealed by the smAFM measurements. The loading rate applied to a biological interaction influences the force at which this interaction is disrupted, with greater loading rates resulting in higher unfolding forces.¹⁵⁶⁻¹⁵⁷ This explains the large difference between the SMD and smAFM unfolding forces as the loading rates are different by orders of magnitude.

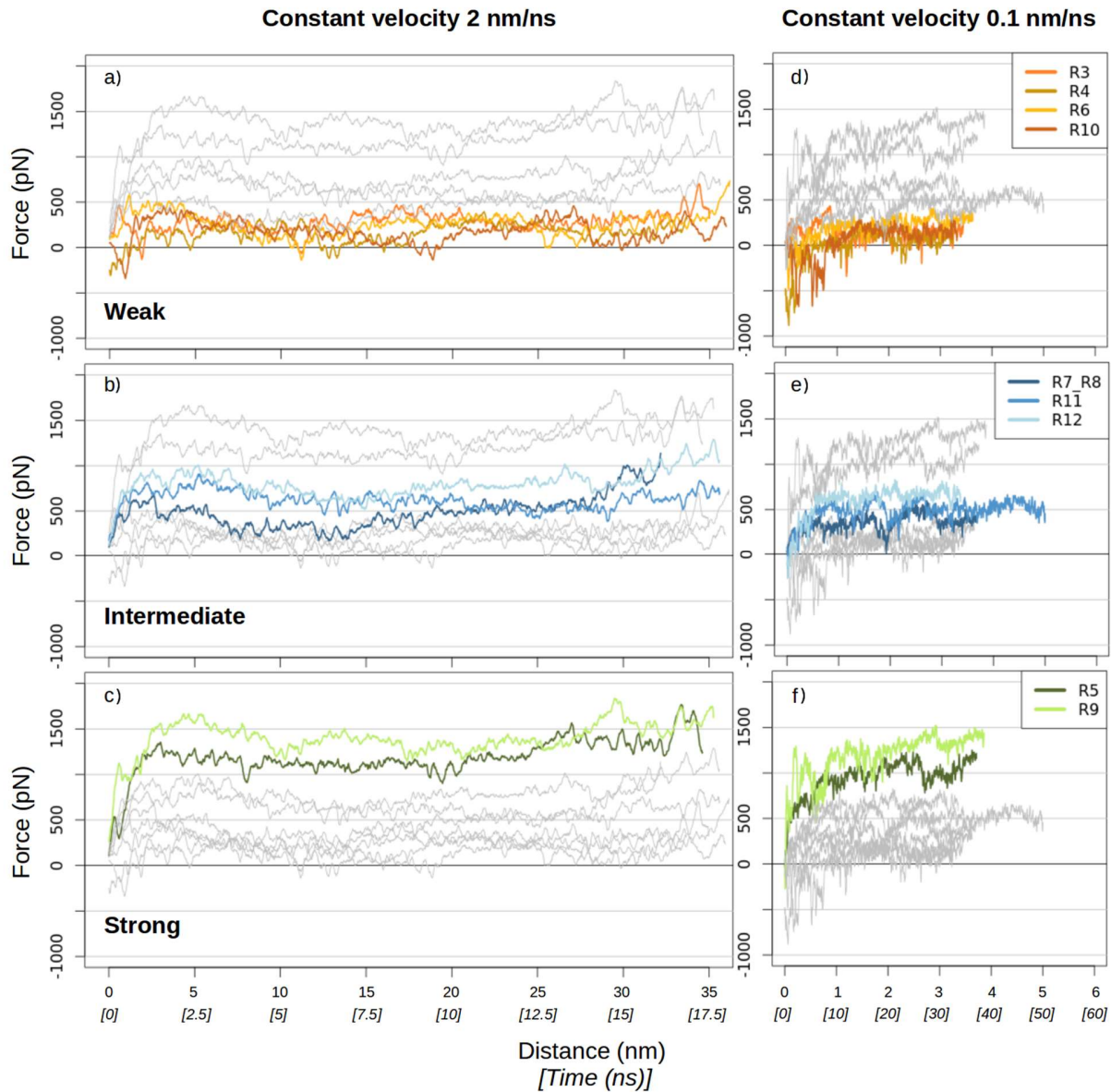


Figure 3.5 Unfolding force magnitude (pN) of talin rod domains in SMD . (a-c) Force against simulation time during constant velocity pulling at 2 nm/ns. (d-f) Force against simulation time during constant velocity pulling at 0.1 nm/ns. Grey lines visualize the force magnitude for all bundles. The tested rod domains show varying mechanical stability represented by different unfolding force magnitude. All rod domains are classified into three groups which are colour highlighted in separate plots against the complete dataset. (a and d) Weak class (orange) contains R3, R4, R6 and R10 bundles unfolding under maximum unfolding force 500pN; (b and e) Intermediate class (blue) contains bundles R7, R11 and R12 unfolding between 500 – 1000pN; (c and f) Strong class (green) with R5 and R9 bundles with unfolding force over 1000pN. Similar classification was observed for both simulation settings at 0.1 nm/ns and 2 nm/ns velocity. This figure was created by Magdalena von Essen.

Due to the character of the simulations, calculated unfolding forces from SMD are only relative and cannot be directly compared to the experimental unfolding forces. Despite the large differences in force magnitude, the unfolding trajectories illustrate very similar unfolding dynamics over the tested domains (Figure 3.6). Moreover, small differences in structural changes result in large changes in the calculated force magnitude. Hence, SMD unfolding force is used here only for pattern analysis and talin rod domain stability classification, but not for providing predictions for the absolute unfolding force. This is a similar approach to that presented in the work by Craig *et al.* concerning the stability of fibronectin Ig domains.¹⁵⁵ It is also good to keep in mind that the cellular processes may happen in the timeframe of milliseconds to hours, not reachable by all-atom simulations.

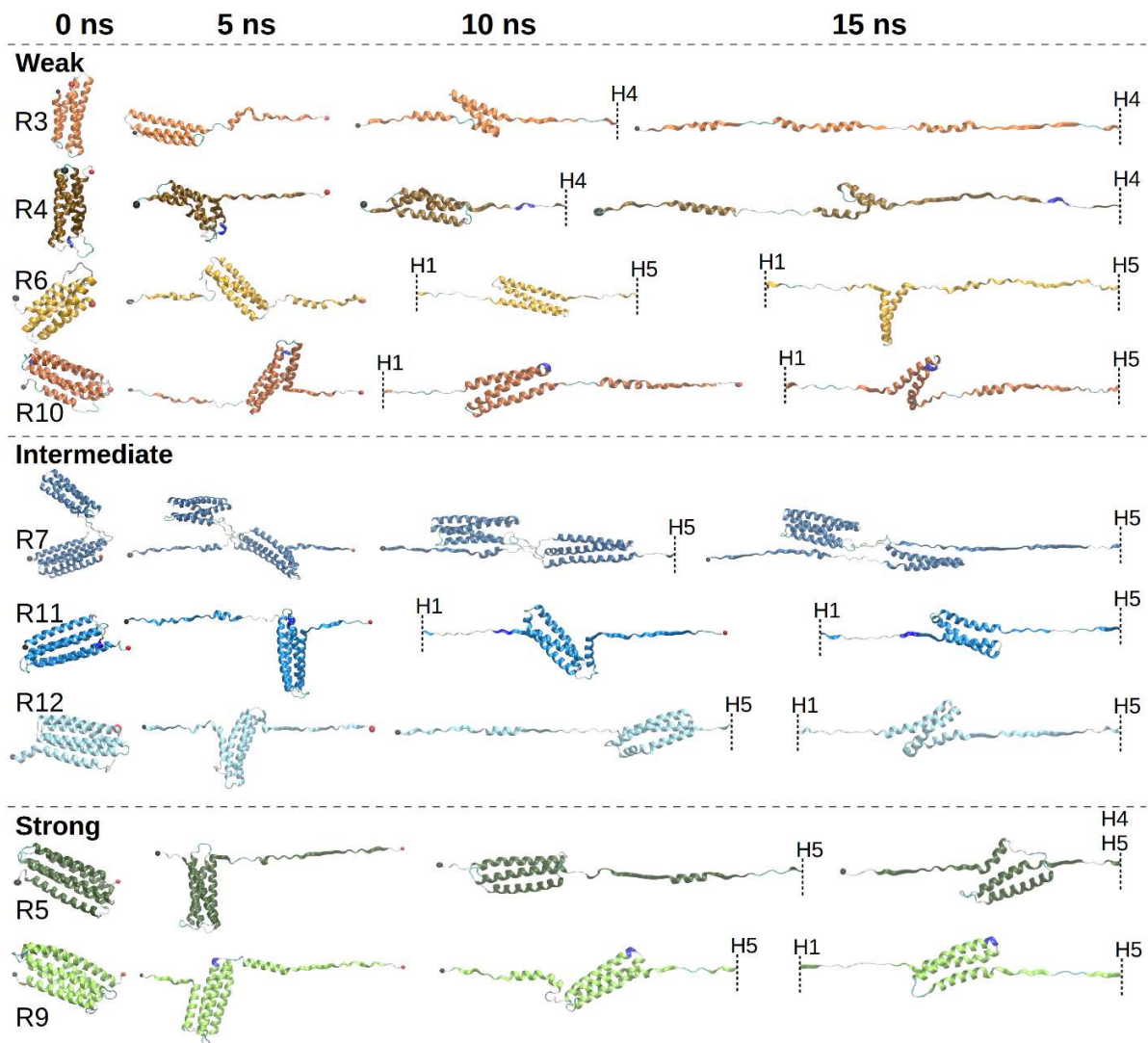


Figure 3.6 Representative structure snapshots of talin rod domains from SMD. Structure snaps were captured at 0, 5, 10 and 15 ns of the constant velocity pulling at 2 nm/ns. The three stability groups (weak - orange, intermediate – blue, strong - green) show mild yet distinct differences in the domain unfolding. Fully unfolded helices are cut away and presented by dashed line with a helix identifier. This figure was created by Magdalena von Essen.

Based on the relative force magnitude and unfolding trajectories, the tested talin alpha-helix bundles are classified into three stability groups: weak, intermediate and strong. The weak class contains the easily unfolding four-helix bundles R3 and R4. The R3 bundle contains a belt of polar residues in its hydrophobic core unique among the talin rod bundles. This polar pattern may cause R3 destabilization and unfolding

under low mechanical load. Previous studies showed that replacement of the polar belt residues by hydrophobic amino acids increased the mechanical stability of R3.^{42, 58} Bundle R4 also unfolded gradually without noticeable resistance in SMD. In all three 2nm/ns simulations, the helicity in H2 (Ala960 – Pro964) and H3 (Pro996 – Met1000) of R4 was greatly disturbed by twisting and bending. This effect was observed during pulling simulations, however the areas were not affected by the mechanical force directly. Interestingly, such proline occurrence is rather uncommon across other talin helices. Furthermore, both proline residues are highly conserved between species for both talin-1 and talin-2, as determined by multiple sequence alignment. The weak class additionally contains two five-helix bundles, R6 and R10, which unfold under low force in SMD. Both R6 and R10 bundles have helix H1 considerable shorter compared to other bundle helices. This may contribute to the lower domain mechanostability. The short H1 was separated in both domains early in the simulation followed by gradual unfolding of H5 at the C terminal end. In both cases 3-helix intermediate (H2-H4) was observed to be stable between approx. 8-11ns for R6 and approx. 9-12ns for R10 (Figure 3.6). Whether this intermediate represents a functionally important state remains unclear.

The intermediate class contains domains R7, R11 and R12 with domain R7 fluctuating at the border of the soft and intermediate classes during the first 9 ns of 2 nm/ns simulation. All domains in this class consist of five-helix bundles where all the helices have similar lengths. In the R11 bundle, the highly conserved residue Met2121 in H5 (the long connecting helix between R11 and R12) was identified as a possible gatekeeper in all three simulations. Met2121 is located in the IBS2-A region⁴⁴ with its hydrophobic side-chain pointing towards the hydrophobic core of R11. It creates nonpolar contacts with H2 and H4 and possibly contributes to the stability of the bundle and thus maintains the IBS2-A. Similarly to unfolding trajectories of R6 and R10, possible 3-helix intermediates consisting of H2-H4 core was also observed in R11 and R12 simulations (Figure 3.6).

Finally, the strong group contains domains R5 and R9. Both of these two bundles are five-helix domains with similar helix lengths. Furthermore, in strong bundles, additional characteristic twisting of the helices

around the bundle axis was observed in structural alignments when compared to the bundles of intermediate class. Greater displacement of helix ends at the bundle top and bottom was observed for R9 bundle, which also exhibits the greatest mechanical stability. On the other hand, the smallest differences between the top and bottom helix positions can be seen for bundle R11 which is the softest of the presented bundles (R5, R9, R11, R12). Such specific twisting of the bundle is likely to increase the contact areas between its helices and increase the unfolding force demand.

To further investigate the differences of the strong (R9) and intermediate (R11) bundles, individual helix alignment was performed against an ideal α -helix. It was noted that the helicity between the two bundles is affected. The average root mean square deviation (RMSD) of atomic positions of structural alignments was 1.810 Å for R9 and 1.599 Å for R11 suggesting that α -helices of R9 bundles are more deformed compared to R11 helices. Two residues were identified as being important for the R9 bundle stability, namely Leu1668 in H1 and Met1803 in H5. These highly conserved residues create large hydrophobic contacts with the core of R9. Furthermore the Met1803 residue is located in close proximity of the R9-R8 as well as R9-F3 interfaces and may contribute to the stability of the auto-inhibition conformation.^{47, 158}

The smAFM force data supports this proposed hierarchy of bundle stabilities. Based on the SMD classification, R4-R6 contains two bundles from the weak group, R4 and R6, and one strong bundle, R5. In smAFM, two unfolding events occur at a lower force (< 20 pN) with one event at a higher force (> 20 pN), well in agreement with the SMD (Figure 3.4b). In case of R9-R10, SMD proposes one weak bundle and one strong and indeed, AFM demonstrates one lower force unfolding event and one with a higher force (Figure 3.4d). Finally, in the R11-R12 consisting of two bundles of intermediate strength according to SMD, both unfolding events seem to occur at a similar force regime (Figure 3.4e). Thus, there is excellent agreement between the mechanical stabilities derived from the bundle structures and those observed experimentally.

3.4 DISCUSSION

The strong links between talin and a cell's mechanosensitivity have highlighted the need for a thorough understanding of its force response. Single-molecule studies focusing on the R1-R3 region of the rod have demonstrated the basic mechanisms of this mechanosensitivity. It has been shown that unfolding of this region can occur at forces as low as 5 pN and that this unfolding leads to an increase in affinity for vinculin.⁵⁷⁻⁵⁸ Despite the comparative lack of previous molecular investigations on the remainder of the rod, cellular studies have indicated that the regions R4-R12 may be actively involved in talin's mechanosensing. A study that measured the distance between talin's N- and C-termini showed that this length varied between 50-350 nm.⁶⁰ This increase in length would require the unfolding of more than just the R1-R3 region, as this region alone could only contribute up to 150 nm of added length. Additionally, super-resolution microscopy of focal adhesions has shown that vinculin binding occurs along the full rod, including R4-R13,⁶¹ and this indicates that the cryptic VBSs of the full rod are being revealed through structural rearrangement. It is thus important to fully understand the mechanical hierarchy of the talin rod substructures in order to enable further studies needed to better understand the physiological and pathological role of talin.

Here, the bundles R4-R12 have been shown are vulnerable to mechanically-induced structural rearrangement comparable to R1-R3. The application of force to these bundles results in characteristic unfolding events; these events are followed by extension that suggests substantial unfolding of the helical bundle structures. The unfolding of these additional bundles explains how the N-C terminal length varies in cells and also provides a mechanism by which the cryptic binding sites in these bundles become activated. These data and techniques may be used in future to design talin mutants of varying mechanical stability in order to evaluate the contribution of talin-mediated mechanosensing in cellular differentiation or developmental biology processes.

In order to fully appreciate talin's role in cellular mechanotransduction, it has been highlighted that force-induced structural rearrangement occurs in the whole rod domain. Crucially this occurs within a

physiological force range. Recent advances in combined single-molecule cellular studies have revealed the forces acting on talin and other focal adhesion proteins. Single integrin molecules have been shown to experience forces up to 40 pN in magnitude. Additionally cells require this level of tension to be applied to integrins in early adhesion formation in order for the cells to adhere correctly.^{131, 159} When tension sensors were inserted into the talin molecule itself it was shown that the majority of talin molecules experience tension greater than 7 pN with a proportion experiencing greater than 10 pN.⁹² In light of this cellular data it is interesting that all bundles in the rod unfold at forces in the 15 – 30 pN range, within the physiological range of forces applied to focal adhesions *in vivo*. Importantly, one may assume that the unfolding force predicted by AFM is the upper limit needed for the unfolding *in vitro*, as the retraction rate used, 400 nm/s, was higher than the accepted physiological actin retraction rate of 50-100 nm/s.¹⁶⁰ For comparison, Yao *et al.*, when using magnetic tweezers with a loading rate of 5 pN/s, found that the stronger bundles in R1-R3 unfolded at 12 pN and 20 pN, which compares to 19 ± 3 pN and 25 ± 2 pN for the data shown in this chapter.⁵⁸ The loading or retraction rate affects the force of an unfolding event, with lower rates resulting in lower unfolding forces.¹⁵⁶ This explains the forces displayed in this chapter are greater than Yao *et al.* as a higher loading rate has been used. Given that the retraction rate here is higher than physiological and the experiments of Yao *et al.* were conducted at the lower end or below physiological rates, it is likely that *in vivo* the unfolding forces of the talin bundles lie in the narrow range between these values and theirs.

Within this physiological range, a hierarchy of bundle stability can be observed as demonstrated by both the SMD simulations and smAFM unfolding. Stronger rod bundles unfold at consistently higher forces than the weaker bundles. This means that the full talin rod possesses a graduated response to force. Lower forces applied to the rod unfold only the weakest bundles, and as the force increases first the intermediate and finally the strong bundles unfold, revealing or destroying appropriate binding sites in the unfolded domains (Figure 3.7).

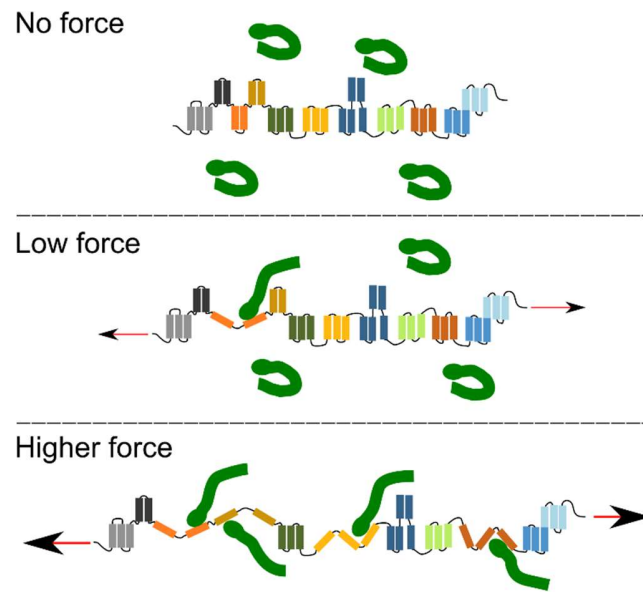


Figure 3.7 Cellular mechanotransduction as a result of graduated talin rod unfolding. Without force, the talin rod remains fully structured and no VBSs are available. Under low force regimes only the very weakest bundle, R3, unfolds revealing its VBS. This activates one vinculin molecule, releasing it from its auto-inhibited state. As the force applied to talin increases, more bundles are unfolded, revealing more VBSs and thus activating an increasing number of vinculin molecules. The helical bundles have been coloured to reflect the mechanical hierarchy shown in Figure 3.5. This schematic represents a simplified version of vinculin-mediated talin mechanosensitivity. The process in vivo is likely to include additional binding partners and more complex modes of force application and unfolding.

The presence of cryptic VBSs along the rod and vinculin's importance in regulating focal adhesion pathways means that talin's graduated force response could result in a similarly graduated cellular response to force. Vinculin natively exists in an auto-inhibited form where its head interacts with the tail domain. The binding of talin's VBSs with vinculin breaks this auto-inhibition and thus releases the vinculin tail, which consequently interacts with actin and numerous other focal adhesion and cytoskeletal proteins.⁷³ Thus increasing the force on talin could lead to greater unfolding of bundles, increased activation of vinculin and therefore amplified vinculin-dependent downstream signalling.

This simple framework for mechanotransduction outlined above ignores the potential involvement of talin's additional binding partners. The bundles that were investigated contain binding sites for numerous

structural and signalling proteins including, RIAM, synemin and Deleted in Liver Cancer 1 (DLC1). RIAM assists in targeting talin to plasma membranes, through its interaction with talin and Rap1.⁴⁹ DLC1 is a potent tumour suppressor protein whose action involves the down-regulation of RhoA activity and thus reduces the contractility of cells.⁷⁶ The binding sites of these proteins overlap the locations of VBSs and thus are thought to compete for binding.^{42, 123} In the case of RIAM, it is expected that the bundled helical structure favours RIAM binding whereas the disruption of the bundle leads to a greater affinity for vinculin. Thus, the influence of a range of physiological forces on talin's structure may modulate switching between favouring different binding partners to control cellular function. The binding to DLC1 and RIAM reduces contractility and increases the formation of lamellipodium,^{78, 161} whereas the presence of vinculin is a hallmark of more stable, mature adhesions. This process of mechanotransduction, through the exposure or disruption of binding sites, may be only one of the methods by which talin's behaviour is controlled by force. Recent work in *Drosophila* development has shown the importance how two talin molecules are orientated with respect to each other when in its dimerized form.⁵³ Crucially, simulations have demonstrated that the orientation of the talin dimer, as dictated by its N-terminal dimerization domain, is controlled by the application of force.¹⁶² Thus the ability of talin to interpret forces and consequently direct cell behaviour may be occurring on multiple levels of structure.

The current study communicates the role of alpha-helical proteins in mechanosensing. It is worth noting that the vast majority of the previous studies focusing on mechanostability of the proteins are conducted using proteins mainly containing beta-sheet secondary structures. Alpha-helical proteins, however, may represent a highly important class of mechanosensing proteins.¹⁶³⁻¹⁶⁴ Interestingly, twisting of the helical structures around the bundle axis, similar to the description for the R9 bundle, can be observed for other alpha-helical proteins with an important role in mechanobiology. A broad variety of helical organization and degree of bundle twist can be detected over many mechanotransducers and structural proteins, such as spectrin, alpha-actinin or nuclear lamin. However, the role of such structural characteristics on the mechanostability is yet to be fully determined. The unique properties of the alpha-helices render some

challenges in terms of the SMD. The helices stretch substantially upon unfolding and the interactions between helices are dictated by entropy-mediated interactions, thus causing possible bias in the relevance of the absolute force determined. However, despite these limitations, the SMD simulations are useful and reliable for the determination of relative mechanical stabilities. Especially when used in conjunction with smAFM, a combination that has not been provided previously. It may be possible to further develop the SMD methodology to better serve the needs of alpha-helical protein research, but this is out of the scope of the current study. It is also worth noting that although the separation of the rod into fragments facilitated the study of its biomechanical properties, it is possible that interactions between bundles in separate fragments may alter their stability and behaviour under load. Future studies will be required to elucidate the complete picture of the unfolding of the full-length talin rod, and precisely how this integrates with its role in mechanotransduction.

3.5 CONCLUSIONS

The combination of SMD and AFM single molecule experiments provide a valuable insight into the molecular behaviour and biological mechanisms. In this study it has revealed the range of forces over which the entire talin rod unfolds and the hierarchy of mechanical stability that the different bundles exhibit. While the contribution of these methods for biological predictions and clinical significance discovery is indisputable, it is a simplified representation of the complex biological system, and does not take into account the other cellular components and the close proximity of the lipid bilayer. However, as compared to cellular experiments, the benefit is that the contribution of other factors can be excluded and thus enable better testing systems to be built using cell and animal models.

Based on the results of this chapter, I hypothesize that talin acts as a delicate force meter sensitive to a range of small mechanical forces. Such fine mechanosensing could control the affinity switch towards ligands triggering biochemical cascades that lead to strong surface adhesion or active spreading and locomotion. Given that the observed range of bundle stabilities depends on small structural differences, I also propose that changes in the mechanical stability of the talin rod bundles could be modulated by a small number of single point mutations. These alterations may lead to misinterpretation of the mechanical impulse and wrong cellular response. Hypothetically, such false interpretation could lead into pathological changes in cell differentiation and tissue growth leading to disease development.¹ Work by others has shown that talin expression is vital for cell differentiation and embryogenesis. The effect of mutations on talin mechanostability and cellular behaviour, however, has yet to be demonstrated. In the light of this discussion it is clear that understanding the principles and mechanisms of talin-mediated mechanotransduction may provide crucial insights into biology in health and in disease.

4 INVESTIGATING THE 3-HELIX INTERMEDIATE STATE

This chapter has been adapted from work provisionally accepted for publication in PLOS Computational Biology, under the title *Mechanical unfolding reveals stable 3-helix intermediates in talin and α -catenin*. It was a joint work undertaken with Vesa Hytonen's group at the University of Tampere. They are responsible for the SMD results shown. Results were interpreted, and conclusions drawn collaboratively.

4.1 INTRODUCTION

Despite the overall pattern of unfolding being as described in the previous chapter, particularly as demonstrated in Figure 3.3, it was possible to discern the presence of a mechanical intermediate in individual traces for both smAFM and SMD (Figure 4.1). SMD simulations indicated that this intermediate is formed from the collapse of a 4- or 5-helix bundle into a 3-helix state that demonstrates some degree of mechanical stability. Given the possible importance of talin's unfolding to its biological function, further examination of the nature of this mechanical intermediate and its possible function is necessary. The α -helical structure of talin's rod bundles also shares similarities with other proteins and so it was examined whether this unfolding pattern is common between them.

α -helix folds are highly abundant among the structural proteins located at the cell-cell and cell-ECM contacts,¹⁶⁵⁻¹⁶⁷ among the essential muscle costamere complexes,¹⁶⁸ as well as among the structures interconnecting the cytoskeleton¹⁶⁹⁻¹⁷⁰ and cellular organelles.¹⁷¹ Despite the numerous studies on mechanotransduction and the mechanosensitivity of proteins, the mechanisms associated with forced protein unfolding and mechanosignalling are not well understood especially where α -helical proteins are concerned. One reason for such a lack of understanding might be that α -helices are, in general, mechanically weaker compared to β -strand folds¹³⁶ and are therefore challenging to study experimentally.

The low mechanical stability of α -helical proteins requires sophisticated experimental methods capable of force measurement in the range of piconewtons.¹⁷²

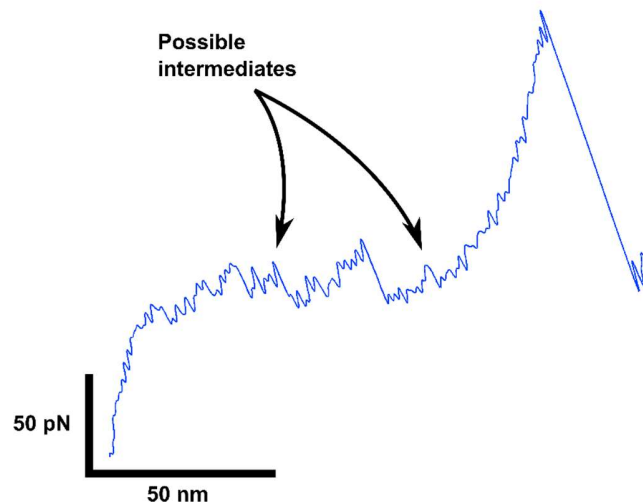


Figure 4.1 Presence of mechanical intermediate. A single smAFM trace (R9-R10) with highlighted unfolding events that are thought to evidence a stable mechanical intermediate.

Among the α -helical structural proteins, several distinct folds have been identified. Talin-like proteins contain 5- and 4-helix bundles.⁴² Proteins of the catenin family contain a 4-helix conformation with long α -helices interconnecting two bundles.¹⁷³ Both of these multi-helical protein folds respond to mechanical load by a dissociation of the bundles leading to mechanoregulatory function. The spectrin fold is formed of a 3-helix conformation with a long α -helix connecting the neighbouring domains, forming a rigid rod of interacting 3-helix bundles. The spectrin-like conformation accounts for structural reinforcement in the cellular scaffolds.^{165-166, 169} Finally, single long α -helices and coils can also be found among structural α -helical proteins (PDB id 5KHT).

Analogous to focal adhesions, adherens junctions are fundamental mechanosensitive structures through which cells communicate with adjacent cells, rather than the ECM. Adherens junctions are associated with the cadherin super-family of transmembrane proteins, which are connected through catenin-rich

protein complexes to the actin cytoskeleton. The cadherins bind to the cytoplasmic protein β -catenin, which in turn binds to the filamentous F-actin binding adaptor protein, α -catenin.¹⁷⁴⁻¹⁷⁵

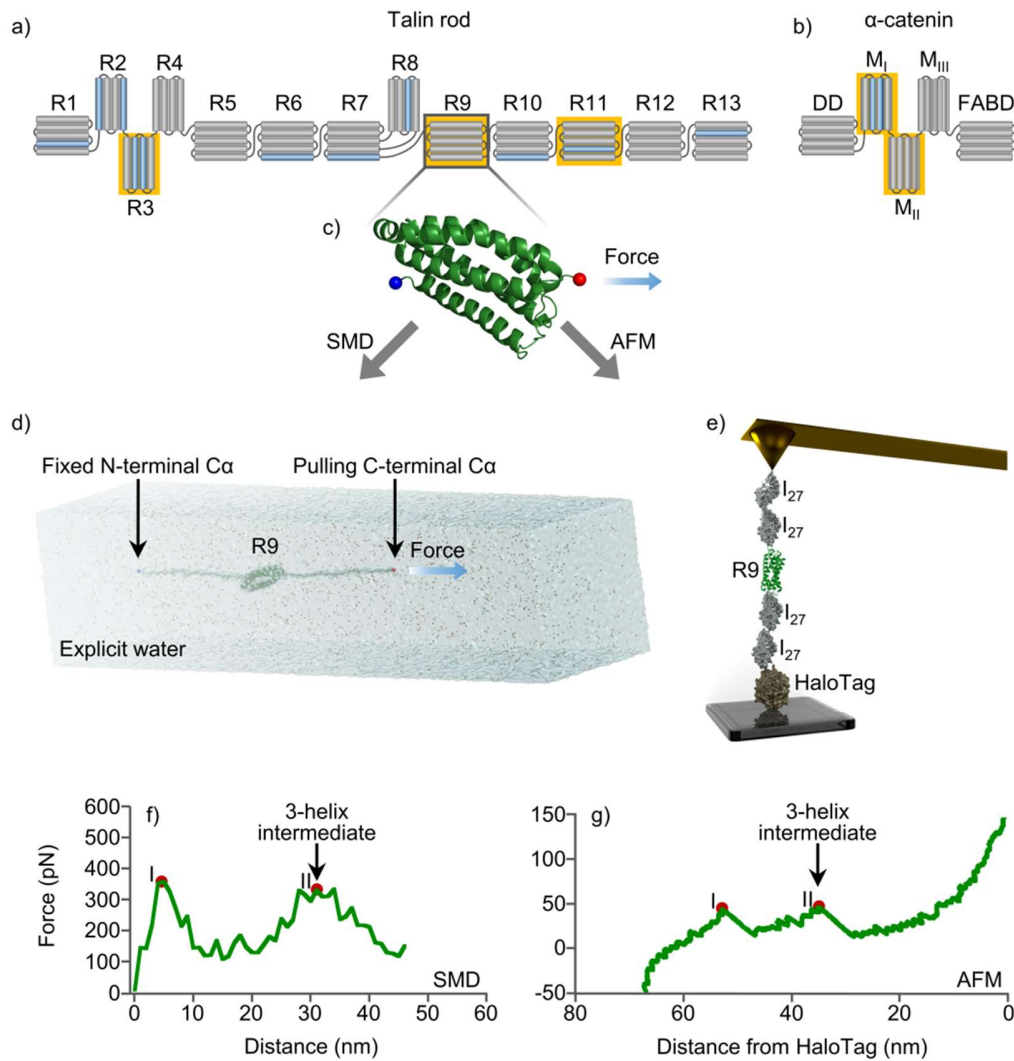


Figure 4.2 α -helix bundle mechanical stability. (a) Schematic representation of talin rod bundles and (b) α -catenin domains. Vinculin binding sites coloured in blue. (c) Cartoon illustration of R9, where Ca of N-terminal residue coloured in blue and Ca of C-terminal residue is red. (d) SMD water box and (e) smAFM setup, that were used for end-to-end stretching of studied proteins. Unfolding force profiles of talin rod R9 in (f) SMD and (g) smAFM experiments, where after collapsing of 5-helix bundle (peak I) the stable 3-helix intermediate was found (peak II). α -helix bundles used in unfolding experiments highlighted with yellow. (a-f) these panels were created by Vasyi Mykuliak and Magdalena von Essen.

Similar to talin, α -catenin recruits vinculin, providing the mechanical connection between cell-cell adhesions and the cytoskeleton. α -catenin contains 5 α -helix domains: the dimerization domain (DD) functioning as β -catenin binding domain, three modulation domains I, II and III (M_I , M_{II} and M_{III}), and an F-actin binding domain (FABD) (Figure 4.2b). Vinculin binds the M_I domain of α -catenin while the two adjacent domains (M_{II} and M_{III}) inhibit vinculin binding to M_I . It has been demonstrated that vinculin is recruited in a force-dependent manner to the cadherin/catenin complex upon force-dependent unfolding of α -catenin. This process resembles the binding of vinculin to the talin rod domain upon tension-dependent unfolding. This tension-dependent unfolding is thought to be central in cell-cell adhesion mechanosensing.^{171, 176-177}

It has been previously hypothesized that the force-dependent unfolding of the vinculin binding domains of the talin rod and the α -catenin domains includes stable intermediate conformations.^{147, 178-179} However, this has never been previously studied in detail in computational simulation or in experimental setup suitable for analysis of single-molecule unfolding events. α -helical proteins are associated with key physiological and pathological processes in mechanobiology.¹⁸⁰⁻¹⁸² A number of diverse diseases from heart¹⁸³ and muscle¹⁸⁴, diseases of bone, vascular and nervous system, or skin¹⁸⁵ have been associated with mechanobiology. Hence, better understanding of their unfolding characteristics will open the possibility of answering a plethora of questions in biology and medicine. In this chapter, the molecular mechanisms of the unfolding of two α -helical proteins, talin and α -catenin, are investigated on an atomistic level by a combination of steered molecular dynamics (SMD) simulations and single-molecule atomic force microscopy (smAFM).

in the light of the results presented in Chapter 3, two talin subdomains were selected on either end of the mechanical sensitivity scale for the current study; weak R3 and strong R9. Talin R9 is an exceptional subdomain responsible for talin autoinhibition while it does not contain VBS. For that reason, the subdomain R11 was included to investigate the unfolding intermediate with respect to VBS activation.

4.2 METHODS

4.2.1 SMD Simulations

The following structures from RCSB Protein Data Bank were used as the protein models for the individual talin rod subdomains: R3 (id 2L7A residues 796 to 909), R9 (id 2KBB) and R11 (id 3dYJ residues 195 to 2140). Talin R9 tandems were constructed using PyMOL, by creating a peptide bond between the last residue of the first R9 monomer and the first residue of the second R9 monomer. α -catenin including M_I and M_{II} domains (id 4IGG residues 275 to 506) was used in these simulations. The point mutations introducing cysteine residues into the talin rod subdomains in order to form the disulphide bonds (clamps) preventing the unfolding of 3-helix state, were designed and mutated using PyMOL.

MD and SMD simulations were performed using Gromacs ver 2016.1^{149, 186} at the Sisu supercomputer, CSC, Finland. The CHARMM27 force field¹⁸⁷ and explicit TIP3P water model¹⁸⁸ in 0.15 M KCl solution were used and the total charge of the system was adjusted by K⁺ and Cl⁻ ions. The energy minimization of the system was performed in 10 000 steps using the steepest descent algorithm. The system was equilibrated in three phases using harmonic position restraints on all heavy atoms of the protein. The first phase of the equilibration was performed with NVT ensemble for 100 ps using the Berendsen weak coupling algorithm¹⁸⁹ to control the temperature of the system at 100 K. Integration time step of 2 fs was used in all the simulations. Following the NVT, the system was linearly heated from 100 to 310 K over 1 ns using an NPT ensemble at 1 atm of pressure. During this process, the Berendsen algorithm was used to control both temperature and pressure. For the final phase of equilibration and for all subsequent simulations, an NPT ensemble was maintained at 310 K using the V-rescale algorithm¹⁹⁰, and 1 atm as implemented in Gromacs 2016.1. The temperature coupling was applied separately for the protein and the solution parts. Each system was equilibrated up to 30 ns, with subsequent monitoring of the root mean square deviations (RMSD) of C α atoms, considering the first approx. 5 ns as relaxation step. Hence, snapshots at 5 ns were used as starting structures for SMD simulations. Pulling vector was set between C α of the first and the last residue of the appropriate domain. The movement of C α of N-terminal

residue was restrained with harmonic potential, while C α of C-terminal residue was subjected to the constant velocity or constant force pulling. The pressure control was turned off for the pulling dimension (z-axes) in all SMD simulations as described in previous work¹⁴⁷. The constant velocity pulling SMD simulations were performed at 2 nm / ns with the spring constant set to 1000 kJ/mol nm². In the constant force pulling SMD simulations, different force regimes were applied, 160 pN, 170 pN, 180 pN and 200 pN for R3 and 200 pN, 220 pN, 230 pN, 250 pN and 300 pN for R9. The system size in SMD was 227 thousand atoms for R3, about 800 thousand atoms for R9, R11 and α -catenin M_I-M_{II}, and about 1.2 million atoms for R9 tandems.

4.2.2 Generation of polyprotein constructs and ligand-functionalized surfaces

The polyprotein constructs were created and expressed as described in 3.2.1 (Appendix 1). Halo-ligand surfaces were prepared as described in 3.2.2

4.2.3 AFM Experiments and Analysis

smAFM data capture was carried out as described in 3.2.3. Data analysis was carried out using Igor Pro (Wavemetrics). Unfolding peaks were identified by adjustable smoothing with a moving box average and then by searching for local maxima. The force of the peaks along with their unadjusted distance from the HaloTag benchmark was measured (Appendix 2).

4.3 RESULTS

In order to probe the existence of stable intermediates in the unfolding trajectory of α -helical mechanosensitive domains, two mechanoregulatory proteins, talin and α -catenin were selected (Figure 4.2). The mechanically stronger talin 5-helix bundles R9 and R11 were specifically chosen, along with the mechanically weaker 4-helix bundles of talin R3 and α -catenin modulation domains I to II (M_I - M_{II}).

4.3.1 smAFM analysis of α -helical bundles indicates unfolding through intermediate states

We utilized smAFM to characterize the unfolding patterns of R9 and R11 constructs and captured the 3-helix intermediate that was provisionally identified in Chapter 3 (Figure 4.3**Error! Reference source not found.**). Two unfolding events were detected for each of the bundles, implying that unfolding occurs through a mechanically stable intermediate. Overall the bundle stability was higher in the case of R9 than R11, consistent with the results of the previous chapter. The distance between the two unfolding events is 20-25 nm, which is consistent with the collapse from 5 helices to 3 helices, supporting the previous indications on the nature of the intermediate state (Figure 3.6). Likewise, the distance from the second unfolding event to the HaloTag ruler of 25-35 nm is consistent with the subsequent collapse of the 3-helix intermediate.

We also tested the R9 tandem construct to examine if the 3-helix intermediate could be detectable within a model of a polyprotein (Figure 4.4**Error! Reference source not found.**a-d). 4 unfolding events were observed, consistent with a pattern of two bundles unfolding via a stable intermediate. The distances between the first and second event (~20 nm), second and third (~30 nm), third and fourth (~20 nm) and fourth and HaloTag (~30 nm) possibly indicating that one α -helix bundle collapsed completely through a 3-helix intermediate before the second α -helix domain started unfolding. However, it is difficult to be certain, given the error margin of the peak locations and the likely stochastic nature of the unfolding

process. When a 3-helix disulphide clamp was introduced into one of the tandem R9 domains (Figure 4.6), there was a reduction in the number of unfolding events from 4 to 3 (Figure 4.4**Error! Reference source not found.**e-h). This, along with the reduction of the overall unfolding length from 105 nm to 85 nm, demonstrates that the disulphide clamping was able to protect the 3-helix intermediate of the R9 from mechanical unfolding.

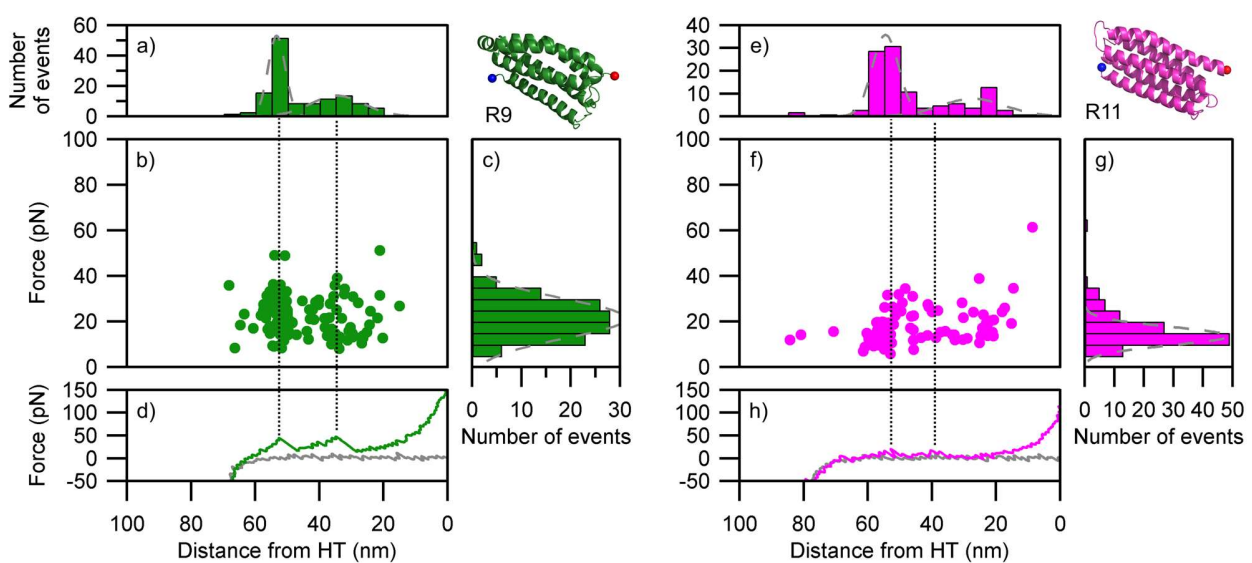


Figure 4.3 Unfolding patterns of the α -helix monomers as determined by smAFM. (a-d) R9 (87 traces). (e-h) R11 (79 traces). (a & e) length histogram indicating the distance from unfolding event to HaloTag standard; (b & f) scatter plot showing force vs position for all unfolding events; (c & g) histogram the force associated with unfolding events; (d & h) representative force extension retraction curve (green & magenta line) showing how the unfolding events relate to scatter plot and length histogram (black dotted line), grey line represents approach curve for AFM tip. Grey dotted lines on (a, c, e & g) histograms represent Gaussian fits.

The forced unfolding of α -catenin modulation domains I to II (M_I - M_{II}) by AFM produced a pattern consistent with it also collapsing through an intermediate state (Figure 4.4**Error! Reference source not found.**i-l). There were two unfolding events with a maximum unfolding length of ~ 50 nm which implies that there is no unfolding event with respect to the reduction of both 4-helix bundles into 3-helix states. This is in agreement with the previous results showing the lower mechanical stability of the 4-helix

bundles. The two events observed with unfolding lengths of ~ 25 nm seem to correspond to the mechanical unfolding of 3-helix intermediate states.

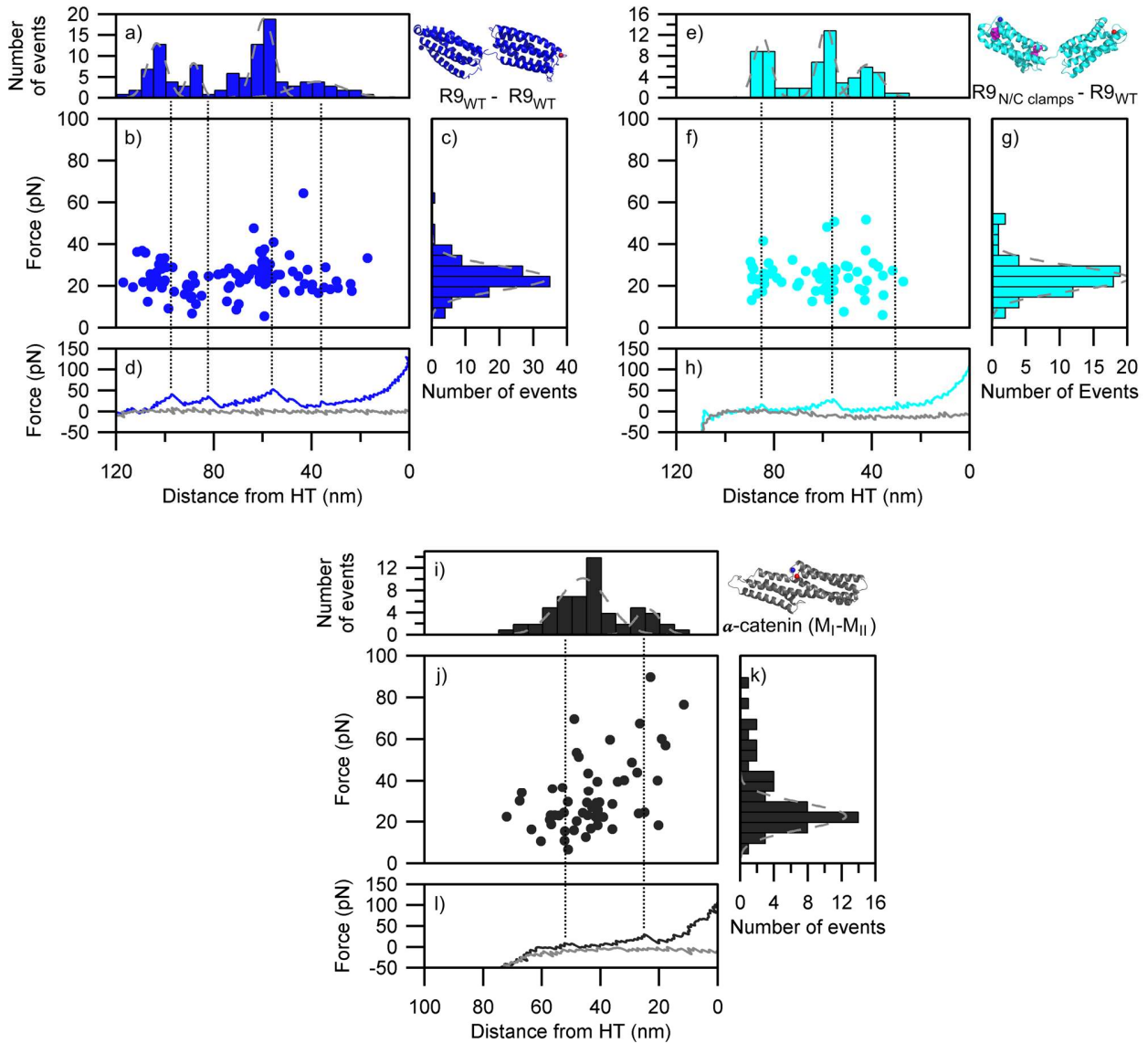


Figure 4.4 Unfolding patterns of tandem α -helical bundles as determined by smAFM. (a-d) Talin R9 (wt)–R9 (wt) (47 traces). (e-h) R9 (N/C-clamps)–R9 (wt) (34 traces). (i-l) α -catenin (M1–M11) (40 traces). (a, e & i) length histogram indicating the distance from unfolding event to HaloTag standard; (b, f & j) scatter plot showing force vs position for all unfolding events; histogram the force associated with unfolding events; (d, h & l) representative force extension retraction curve (blue, cyan & l) showing how the unfolding events relate to scatter plot and length histogram (black dotted line), grey line represents approach curve for AFM tip. Grey dotted lines on (a, c, e, g, i & k) histograms represent Gaussian fits.

4.3.2 Constant velocity pulling simulations reveal that talin rod bundles unfold through stable 3-helix intermediates

In order to investigate the nature of the intermediate state that led to the presented smAFM data, two talin rod 5-helix bundles, R9 and R11, were subjected to end-to-end SMD stretching with constant velocity pulling simulation at 2 nm/ns. Previously, talin 5-helix bundles were more mechanically stable in SMD simulations compared to 4-helix bundles. According to the previous results, R9 was one of the strongest rod bundles. Overall, unfolding of R9 and R11 showed two force peaks (Figure 4.5a), which correspond to breaking of the 5-helix bundle and 3-helix intermediate. This unfolding intermediate consists of core helices (H2–H4) after the dissociation of H1 and H5. For R9 the maximum unfolding forces detected in constant velocity pulling simulation at 2 nm/ns were 348 ± 24 pN and 349 ± 39 pN (average force $\pm$ standard deviation). Unfolding peak forces for R11 were very similar to those for R9 (Figure 4.5a). Representative snapshots of the unfolding trajectories are shown in Figure 3, indicating changing of the bundle conformation during unfolding.

To confirm the unfolding intermediate, point mutations were included in the R9 bundle forming disulphide bonds (clamps) in order to block the unfolding of the 3-helix core. These clamped R9 mutants were subjected to end-to-end pulling in simulation and experimental setup. Three constructs were prepared including L1698C and A1748C cysteine mutations that protect 3-helix core from N-terminus (N clamp), A1720C and A1779C mutations that prevent unfolding of the 3-helix core from C-terminus (C clamp), and R9 construct with both N- and C-terminal disulphide clamps (N/C clamps) (Figure 4.6). All R9 clamp mutants showed very similar unfolding of 5-helix state compared to the wild-type R9 (Figure 4.5b). The unfolding of the 3-helix state was effectively blocked in the R9 equipped with N/C clamps, while the constructs with only one disulphide clamp allowed unfolding of either H2 (R9 with C clamp) or H4 (R9 with N clamp) of the 3-helix core but did not compromise the stability of the 3-helix intermediate (Figure 4.5b).

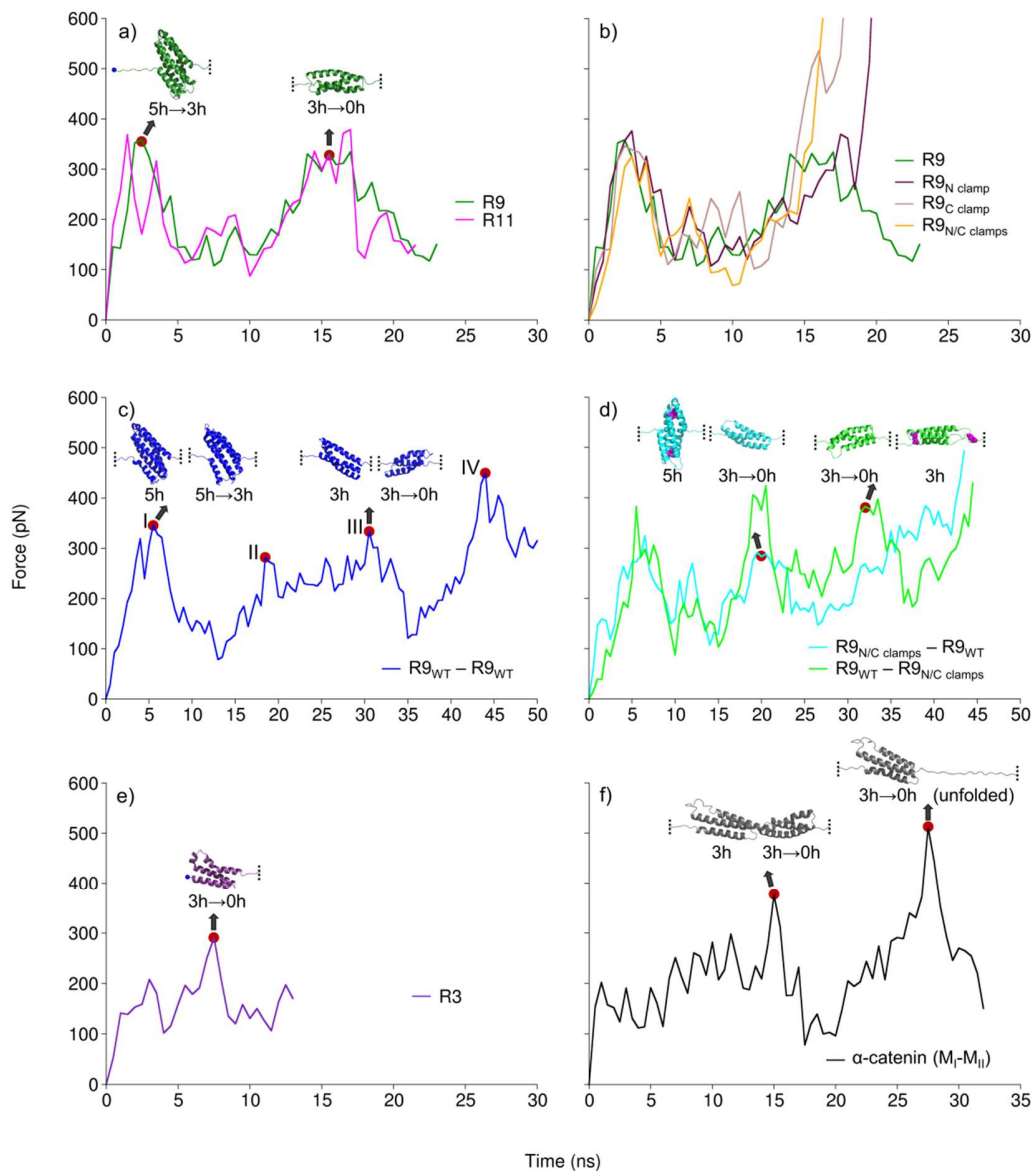


Figure 4.5 Unfolding force profiles of the studied protein constructs in constant velocity SMD. (a) Unfolding forces for 5-helix talin rod R9 and R11 have similar profiles and show two peaks, which correspond to breaking of 5-helix and 3-helix state respectively. (b) R9 constructs with disulphide clamps have similar force profiles to wild-type R9 but unfolding of 3-helix state was blocked. Tandem constructs for (c & d) R9 and (f) α -catenin (MI-MII) were unfolded through 3-helix state for both monomers simultaneously. (c) R9 (wt)–R9 (wt) tandem showed four peaks, corresponding to breaking of the 5h & 5h→3h (peak I), 5h→3h & 3h (peak II), 3h & 3h→0h (peak III), and 3h→0h & 0h (peak IV) respectively. (d) Tandems with the clamped 3-helix state in one monomer showed three peaks, lacking the peak for unfolding of the clamped 3-helix state. (e) Unfolding force for 4-helix R3 bundle has one peak that corresponds to collapsing of 3-helix state. (f) 4-helix α -catenin showed one peak for breaking the 3-helix state in each domain. Cysteine residues in (d) R9 tandems, that form disulphide clamps shown as magenta spheres. Structure snapshots correspond to force peaks highlighted with red dots. This figure was created by Vasyl Mykuliak and Magdalena von Essen.

In additional experiments, the talin R3 bundle and α -catenin modulation domains I to II (M_I - M_{II}) were examined, which are all 4-helix bundles. Talin R3 was easily unfolded to the 3-helix state by the separation of H4. The 3-helix intermediate (H1–H3) was more stable compared to the 4-helix bundle, the unfolding peak force for breaking the R3 3-helix state was 276 ± 20 pN (Figure 4.5e). Similarly, α -catenin was unfolded to 3-helix conformation by dissociation of H4 (four out of five simulations) in M_{II} and H1 in M_I (all five simulations). Further unfolding showed two force peaks for breaking the 3-helix state in M_{II} (at 349 ± 33 pN) and M_I (at 461 ± 68 pN) (Figure 4.5f). Collectively these data suggest that both 4- and 5-helix bundles unfold through stable 3-helix intermediate state. Furthermore, 5-helix bundles withstand mechanical load better than 4-helix domains, which are easily unfolded to the 3-helix state.

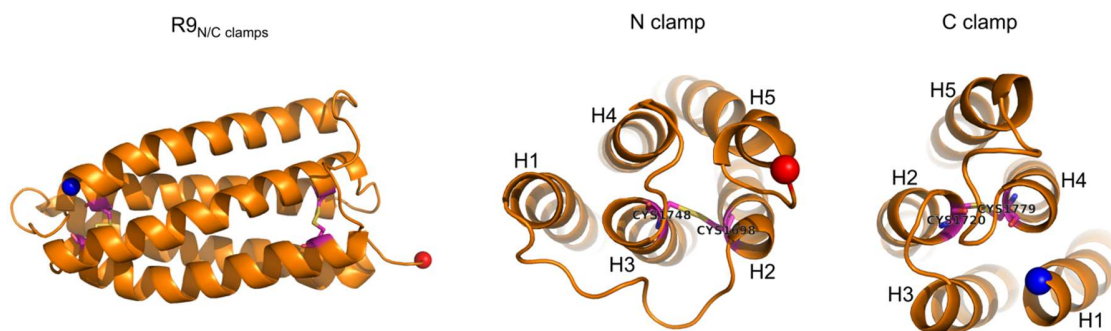


Figure 4.6 Design of disulphide clamps in R9. L1698C and A1748C cysteine mutations prevent unfolding of the 3-helix core from N-terminus (N clamp), while A1720C and A1779C mutations protect the 3-helix core from C-terminus (C clamp). This figure was created by Vasyi Mykuliak and Magdalena von Essen.

Described constant velocity SMD simulations of individual talin rod bundles and α -catenin were run five times each. Repeated unfolding force profiles showed that the results are well reproducible (data not shown) and allowed us to calculate average peak force and a standard deviation (Table 4.1).

Table 4.1. Unfolding peak force in constant velocity SMD

Protein construct	Conformational state	Unfolding peak force (pN)					Average $\pm$ standard deviation
		Replica 1	Replica 2	Replica 3	Replica 4	Replica 5	
R3	3h $\rightarrow$ 0h	281	291	295	244	272	276 $\pm$ 20
R9	5h $\rightarrow$ 3h	358	320	340	385	340	348 $\pm$ 24
R9	3h $\rightarrow$ 0h	331	400	340	374	299	349 $\pm$ 39
R11	5h $\rightarrow$ 3h	368	329	351	349	368	353 $\pm$ 16
R11	3h $\rightarrow$ 0h	378	352	283	327	322	332 $\pm$ 35
α -catenin	3h $\rightarrow$ 0h (M _I)	511	345	491	454	504	461 $\pm$ 68
α -catenin	3h $\rightarrow$ 0h (M _{II})	375	379	350	296	345	349 $\pm$ 33

4.3.3 Talin tandem domain experiments confirm the existence of 3-helix intermediates

To assess the effects of force penetration on the unfolding mechanisms in SMD simulations, the unfolding mechanisms and the existence of the stable intermediates were studied for a linear protein chain consisting of two talin R9 monomers resembling the natural biological assembly of talin. A tandem construct was designed possessing exactly the same mechanical stability, i.e. two talin R9 domains (R9_{WT} – R9_{WT} tandem). Furthermore, the R9 tandems were analysed with disulphide clamps, protecting the 3-helix core from unfolding, in either first or second monomer, with respect to the fixed N-terminal and pulled C-terminal end. Thus, there were two tandem constructs with clamps, R9_{N/C clamps} – R9_{WT} and R9_{WT} – R9_{N/C clamps}, respectively. For R9_{WT} – R9_{WT} tandem, unfolding force showed four peaks, corresponding to the breaking of 5-helix states first, followed by dissociation of the 3-helix states in both α -helix bundles (Figure 4.5c). Because the pulling was applied to C α atom of C-terminal residue, the second R9 in the tandem was closest to the point of pulling and unfolded to 3-helix state first. Both monomers of the R9_{WT} – R9_{WT} tandem unfolded to the 3-helix intermediate within approx. 30 ns of the SMD simulation with 2 nm/ns pulling velocity and unfolded at approx. 50 ns. Both tandems containing one clamped monomer showed three unfolding force peaks (Figure 4.5d) lacking the peak corresponding to the breaking of the disulphide-clamped 3-helix structure. Indeed, the force penetration did affect the two different tandems with clamps resulting in different unfolding trajectories. For R9_{WT} – R9_{N/C clamps} tandem, both monomers

had 3-helix conformations at ~ 30 ns, while for $R9_{N/C \text{ clamps}} - R9_{WT}$, closest to the point of pulling monomer ($R9_{WT}$) unfolded completely before $R9_{N/C \text{ clamps}}$ molecule unfolded to 3-helix state (Figure 4.5d, Figure 4.7).

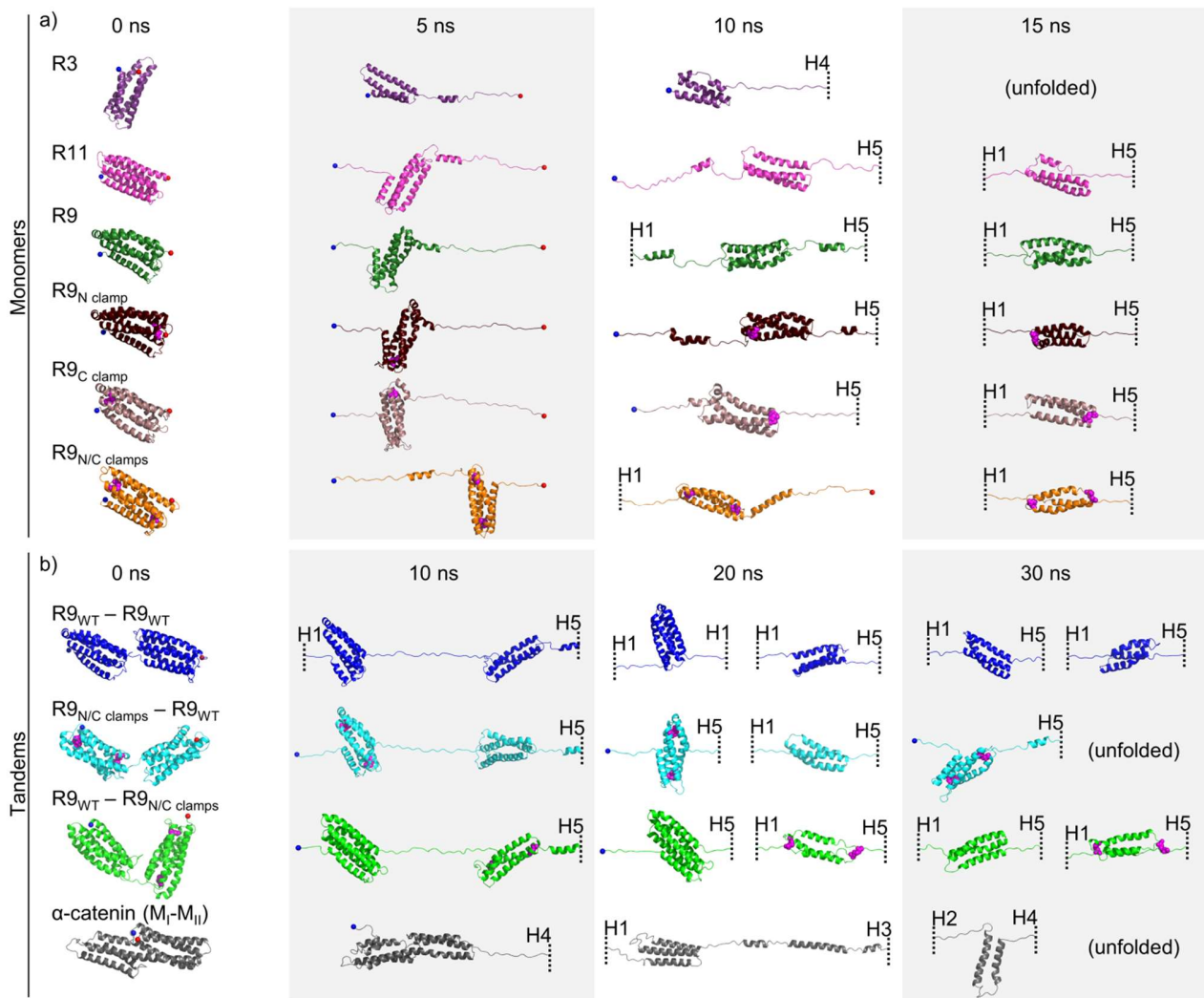


Figure 4.7 Representative structure snapshots from SMD. Talin rod domain bundles and α -catenin (MI-MII) in constant velocity SMD simulations. Structures were taken (a) at 0, 5, 10 and 15 ns for monomers, and (b) at 0, 10, 20 and 30 ns for tandem constructs. Cysteine residues forming the disulphide clamps shown as magenta spheres. Unfolded structures are cut away and presented by dashed line with a helix identifier. This figure was created by Vasyl Mykuliak and Magdalena von Essen.

The investigation of the unfolding of a tandem provided us with a tool of studying the unfolding principles of multiple domains. Furthermore, the use of disulphide clamps in the tandem construct protecting the

stable state provided us with a comparison force trace and additional proof of an intermediate unfolding conformation in both mechanosensitive proteins. Altogether, these findings indicate that the unfolding force required for the unfolding of the 3-helix intermediate state is similar to that needed for the unfolding of 5-helix state.

4.3.4 Constant force SMD confirms the existence of 3-helix intermediates in talin rod R3 and R9

For the comparison of the 3-helix state mechanical stability two mechanically diverse talin rod bundles were selected, i.e. the mechanically weaker R3 and the mechanically stronger R9 for more detailed analysis. Although constant velocity SMD simulations are an excellent tool comparing the results with AFM analysis, they are less sensitive for the assessment of intermediate states as compared to constant force simulations. Therefore, the R3 and R9 bundles were subjected to constant force pulling simulations where, after screening of suitable force regime, constant force ranging from 160 pN to 200 pN for R3, and from 200 pN to 300 pN for R9 (Figure 4.8) was used. In constant force SMD, 4-helix R3 was weak even at 160 pN and rapidly unfolded to 3-helix state (within ~ 3 ns). After the separation of H4, H3 was slowly sliding relative to H1 and H2 (from ~ 3 ns to ~ 23 ns). The 3-helix intermediate did not unfold at 160 pN in 40 ns time window (Figure 4.8a), however, it unfolded completely (at ~ 72 ns) in extended 160 pN simulation (data not shown). R3 was extended also with constant force at 170 pN, 180 pN and 200 pN. Although it was completely unfolded after ~ 18 ns, ~ 13 ns and ~ 11 ns respectively, the stable 3-helix intermediate was observed in all trajectories (Figure 4.8). For strong R9 bundle, constant force of 200 pN was applied and only partial uncoiling of terminal helices (H1 and H5) was observed within 40 ns time window (Figure 4.8b), yet the 5-helix state remained intact. However, R9 unfolded to the 3-helix state (at ~ 86 ns) in extended 200 pN simulation (data not shown). The application of constant force of 220 pN or higher resulted in gradual unfolding of the bundle. During the unfolding, there were two stable states (5-helix and 3-helix states). In order to compare mechanical stability of 5- and 3-helix states, the disrupted

5-helix state of R9 was subjected to stretching with constant force of 200 pN. Although 3-helix intermediate was relatively stable, it slowly unfolded over time under 200 pN while 5-helix bundle resisted the unfolding under the same force magnitude.

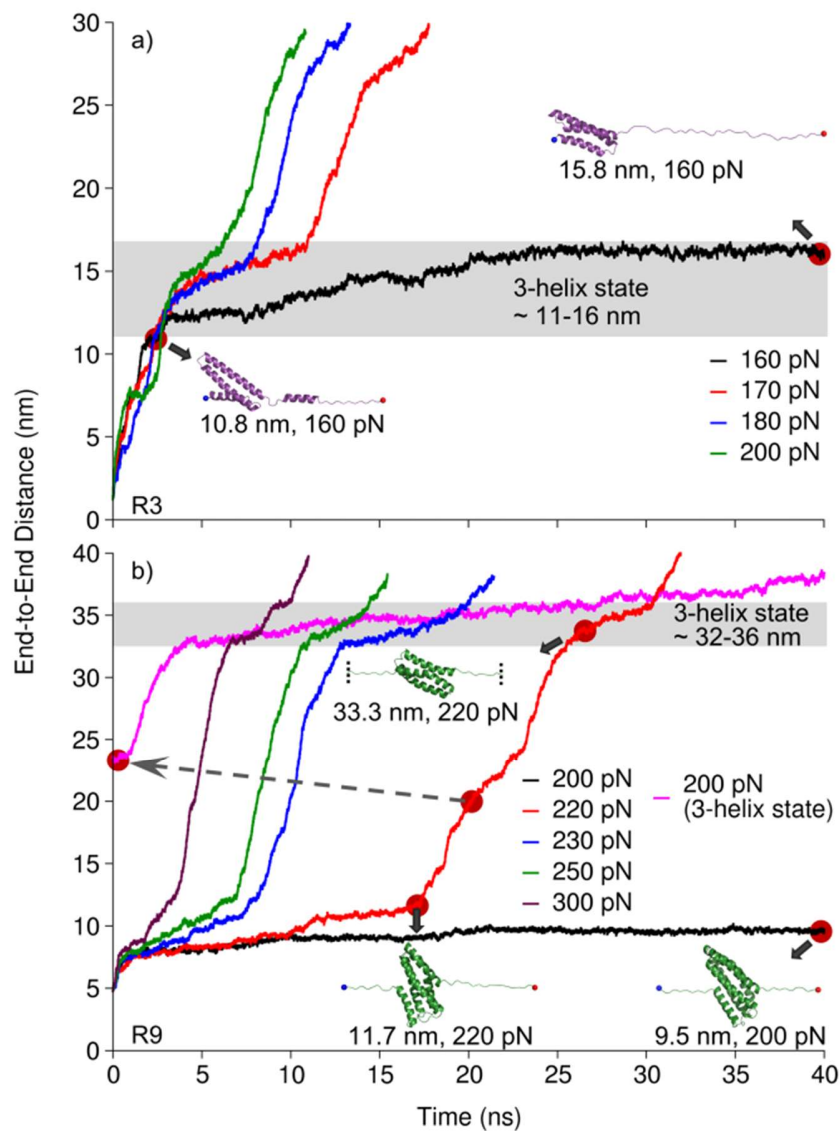


Figure 4.8 Stability of the intermediate states. Talin rod (a) R3 and (b) R9 over time in constant force SMD using constant force pulling at different force regimes. R9 is a strong 5-helix bundle, while stable 3-helix intermediates were observed in both R3 and R9 (grey shading). The pink trace in panel (b) corresponds to end-to-end distance measured under 200 pN applied force, where 220 pN simulation was used as a starting structure after passing the 5h to 3h transition (dashed grey arrow). created by Vasyl Mykuliak and Magdalena von Essen.

These results suggest that 3-helix state in R9 is a stable conformation. However, it is weaker as compared to the 5-helix state of R9. In more detail, the 3-helix state can be unfolded under lower force load once the 5-helix state of the R9 bundle is broken. On the other hand, the 3-helix intermediate state in R3 is the most stable conformation of the R3 bundle.

4.4 DISCUSSION

Numerous studies concerning α -helical mechanosensitive proteins have provided information on mechanically regulated switches between diverse binding partners and their associated functions. Perhaps one of the best known examples of this mechanoregulated protein-protein interaction is the tandem talin-vinculin, where mechanical stress applied to talin rod exposes binding sites for vinculin.⁵⁶⁻⁵⁷

Our observation of SMD trajectories for talin multidomain constructs, described in the previous chapter, revealed a possible stable 3-helix intermediate during the forced domain unfolding. Because of the complexity of the SMD and smAFM data obtained during the multidomain construct unfolding, it was not possible to identify previously the 3-helix intermediate among the force traces directly. However, simultaneous domain unfolding was recognized for bundles of similar mechanostability in smAFM¹⁴⁷ and also in SMD carried out for domains in tandem (von Essen et al., unpublished experiments).

In this chapter, the detailed unfolding mechanisms of α -helical talin rod bundles and α -catenin M_I-M_{II} domains were investigated to probe the presence of any intermediate or partial unfolded states. The results show that the two studied proteins unfold through a stable 3-helix intermediate. Constant velocity pulling used in SMD and smAFM revealed, that the unfolding force profiles for the 5-helix rod bundles R9 and R11 have two peaks, which correspond to the breaking of the 5- and 3-helix states (Figure 4.5a). The talin 4-helix bundle R3 and α -catenin bundles M_I-M_{II} also unfolded through stable 3-helix intermediates (Figure 4.5e, f). In addition, this 3-helix state was recognized as the most mechanically stable conformation for the 4-helix domains (Figure 4.8a).

Other studies have also provided indirect evidence of unfolding intermediates in alpha helical proteins. Investigations of the talin R3 subdomain have revealed a possible 3-helix intermediate capable of rapid or instant activation for vinculin binding. Specifically, the deletion of R3 H4 leads to super-active R3 rapidly localizing in cell-ECM contacts. Rahikainen *et al.*¹⁹¹ showed that one or two destabilizing mutations in R3 H1 were sufficient to facilitate bundle unfolding, increasing the activation of vinculin

binding and resulting in a strong cellular phenotype. The phenotype of the further destabilized state modified with four mutations was comparable to the super-active R3 potentially indicating a 3-helix intermediate. Further evidence of a 3-helix state in R3 domain is found in a recent study by Baxter *et al.*¹⁹² A 3-helix open state has been recognized after the dissociation of H1 from the R3 bundle under high pressure conditions. Similar effects were observed even for the talin R1 bundle, where the deletion of H5 resulted in the exposure of the VBS located in H4 and an active conformation of R1.⁵⁴ The authors also suggest that the deletion of H5, resulting in a 4-helix partial bundle, causes a destabilization of the R1 domain leading to partial unfolding. This observation is in line with the results shown here: the 4-helix fold is a fragile conformation which does not require excessive mechanical force to unfold to a stable 3-helix state (Figure 4.5e). Finally, even previous computational studies suggested that only partial unfolding of talin subdomains described by minimal protein extension is sufficient for VBS activation. In more detail, R1 VBS was activated through torsional conformational change of the hydrophobic core orientation within R1 subdomain during an extension of less than 2 Å.¹⁹³

Inspection of the molecular characteristics of 5- (4- in R3) and 3-helix states did not reveal any dominant differences between these assemblies in terms of interactions or packing. The hydrophobic interactions appear to be the main factor in maintaining both these states. In the previous chapter, two conserved residues were shown to be important for maintaining R9 5-helix bundle stability, namely Leu1668 in H1 and Met1803 in H5. Further studies including experimental investigation of subdomains carrying mutations targeting the 3-helix core fragment would be needed to evaluate the contributions of individual residues for the mechanical stability of the 3-helix intermediate state.

Further unfolding of the 3-helix intermediate was observed in the experimental and simulation setup. Whether the complete unfolding of an α -helical domain takes place *in vivo*, or whether the 3-helix state is the final unfolding conformation remains unclear. Hints of both of these options can be found in the literature. As discussed earlier from Rahikainen *et al.*¹⁹¹, the deletion of terminal helix in R1 and R3 is sufficient for VBS activation and vinculin recruitment. Thus, I speculate that the 3-helix state is capable

of vinculin binding. Vinculin binding to unfolded talin or α -catenin domains inhibits domain refolding under low mechanical load.^{58, 62, 177} Simultaneously, vinculin binding to the 3-helix state bundle may protect it from complete unfolding. Studies by Margadant et al.⁶⁰ show that the maximal length of talin is approx. 400 nm in living cells. This also supports the notion of partial unfolding even in the absence of vinculin. Interestingly, recently published work by Ringer et al.¹⁷² revealed a force gradient across the talin rod domain. In the presence of vinculin, greater force was measured at the N-terminal end than at the C-terminal end resulting in the bundle unfolding and activation for vinculin binding. As vinculin binds to activated talin and to actin, the force acting on the talin rod is divided and reduced towards the C-terminal end. I speculate that the reduced force might be insufficient to unfold the stable 5-helix subdomains located at the C-terminal end of the talin rod. However, here it is shown that the intermediate resulting from 4-helix unfolding was mechanically weaker compared to the intermediate of a 5-helix bundle. Thus, the complete unfolding of 4-helix bundles at the N-terminal end of the talin rod may be possible. Based on the work by Yao et al., it can be assumed that complete yet reversible unfolding of R3 domain takes place under low force load. It was shown that under 4.8 pN of constant force load exerted on the full length talin, R3 occupies two distinct conformations with elongation of approx. 19 nm. However, which states these in fact are remains unclear.¹⁹⁴ It may be hypothesized that only the end-to-end attachment to the pulling device and initial elongation under low force load is sufficient to collapse R3 into an activated 3-helix state. Such immediate conformational change would not necessarily result into an observable difference in the total end-to-end elongation compared to the R3 conformation in solution.¹⁹³ Similar elongation of approx. 19 nm between the 3-helix intermediate and the completely unfolded state was shown in the SMD results (Figure 4.8a). Here it is evident that the 3-helix intermediate remained stable even though the end-to-end distance increased by 5 nm. This distance increase was caused by the uncoiling of H4.

Based on these observations of the talin unfolding mechanisms, I propose a model of multidomain α -helical protein unfolding under mechanical load, shown in Figure 4.9. In the absence of mechanical force,

α -helical bundles remain in a folded conformation, capable of binding their ligands such as RIAM (R2, R3, R8 and R11) and DLC1 (R8) in the case of talin.¹⁹⁵ At low mechanical load, soft α -helix bundles, namely 4-helix domains, unfold to stable 3-helix intermediates. Since the activation of vinculin binding sites (VBSs) requires the unfolding of talin bundles,¹⁹⁶ the formation of 3-helix states suggests that VBSs located at the terminal helices become available for vinculin. At the same time, the partial unfolding of the bundles leads to a disturbance of the binding sites for other binding partners located on the bundle surface, which abrogates their interaction.⁴² With increasing force, additional bundles collapse to a 3-helix conformation switching from the mechanoregulatory role to a structural reinforcement role, similar to that of spectrin. In other words, it is possible that talin reacts to a range of small mechanical forces by the dissociation of certain bundles leading to a change in binding to other proteins. Such mechanoregulation would take place until the bundles reach a stable 3-helix spectrin-like conformation. At this point, the talin protein would assume a structural reinforcement role. Finally, at a high force, completely unfolded bundles would lose the ability to support ligand binding as well as structural function.⁵⁸ Such a model enables rich mechanosignalling through talin. A recent study by the Barsukov group has proposed the talin protein as being a hub for several different binding partners¹⁹⁵ where the proposed 3-helix intermediate state could be an essential component of binding regulation. Moreover, it has been recently shown, that the mechanical load across talin is not homogeneous, providing further variation in the regulation of talin functions.¹⁷² Since the talin rod experiences a force gradient, once vinculin is bound, the local stress may become modulated and insufficient to unfold the 3-helix state. The 3-helix state may thus represent abundant talin rod subdomain conformation in living cells.^{60, 172} Experimental work is essential to confirm and refine these models.

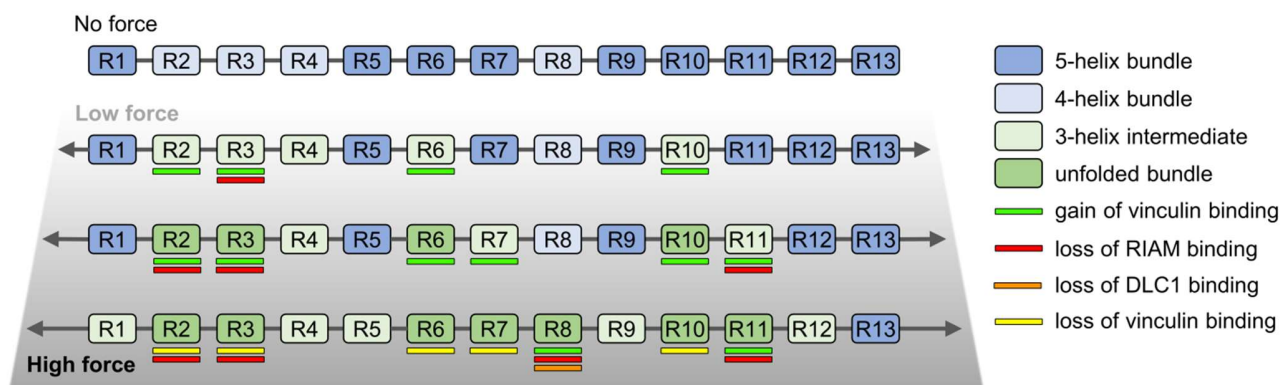


Figure 4.9 Schematic representation of talin rod unfolding through stable 3-helix intermediates. Without force, the talin rod subdomains remain intact and no VBSs are exposed. Under low load weak 4-helix bundles unfold to stable 3-helix intermediates. As the force increases, some of the 5-helix bundles unfold, forming the 3-helix intermediates along talin rod structure. Force-regulated unfolding of the talin rod changes affinity to different binding partners. RIAM and DLC1 are known to bind folded bundles, while recruitment of vinculin requires partial or complete unfolding of rod domains. VBSs that are located at terminal helices of the R2, R6, R7, and R10 bundles become available after unfolding to the 3-helix state (panel “Low force”).

While the activation of talin binding sites by mechanical force has been long studied, the detailed mechanism of the forced unfolding has not been previously discussed. The existence of a stable 3-helix intermediate may offer yet another level in the mechanoregulation process and in the cell's response to mechanical stimuli. The existence of the unfolding intermediate also adds additional complexity to the assessment of the impact of mutations in the case of diseases. α -catenin truncating mutations have been detected in patients with hereditary gastric cancer¹⁹⁷ possibly increasing the disease susceptibility. Furthermore, α -catenin mutations have been directly associated with macular dystrophy.¹⁹⁸ The understanding of molecular mechanisms would shed light on the disease development and guide new treatment solutions. I may also speculate that the existence of a 3-helix intermediate, whether undergoing full unfolding or not, may provide an additional structural support. It is also possible that the 3-helix state functions as a molecular bumper reducing the impact of functional mutations present in the mechanosensitive protein. In other words, with additional level of mechanoregulation, the mutation effect on the cell's behaviour may be defused with only moderate effect on the cell's fitness. Such a theory may

be of importance in the case of talin which has been presented as a vital protein in cell and tissue biology. Yet, despite its important roles, only one mutation has been recognized as disease causing, in the talin-2 isoform located outside of the mechanosensitive region.¹⁹⁹

4.5 CONCLUSION

We show that α -helical proteins unfold via stable 3-helix intermediate states, representing biologically active states. smAFM and disulphide clamp mutations were used to confirm the models obtained with SMD. These results suggest that talin is a central scaffolding hub in focal adhesions with multiple discrete unfolding states, acting as a sophisticated mechanosensor and an important regulatory switch. I further propose that the mechanical stability of α -helical domains as well as the mechanical stability of their unfolding intermediates should be considered when studying mechanoregulation models of α -helical proteins.

5 USE OF CLAMPED MUTANT TO TEST TALIN MECHANOSENSITIVITY

This chapter is adapted from work currently under review for publication in PLOS Biology, under the title *Talin unfolding regulates traction forces and cell motility*. We originally conceived the idea, with Vesa Hytonen's group at the University of Tampere subsequently included owing to their experience with the respective cell types. Experiments on cell migration were conducted by Rolle Rahikainen under the supervision of Vesa Hytönen. Data analysis and interpretation were done jointly.

5.1 INTRODUCTION

The previous chapters have demonstrated that the talin molecule is vulnerable to tension-induced disruption of its three-dimensional structure. This leads to the question of what role this structural rearrangement has in cells and through what pathways is this mediated. The mechanically-induced unfolding of mechanosensitive proteins, such as talin, has provided a new mechanism for force transduction through the disruption of the tertiary structure of these proteins, which may lead to exposed cryptic sites for ligand binding, molecule activation, or cleavage of domains by proteases.^{57, 200-201} However, there is still an outstanding gap in our understanding of how this mechanically induced protein unfolding triggers distinctive/alternative large-scale downstream signalling in cells to differentially control their behaviour. In this chapter, the single molecule techniques evidenced so far are extended and combined with protein engineering and cellular biophysical approaches to characterise how protein unfolding can regulate cell mechanics.

These findings of the previous chapters open a plethora of possibilities to explore how talin interacts with other molecules to control cell function. Deleted in liver cancer 1 (DLC1) – a RhoGAP protein that negatively regulates the activity of RhoA-C¹³³ and is a potent tumour suppressor, whose loss has been implicated with numerous cancers¹³⁴ – is a notably interesting talin binding partner as mentioned in the

introduction. In order for its regulatory role on the Rho family of GTPases to have an impact on cellular function, DLC1 requires to be localised in focal adhesions, and this localisation is mediated by its interaction with talin.¹²³ DLC1 binds to the R8 helical bundle of the talin rod. This bundle is unique in that it does not lie in the chain between two other bundles in the talin rod, but exists rather as a protrusion of the R7 bundle (Figure 5.1). The nature of DLC1's binding to R8 is such that it forms an interaction with two of the helices in R8, effectively creating a five helix bundle out of R8's four helices and one of DLC1's.¹⁹⁵ This proposed mechanism of interaction suggests that if the R8 bundle structure is disrupted, it would abrogate the binding to DLC1. Given that the previous research has shown that the R8 bundle is vulnerable to force-induced unfolding,¹⁴⁷ I hypothesized that unfolding of the R8 region of talin by intracellular tension may modulate DLC1's recruitment to focal adhesions mediated by talin; and this triggers differential downstream pathways in cells (Figure 5.1).

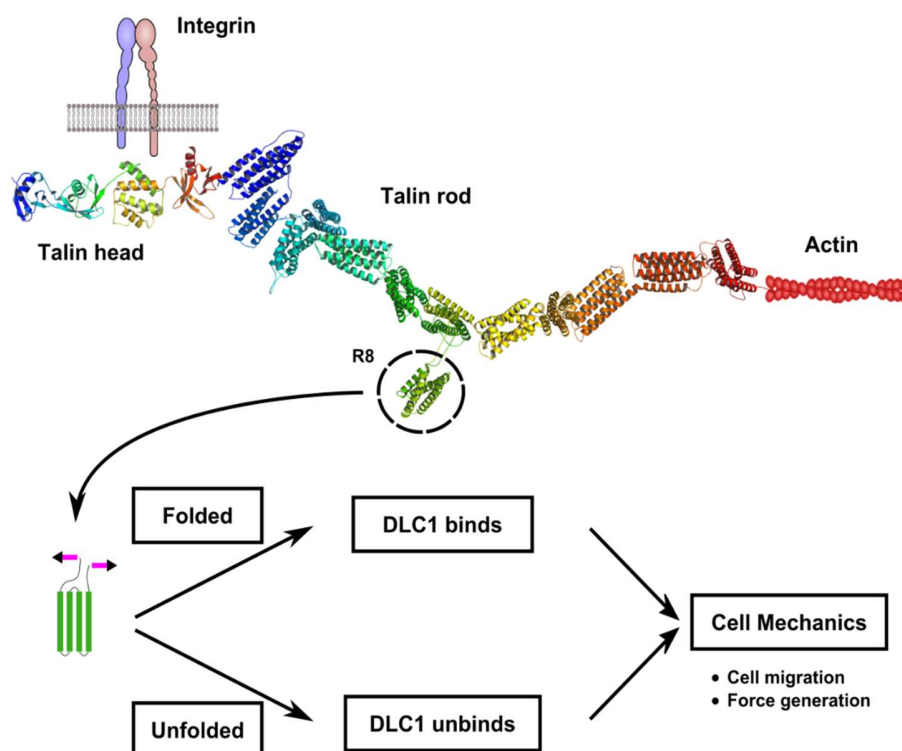


Figure 5.1 The unfolding/folding state of the R8 region of the talin rod modulates cell mechanics through DLC1 recruitment to focal adhesions. Talin simultaneously links integrins with the actin cytoskeleton. The R8 region protrudes out

of the talin molecule structure and a disulphide bridge was added to the linker area of R7-R8 to prevent R8 unfolding under mechanical load. The folded R8 region will promote DLC1 binding to talin and localisation to focal adhesions. This activates the negative regulation of the RhoA pathway that decreases myosin activation, actomyosin contractility, force generation and migration. On the other hand, force application on the native talin molecule would unfold the R8 region resulting in the unbinding and deactivation of DLC1, which would thus not be localised to focal adhesions and would lose its capacity to regulate the above-mentioned pathway.

To experimentally test this hypothesis, two polyproteins were engineered, amenable for mechanical manipulation using single molecule Atomic Force Microscopy (smAFM). The first polyprotein (R7-R8) contained the region R7 of the talin molecule and its linked R8 bundle flanked by two units of I27 domains and a HaloTag as previously reported.¹⁴⁷ Next, a mutant of the R7-R8 subdomain was designed (R7-R8_clamp) to make the R8 bundle resistant to tension-induced unfolding. To do this, I took advantage of the unique structure of the R8 bundle and its attachment to the rest of the talin rod. The long linkers that connect the opposite ends of the R8 bundle's amino acid chain to the R7 bundle are in close proximity to one another (<5Å between the backbone carbons).⁴⁶ I hypothesised that the introduction of a cysteine residue into each linker (by single point mutation to amino acids Q1459 and S1583) would create a disulphide bridge between the linkers, creating a clamp to hold the R8 bundle together (Figure 5.2). The advantage of the use of the R8 mutant is that it will allow the study of the talin unfolding without abrogating binding sites or removing entire subdomains.

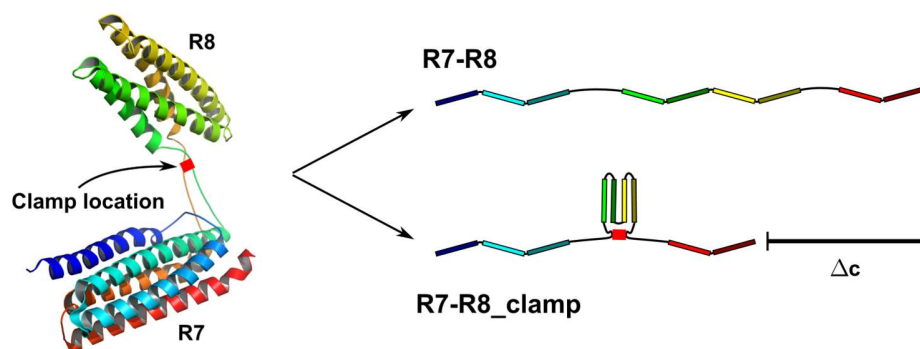


Figure 5.2 Position of disulphide clamp. Representation of the R7-R8 bundles showing the location of the clamp and how the clamp leads to a difference in unfolding length (Δc).

5.2 METHODS

5.2.1 Generation of polyprotein constructs and smAFM

The smAFM experiments were carried out as described in 3.2.1, 3.2.2 and 3.2.3 (Appendices 1&2).

5.2.2 Cells and culture conditions

The wild type MEF cell line was a kind gift from Dr. Wolfgang Ziegler and has been previously described by Xu & Baribault, 1998.²⁰² The TLN1-/-TLN2-/- MEF cell line has been previously described by Theodosiou *et al.*²⁰³ Both cell lines were maintained in high-glucose DMEM supplemented with 10% FBS and 1% GlutaMax (Thermo Fisher Scientific, USA). A humidified 37 °C incubator with 5 % CO₂ was used for culturing both cell lines.

5.2.3 Plasmid constructs for use in MEFs

In all cell experiments other than the FRAP, a GFP-Talin1 plasmid was used. The GFP-Talin1 plasmid was a gift from Anna Huttenlocher (Addgene plasmid # 26724).²⁰⁴ This was used to create the R8 mutant version of the plasmid, along with versions of both without the GFP tag (for use in FRAP). The GFP-DLC1 plasmid was a gift from Irene Ng.²⁰⁵

5.2.4 Cellular transfection

The TLN1-/-TLN2-/- MEF cell lines were transfected with a Neon transfection system (Thermo Fisher Scientific, USA). Cells were trypsinized and counted before being resuspended in the provided resuspension buffer, R1, at a concentration of 10⁶ cells per 100 µl transfection needle. 5 µg of DNA was added to each transfection volume before the following electroporation parameters were applied: 1400 V, 30 ms and 1 pulse.

5.2.5 FRAP

The GFP-DLC1 FRAP experiments were conducted on glass bottom Petri dishes (Mattek) coated with human plasma FN ($10\text{ }\mu\text{g ml}^{-1}$; Sigma) and incubated at $37\text{ }^{\circ}\text{C}$. For the DTT condition, dithiothreitol (Sigma) was added at a concentration of $100\text{ }\mu\text{M}$ 30 min prior to the start of the experiment. Confocal photobleaching and TIRF imaging were carried out using an inverted microscope (Eclipse Ti; Nikon). Five TIRF images were taken at 5 s intervals prior to bleaching for reference. Specified regions of the cells were then bleached using the confocal laser at 100% power. TIRF images were taken at 5 s intervals for 100 s to capture fluorescent recovery. Images were analysed with FIJI, with the fluorescent signal normalised between the pre-bleach intensity and background. Statistical analysis was then carried out using Prism (GraphPad). Data was pooled from repeats. The significance between curves was measured using extra sum-of-squares F test on the best fit lines.

5.2.6 MLC-2 immunostaining

Immunofluorescence staining was done on coverslips coated with $10\text{ }\mu\text{g/ml}$ fibronectin (Sigma, F0895). Following pertinent treatment cells were fixed with 4% PFA (Sigma, P6148) in PBS for 10 minutes then blocked and permeabilised with 2% BSA-0.1% Triton (Sigma, T8787) in PBS for 30 minutes. After blocking, cells were incubated with primary antibodies prepared in blocking solution for 1 hour at room temperature in a humidified chamber. Then cells were washed in PBS and incubated with Alexa Fluor 488 conjugated secondary antibodies and Phalloidin (Invitrogen, A22283, 1/1000 dilution) prepared in PBS for 30 minutes at room temperature. Finally, coverslips were washed in PBS and mounted in mounting reagent with DAPI (Invitrogen, P36931).

5.2.7 Micropillar video microscopy and traction force measurements

The micropillar arrays are based on our protocol as described previously.²⁰⁶ Pillar arrays were coated with human plasma FN (10 µg ml⁻¹; Sigma) and incubated at 37 °C for 1 h before measurements. Cells that had been trypsinised before measurements were suspended in culture media and plated onto the pillar substrates. Time-lapse imaging of the pillars was conducted with an inverted microscope (Eclipse Ti; Nikon) operating in bright-field mode with the samples held at an ambient temperature of 37 °C. Image sequences were recorded with a sCMOS camera (Neo sCMOS Andor) at 0.5 Hz using a × 40 (0.6 NA, air; Nikon) objective over 100 mins. The position of each pillar in the time-lapse videos was tracked using a custom MATLAB program to track the centre of a point spread function of the intensity of the pillars across all frames. By selecting a location free of cells, tracking of a small set of pillars allowed a measurement of the stage drift to be obtained and corrected for in the data set. The time-dependent displacement of a given pillar was obtained by subtracting the initial position of the pillar (zero force) from the position in a given frame. Traction forces were obtained by multiplying the pillar displacements by the pillar stiffness, the pillar forces were then summed to provide the cellular force.

5.2.8 Gel contraction

To analyse the ECM remodelling ability of MEFs, collagen-I (BD Biosciences, 354249, stock concentration 9.37 mg/ml) and matrigel (BD Biosciences, 354234, stock concentration 9 mg/ml) mixture gels were prepared with 10% 10x DMEM (Sigma, D2429) and 10% FBS (Gibco, 10500), yielding to a final concentration of 4.4 mg/ml collagen-I and 2.2 mg/ml matrigel. The gel mixture was neutralised with 1 M NaOH (Sigma, S8045), then 5x10⁵ cells were embedded in gels in culture media. 80 µl gel volume was added per well of a 96-well plate which was pre-treated with 2% BSA (Sigma, A8022) for 1 hour, washed with PBS and air dried for 10 minutes. Gels were set 1 hour at 37°C then incubated with culture media for 3 days at 37°C.

5.2.9 Migration assays

Polystyrene 6-well plates were coated with 10 µg/ml fibronectin in PBS (pH 7.4) at 37 °C for 1 h and washed two times with PBS. Transfected *TLN1*^{-/-}*TLN2*^{-/-} MEF cells were allowed to recover for 24 h, trypsinised and plated onto fibronectin coated well plates at a low confluency. Cells were allowed to attach for 90 min, followed by replacement of media (2 ml of fresh media) to remove non-transfected cells. For treating the cells with diamide, diamide powder (Sigma-Aldrich D3648) was dissolved in PBS to a stock concentration of 50 mM (8.6 mg/ml) and further diluted with complete cell culture media to 150 µM concentration. 1 ml of the diamide containing-media was added dropwise to 2 ml of cell culture media in each well 15 minutes before live cell imaging was started. EVOS FL Auto (Life Technologies, USA) equipped with a 20x objective and 37 °C and 5% CO₂ incubator was used for live cell imaging for 12 hours at 120 second intervals. The resulting image stacks were analysed with ImageJ version 1.50e with MTrackJ plugin.²⁰⁷⁻²⁰⁸ For each plasmid construct or diamide treatment, 130 – 200 individual cells were traced from three fully independent experiments.

5.2.10 2D SDS-PAGE analysis

Lysates of diamide-treated wild-type MEF cells were analysed by 2D SDS-PAGE to confirm the effect of diamide treatment on the level of cellular disulphide bonds. Cells at 80% confluency on 10 cm dishes were washed twice with warm PBS and treated for 120 min with 50 µM diamide dissolved in cell culture media. In negative controls, regular media was used instead. After the diamide treatment, cells were washed with ice-cold PBS and treated with 40 mM iodoacetamide in PBS for 5 min. Cells were lysed with 500 µl of RIPA buffer (50 mM Tris-HCl pH 7.4, 1 % NP-40, 0.5 % Na-deoxycholate, 0.1 % SDS, 150 mM NaCl, 50 mM NaF) supplemented with 40 mM iodoacetamide and Roche cOmplete protease inhibitor cocktail. Cell lysates were incubated on ice for 30 min and centrifuged at 14000 g for 20 min at 4 °C to pellet cell debris. The concentrations of cleared lysates were determined with BCA assay and matched by diluting the lysates with RIPA buffer. For 2D SDS-PAGE, 120 µg samples of cell lysates were mixed

with 2x Laemmli sample buffer without reducing agents, denatured at 95 °C for 5 min and loaded onto a medium sized, 1 mm thick 10%/4% polyacrylamide gel. The polyacrylamide gels were run at 25 mA current for 5 h, followed by vertical slicing of the gel with a clean scalpel to separate each lane. The gel slices were incubated in SDS-PAGE running buffer supplemented with 100 mM dithiothreitol for 20 min at room temperature to reduce disulphide bonds in the lysates. After the treatment, the gel slices were briefly washed with running buffer and treated with 100 mM iodoacetamide in SDS-PAGE running buffer for 10 min. The pieces of gel were placed horizontally on top of 1.5 mm thick 10% polyacrylamide gels and ran at 10 mA current for 14 h. Proteins on the gel were visualized by using silver staining.

5.3 RESULTS

5.3.1 smAFM validation of clamp

We used force extension protocol as previously described to study the unfolding of both polyproteins (Figure 5.3a).¹⁴⁷ The wild-type form unfolds in a single step, with a total unfolding length of 104 ± 7 nm, which represents the unfolding length of both bundles. The R8 mutant, by contrast, shows a reduced length of unfolding of 62 ± 6 nm (Figure 5.3b, c). The difference in unfolding length of around 40 nm matches the expected difference from lack of unfolding of the R8 bundle (124aa removal should result in 38-50nm reduction in unfolding length). This confirms that the clamp introduced in the R8 mutant makes this construct resistant to force-induced unfolding.

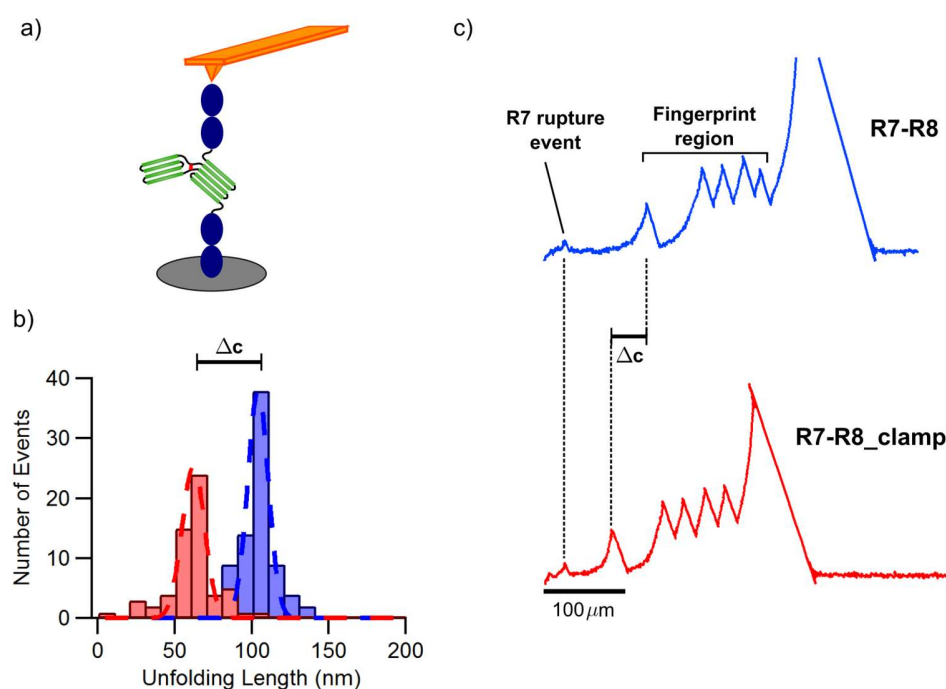


Figure 5.3 The disulphide clamp inserted in the R8 region of talin prevents R8 domain unfolding. (a) Schematic of smAFM setup with construct extended while trapped between a cantilever and piezo-driven surface. (b) Histogram showing the typical unfolding lengths of the wildtype R7-R8 (blue) and R7-R8_clamp (red) subdomains. (c) Representative traces highlighting the shortened unfolding pattern of the R7-R8 (blue) and R7-R8_clamp (red) subdomains.

5.3.2 FRAP and MLC-activation

We then expressed full-length talin constructs, both wild-type (WT) and with the disulphide clamp (R8), inserted within mammalian vectors, in talin null mouse embryonic fibroblasts (MEFs),²⁰³ to determine the cellular effects of preventing R8 unfolding. The redox potential inside the cell is not homogeneous and may vary depending on the culture conditions, and consequently the possibility of low levels of cytosolic sulphides is acknowledged.²⁰⁹ This is mitigated by the use of dithiothreitol (DTT) as a control as will be discussed.

We first tested the dynamics of focal adhesion-associated DLC1 using fluorescent recover after photobleaching (FRAP). Along with the talin constructs the cells were transfected with DLC1 tagged with green fluorescent protein (GFP). A region of the cell was then photo-bleached using a confocal laser. The recovery of the GFP signal into the focal adhesions of the bleached region serves as a measure of the dynamics of the DLC1 in those adhesions (Figure 5.4a). The recovery curves of the GFP signal show a difference in the immobile fraction between WT and R8 mutant (Figure 5.4b). Analysis of these curves displays a larger population of immobile DLC1 in the R8 mutant as compared to wild-type (Figure 5.4c). This implies a lower turnover of DLC1 in focal adhesions where the R8 bundle is unable to unfold and thus favours DLC1-binding.

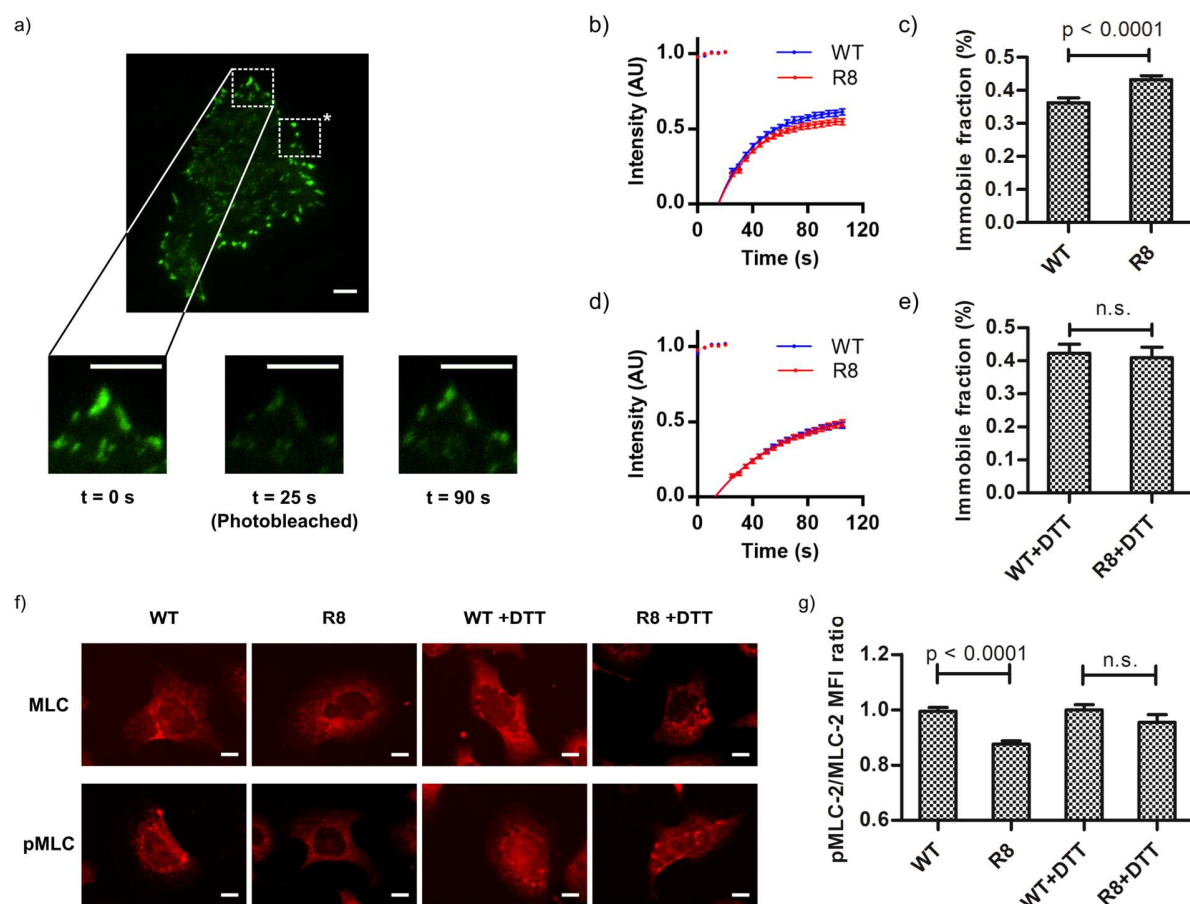


Figure 5.4 Preventing R8 unfolding increases DLC1 binding and downstream activation of mlc-2. (a) Representative images of a cell with GFP-DLC1. Insets show the intensity of the focal adhesions at different time points of the FRAP experiments. Region highlighted with asterisk was not exposed to confocal laser (Figure 5.5). Scale bars are 5 μ m. (b) FRAP curves for the recovery of GFP tagged DLC1 showing a difference in recovery for MEFs transfected with wild-type talin vs the clamped R8 mutant. (c) Immobile fraction data obtained from fit of FRAP curves. (d) FRAP curves for MEFs treated with DTT to remove disulphide clamp showing no difference in recovery. Errors bars represent sem. (e) Immobile fraction data from fit. (f) Immunofluorescence images of MLC and pMLC staining. Scale bars are 10 μ m. (g) Ratio of pMLC-2 staining to total MLC-2 for MEFs transfected with wild-type talin and the clamped R8 mutant, with and without DTT. For all panels, histograms represent mean $\pm$ sem.

To ensure that this effect was due to the presence of a disulphide bridge in the R8 bundle, the FRAP experiment were also conducted in the presence of DTT, a small-molecule that reduces disulphide bonds and thus would disrupt the clamp in bundle R8. When the FRAP experiments were performed in the

presence of DTT there was no difference between the recovery curves (Figure 5.4d), with no significant difference between the immobile fractions (Figure 5.4e).

Given that the localisation of DLC1 to focal adhesions has been shown to affect downstream cell processes,^{123, 210} there may be an effect of the R8 mutant on potential dependent pathways. Myosin light chain 2 (MLC-2) is downstream of the RhoA pathway, and its activation (phosphorylation of serine and threonine residues pMLC-2) is proportional to the level of active RhoA.²¹¹ Greater association of DLC1 with talin in the R8 mutant would downregulate the activation of RhoA and the downstream effector MLC-2. Immunofluorescence quantification of the pMLC-2/MLC-2 levels is used as a surrogate of MLC-2 activation (Figure 5.4f). There was a significantly higher pMLC-2/MLC-2 ratio in the WT cells as compared to those with the clamp (R8), indicating greater activation of MLC-2 (Figure 5.4g). As before, this difference was abrogated by the addition of DTT, showing that the presence of the disulphide clamp that makes the R8 region resistant to unfolding to allow DLC1 localization to focal adhesions was necessary to observe the large scale downstream effect (Figure 5.4g).

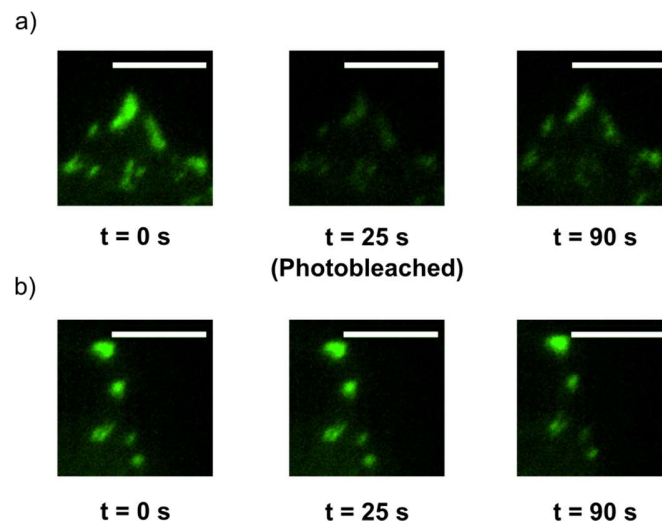


Figure 5.5 Comparison of bleached to non-bleached regions of cells in FRAP. (a) Representative images of a cell region exposed to confocal laser during FRAP, demonstrating photobleaching and subsequent recovery. (b) Images of a region of the same cell not exposed to high-power confocal laser, displaying no apparent photobleaching.

5.3.3 Effect of clamp on cellular traction and motility

Higher MLC-2 activation is associated with greater cellular traction force generation²⁰⁶ and there is expected to be an effect of the R8 mutant on force generation. Cells, expressing both wild-type and R8 mutant talin, were seeded on top of a substrate consisting of an array of flexible elastic polydimethylsiloxane (PDMS) micropillars coated with fibronectin. The deflection of the pillars is optically monitored and is proportional to the forces applied by the cells on the pillars (Figure 5.6a). Cells expressing WT talin applied significantly higher endogenous traction forces in comparison with the group of cells expressing the R8 mutant talin during the 90 minutes of cell spreading (Figure 5.6 4b-c).

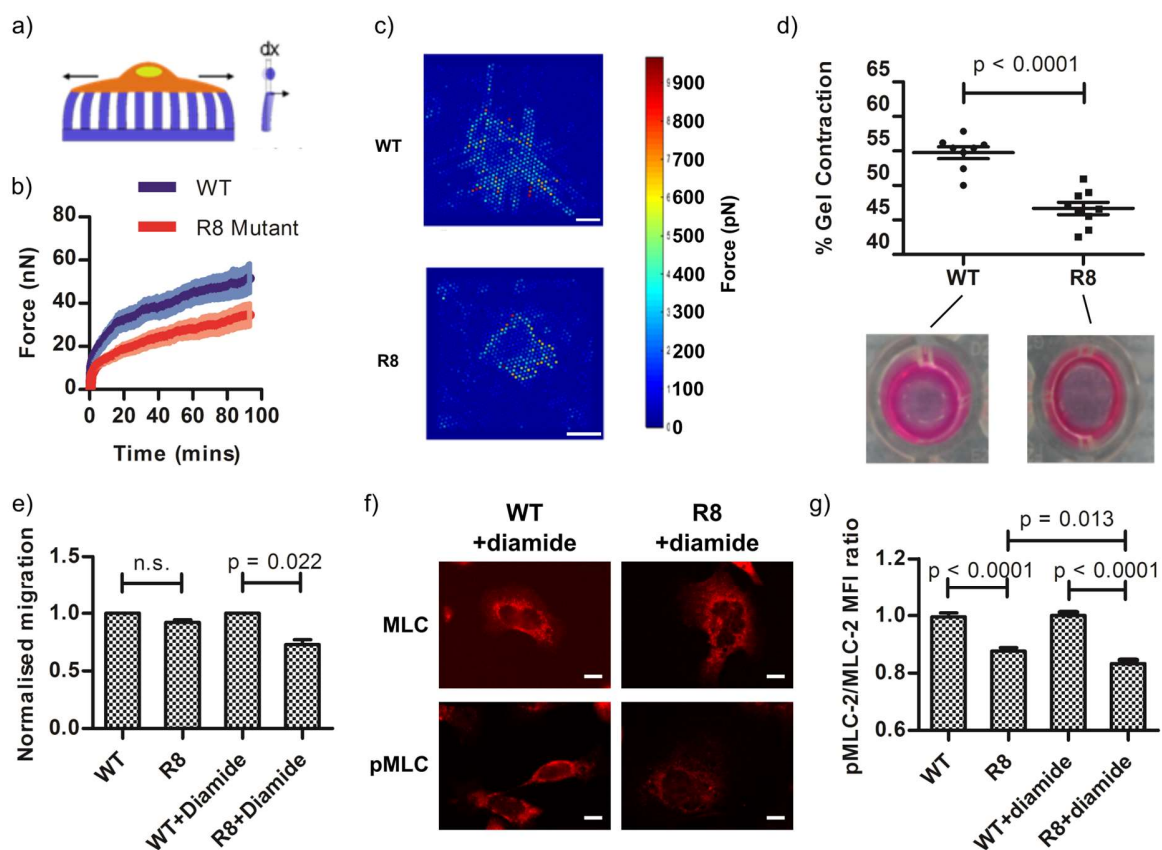


Figure 5.6 Preventing R8 unfolding reduces force application and cell migration. (a) Schematic of elastic pillars setup. (b) Curves of total cell force applied to pillars plotted against the time after initial spreading. (c) Heat maps of force applied to individual pillars by transfected MEFs. Scale bars are 15 μ m. (d) Scatter plot of the percentage change in gel size for collagen matrigels embedded with transfected MEFs for 72 h. (e) Normalized migration rate over 12 h for transfected MEFs, with and without diamide to enhance the disulphide clamp. (f) Immunofluorescence images of MLC and pMLC staining for diamide treated

cells. Scale bars are 10 μm . (g) Ratio of pMLC-2 staining to total MLC-2 for transfected cells, with and without diamide. For all panels, histograms represent mean $\pm$ sem.

The capacity of cells to apply forces on their substrate correlates with their ability to contract collagen gels and to remodel the ECM.²¹²⁻²¹³ To examine the ability of both group of cells (containing WT and R8 talin) to deform matrices, cells were embedded within collagen matrigel gels and, after 48 h, the WT cells contracted the gels to 47% of their initial size, whereas the mutant cells only contracted them to 55% of their initial size validating the previous results that showed that R8 cells displayed significantly lower traction forces (Figure 5.6d).

The localisation of DLC1 to adhesions has also been shown to negatively affect the ability of the cells to migrate.¹²³ The migration speeds of the wild-type and R8 mutant cells were measured over twelve hours, revealing a non-significant decrease in migration rate between wild-type and mutant (Figure 5.6e). Cells were treated with 50 μM diamide to shift the redox balance toward the oxidative range in the cellular cytoplasm so that more of the mutant protein would exhibit the disulphide bond preventing R8 unfolding and DLC1 unbinding. When the migration speed of these cells was tested there was a significant decrease in migration speed in the cells containing the R8 mutant in comparison to wild-type (Figure 5.6e). As a further test of the efficacy of diamide in enhancing the effect of the disulphide clamp, the pMLC-2/MLC-2 staining was performed for cells treated with diamide. In this case again the diamide increases the effect, with a greater decrease in pMLC-2/MLC-2 ratio for diamide treated R8 mutant cells than for those untreated (Figure 5.6f). 10 μM diamide concentration had clear effect in this assay. The oxidative effect of the diamide was confirmed for the disulphides by treating WT MEF cells with diamide and then analysing the cell lysate with 2D electrophoresis (Figure 5.7), comparing oxidizing and reducing conditions as described in previous study.²⁰⁹

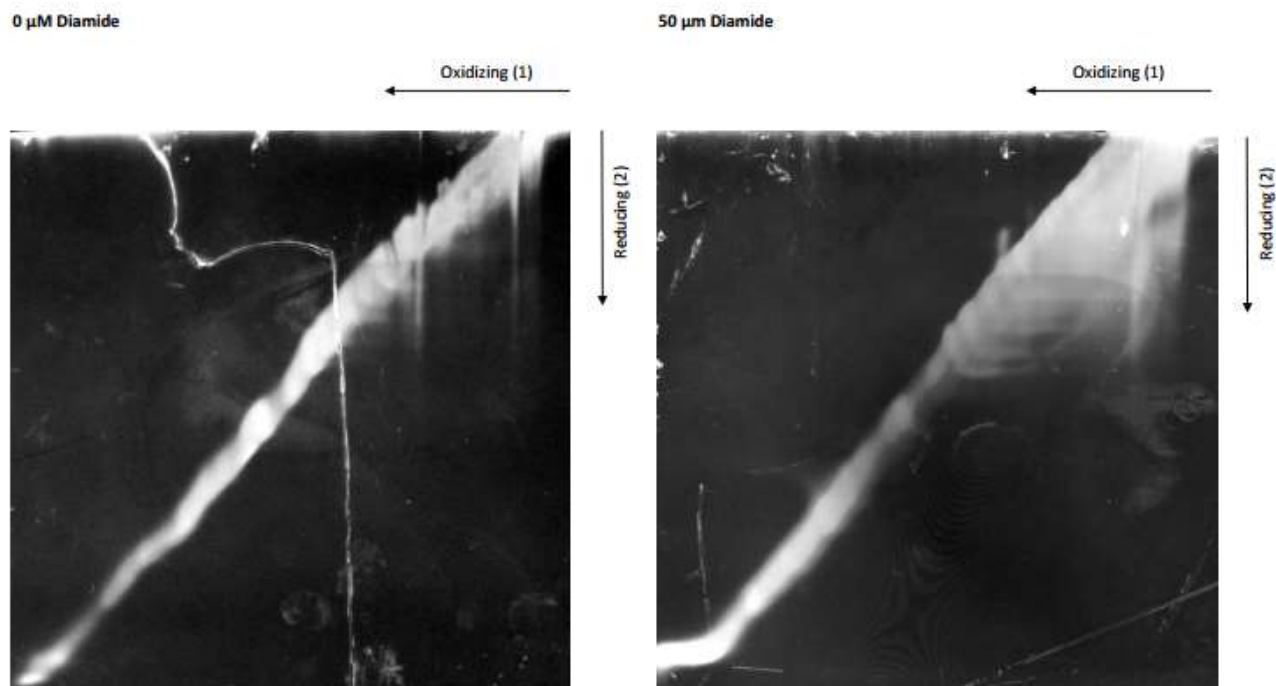


Figure 5.7 2D SDS-PAGE analysis of diamide treated fibroblast cells. To confirm the increase in the level of cellular disulphide bonds after a treatment with 50 μM diamide, lysates of diamide treated fibroblast cells were analysed with 2D SDS-PAGE. In the 50 μM diamide condition a large protein signal is detected beneath the diagonal line. This signal indicates the presence of oxidized disulphide bonds in the original sample that are reduced in the second step of the 2D SDS-PAGE. The signal below the diagonal is much less pronounced in the 0 μM condition, indicating a much lower proportion of proteins containing disulphide bonds. The proteins in total cell lysates were first separated without reducing agents (horizontal axis), followed by excising each gel lane to separate pieces and treating the gel lanes with dithiothreitol to reduce the disulphide bonds. The proteins on the reduced lanes were separated in a second SDS-PAGE run in the other direction to reveal the differential migration of disulphide-containing proteins in oxidising and reducing conditions. Proteins that do not contain disulphide bonds migrate at similar rates in both gel runs and end up on a diagonal band on the gel. Intra- or intermolecular disulphide bonds affect the rate of protein migration on gel, typically slowing down the rate of protein migration on the gel. Thus, proteins with intra- or intermolecular disulphide bonds migrate at a faster rate after breaking the disulphide bonds by reducing conditions, and appear below the diagonal line at the 2D SDS-PAGE analysis. Analysis of lysates of TLN1^{-/-}TLN2^{-/-} treated with 0 μM and 50 μM diamide revealed a clear increase in the level of cellular disulphide bonds after the treatment with 50 μM diamide. The images presented are representative of two fully independent replicates. This figure was created by Rolle Rahikainen.

5.4 DISCUSSION

From these results, it has been shown how it is possible to dramatically change the unfolding characteristics of a protein subdomain through the introduction of only two point-mutations. In this case the technique allowed us to probe the hypothesis: that the DLC1 localisation to focal adhesions is modulated by the unfolding of talin's R8 domain, constituting a novel force-controlled molecular switch. Through stabilising the R8 bundle with a disulphide bridge, the dynamics of focal-adhesion associated DLC1 were altered (Figure 5.4c). This led downstream to reductions in MLC-2 activation (Figure 5.4g), force generation (Figure 5.6b, d) and cell migration (Figure 5.6e).

In the widely accepted model talin-mediated focal adhesion activation, as force is applied to talin in maturing adhesions by the actin contractile machinery its helical bundles are disrupted. This allows vinculin to bind which leads to focal adhesion maturation.^{56, 73} It also, based on these findings, disrupts the binding of DLC1, reducing its negative effects on Rho activity, which enhances the generation of forces at growing adhesions. Thus, in early adhesions this works cooperatively with the increase in vinculin binding to mature the adhesions. However, the weakness of the model as it stood, with the vinculin-talin interaction at its core, was that it failed to account for any form of negative feedback. As the adhesions grew, talin recruited more vinculin, which increased force generation, which led to greater recruitment of talin and vinculin and so on.¹⁹¹

These results provide us with a further refined model of talin-mediated focal adhesion activation that works synergistically with its binding to vinculin initially but has a more important role to play in mature adhesions. Here is where the new model of a mechanical switch involving DLC1 does provide a mechanism of negative feedback and thus control. In the maturing adhesions, it is as before: force on talin unfolds the R8 domain reducing DLC1 localisation and increasing Rho activity. However, as the adhesion matures and recruits more proteins to the complex the force on individual talin molecules is reduced: this is because, although the total force applied to the adhesion is likely increasing, the presence of an increasing number of mechanical linkages, such as talin and vinculin, means that the force on each

linkage is reduced, like springs in parallel. The reduction in force on each linkage has been demonstrated in cellular experiments where the force on linkages has been measured by FRET tension sensors and shown to be lower in more mature, central adhesions than earlier, peripheral ones.²¹⁴⁻²¹⁵ This reduction in force on talin means that the R8 switch now reverts to DLC1 binding. This hypothesis is supported by studies showing a greater localisation of DLC1 to mature adhesions.⁷⁷ This reduces force generation and controls the maturation process of the adhesions. This is an attractive model not just in light of the results presented but also when considered with respect to the role of DLC1 deletion in cancers. The role of highly activated and contractile cells in the cancer progression has been investigated before, and it is possible that DLC1's role in cancer (by its absence) is mediated through the subsequent lack of control over focal adhesion activation. Further research is needed to investigate this link between cancer, DLC1 and this proposed talin-R8 mechanical switch.

5.5 CONCLUSION

These results demonstrate the existence of force-mediated binding between talin and DLC1 while also laying out a method for further investigations of talin or other mechanosensitive proteins. Previously mutation studies of these types of proteins focussed on the removal of sub-domains, whereas here I report a way to force these mechanical switches into a particular state. Small differences can also be amplified by the addition of reagents that alter the redox state of the cells to favour the fixed state. In this way the local effects of these switches can be established along with the downstream phenomena which they control. The regulation of signalling pathways by modulating protein structure and ligand binding may be a cellular adaptation cells have developed for rigidity sensing. Further studies utilizing other mechanosensitive protein would validate this.

6 OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 CONCLUSIONS

This research work has focussed on the focal adhesion protein, talin. As a key component of the integrin-associated complexes that connect the actin cytoskeleton to the ECM, it has become one of the most studied mechanosensitive molecules.⁵⁵⁻⁵⁷ These prior indications into the modes of mechanical activation of talin, along with its highlighted role in a variety of disease states,¹ means that the study of its role in cellular mechanotransduction could have far-reaching implications.

The first body of research results demonstrated that all the α -helical bundles of the talin rod are vulnerable to force-induced disruption at physiological levels of tension.⁹² This means that all the binding partners for these bundles have the possibility of having their interactions modulated by the mechanical force applied to focal adhesions. These results are consistent with two key pieces of prior cellular work: that of *Margadant et al.* who demonstrated the length to which talin extends in cells;⁶⁰ and also with *Lie et al.* who demonstrated that by truncating the talin rod the positioning of vinculin in focal adhesions is shifted, implying vinculin binding to regions other than R1-R3.⁶¹

It is also consistent with other work that I was involved in for the Cellular and Molecular Biomechanics Laboratory, where we examined the length scales over which cells are able to sense the mechanical properties of their surroundings. In these studies, we attached RGD motifs (the ECM binding partner for integrins) to inert, flexible PEG linkers of varying lengths. These were then attached to rigid, glass surfaces. By measuring the response of cells to these surfaces we were able to determine the length range over which cells detect mechanical properties is between 30-300nm, a length range that is consistent with the unfolding properties of talin.²¹⁶

The results of Chapter 3 were also supported by subsequently published research by Yao *et al.*, using magnetic tweezers instead of smAFM. Despite the use of a different technique, there results were highly

consistent with those presented here showing that the talin rod bundles unfold at physiological forces and with a hierarchy similar to ours.¹⁹⁴ The forces they measured were lower than ours, but this is to be expected given the difference in loading rates and the effect this has on binding strength.¹⁵⁶

This work builds upon the structural insights that had already been established and are discussed in the introduction. The focus of previous work on the R1-R3 subdomains had revealed key aspects of talin's mechanosensitivity, but failed to take into account the remaining 10 subdomains and what their role might be.⁵⁷⁻⁵⁸ Here it has been demonstrated that, although more stable than the R3 bundle, the remaining bundles are all able to be disrupted at physiological forces and thus contribute to talin's mechanosensitivity. Any further work that looks to expand on the body of talin research and understand its role must take the mechanical weakness of the remaining subdomains into consideration. In the context of cancer biology, for example, models on cancer progression in response to stiffness must be investigated in light of binding partners along the entire rod. Abrogation of these binding sites on stiff substrates, through the greater tension applied to talin, may account for numerous phenomena observed in these diseased phenotypes.

Chapter 4 presents the results of a study undertaken as a direct result of findings from the previous chapter. SMD experiments had highlighted the possibility that α -helical bundles unfold through a stable intermediate state, consisting of 3 α -helices. The functional role of this intermediate step is as yet unknown. It could be related to talin's force-mediated binding interactions or could be part of its function as a molecular shock absorber,¹⁹⁴ unfolding and refolding to dampen the effect of transmitted forces. Once the functional importance of this intermediate step is established it should be possible to relate it to talin's roles in both healthy and diseased phenotypes.

In the final piece of work, it was shown how an appreciation of talin's unfolding dynamics can be used to design cellular experiments to explore its role in modulating protein recruitment to focal adhesions. The R7-R8 domain provided a unique structure to exploit for this purpose.⁴⁶ The insertion of R8 into R7 via flexible linkers resulted in a convenient location for the novel disulphide clamp to test mechanosensitivity.

Prior mutation studies on talin have involved the mutation of whole binding sites or the removal or relocation of entire bundles.^{61, 191} By contrast, disulphide clamping requires only two, single-amino acid mutations to dramatically stabilise the R8 bundle and maintain the structure that supports the binding of DLC1.¹⁹⁵

This protein engineering allowed us to study an aspect of talin's role that, although revealed in the literature, had not been studied in light of talin's mechanosensitivity: its interaction with DLC1. This has provided a perfect mechanism to explain the greater metastatic response of cancer cells to higher rigidity substrates.² Higher rigidities are likely to cause greater tension and thus extension of the talin molecule, owing to the increased anchoring of integrins to which its head is bound. This will increase the likelihood of disruption to the R7-R8 subdomain.¹⁹⁴ This disruption, as evidenced in Chapter 5, reduces DLC1's affinity for talin and subsequently increases traction forces and migration. Given the important role that migration and cellular rigidity has in cancer progression, this in turn increases the metastatic potential of the cells.²⁰⁶ This model was outlined originally in Figure 1.4, and this work on talin and DLC1 has confirmed an important aspect of this overall picture.

The hope is that this work provides both an insight into talin's role in mechanotransduction and also a template for the study of mechanosensitive molecules. It is necessary to have a fully-integrated approach from the atomic-level, through to the cellular-level, and possibly in the future the tissue- and organ-levels. This way the molecular insights are able to influence the cellular experiment design, as in the design of the clamped mutant here, and the cellular experiments help to shape the focus and justification for the molecular studies, as here the focus on talin's possible role in cancer necessitated the study of those regions and interactions that could be responsible.

6.2 FUTURE PERSPECTIVES

The hope is that this work will have an influence on both the immediate field of talin-mediated cellular mechanotransduction and also on the wider mechanobiology community. In the context of talin mechanosensitivity, it is important to note how the work with DLC1 demonstrates the possibility of a negative feedback loop with this mechanotransduction pathway. As has been highlighted earlier in this work, the interaction with vinculin is one largely of positive feedback: force activates talin; talin recruits vinculin; vinculin increases force activation and recruitment of more talin.^{57, 72-73} By contrast, the association of DLC1 with focal adhesions reduces tension.¹³⁵ Thus the association of DLC1 with talin, and the multiplexing of forces across multiple linkers, can serve to reduce this activation cycle.

The link with DLC1 may also help to serve future research into talin's role in the progression of cancer. As described earlier, the links between both talin and DLC1 and numerous cancers have been well documented, and the research continues to increase with more recent works showing further links to breast, liver and ovarian cancers.^{217 218-219} A full understanding of the interaction between these two proteins and their upstream and downstream partners should help to direct cancer research and eventually cancer therapies.

This link between talin, its mechanosensitivity and cancer needs further research to establish its importance. There will be, however, technical issues that must be overcome to establish this link. The most prominent of which is the integral role that talin plays in a variety of important processes and thus the adverse effect that interfering with its levels will have, potentially masking its effect on cancer progression or preventing study altogether. For example, knocking down Talin 1 entirely could be useful for studying the effect of its levels on cancer progression; doing so, however, results in aberrant embryogenesis and embryo death around 8.5-9.5 days *post coitum* in mice models.²²⁰

One way to circumvent this particular issue would be to utilise a Cre-lox system for inactivation of the talin gene: these systems allow for specific genes to be inhibited in response to an external stimulus.

This would allow for the reduction of talin levels in specific tissues after the mice had developed beyond the crucial developmental stages. This could then be combined with the induction of a talin-related cancer into these Cre/lox mice. As an example, hepatocellular carcinoma has been linked with increased talin levels,⁸⁵ and can be induced in mice by dosing them with diethylnitrosamine (DEN).²²¹ By using both DEN and Cre/lox in conjunction, hepatocellular carcinomas could be induced and talin levels depleted in the same tissue. In this manner the effect of talin levels on cancer progression could be directly established.

As well as studying the effect of reduced talin levels in cancer cells on their metastatic potential, it would also be valuable to understand the effect of reducing talin levels on the cells that surround a tumour but are not themselves cancerous. Prior research has established that mechanical activation of cancer-associated fibroblasts (CAFs) can lead to increases in metastatic promoters. This metastatic promotion can occur either mechanically, with CAFs increasing the stiffness of the tumour ECM, or chemically through the release of cytokines, such as TGF- β .^{206, 222} To study the effect of talin levels of this CAF-promoted metastasis, a Cre/lox system as described above could be used to reduce the talin levels in particular tissue. Cancer cells could then be injected into the same tissue to form a tumour. The difference this time would be that the cancer cells would have normal levels of talin but the CAFs would have their talin levels reduced. Again, one would expect to see a reduction in metastatic potential when talin levels are reduced, although perhaps not as great as if the talin depletion is in the cancer cells themselves.

In order to study the role of talin's mechanosensitive interaction with DLC1 in cancer progression, similarly advanced biological tools must be utilised to overcome the issues as stated before. In this thesis, I have demonstrated the effectiveness of a disulphide clamp in protecting R8 from tension-induced disruption. Given that this increases DLC1 binding and has a negative effect on cancer-associated pathways, one would expect that a similar mutation in a tissue of a mouse would reduce metastatic potential in that tissue. Extending this mutation from single-cell studies to *in vivo* is a complex experiment, however, as a majority of cells in a tissue need to be mutated rather than a population of cells *in vitro*. Recent biological research techniques provide a possible mechanism by which this could be achieved: CRISPR/Cas9 gene

editing, a system that allows for the editing of specific genes of a fully developed organism such as mouse.²²³ Through this method, the R8 disulphide mutation could be created in specific tissues. This could, as above, be combined with *in situ* cancer induction, such as hepatocellular carcinomas through DEN dosing, or with the injection of cancer cells to determine the effect of talin's mechanosensitive interaction with DLC1 on cancer progression.

The relevance of this work should also be felt in the wider field of mechanobiology, which is increasingly being accepted as a key driver and regulator of health and disease. As well as the conditions discussed in the introduction, talin has also been more recently identified in a range of other disease states, including Alzheimer's and multiple sclerosis.²²⁴⁻²²⁵ The discovery that the full talin rod is capable of being unfolded at physiological forces amplifies its mechanosensitive role in various cellular processes as it means that all the binding sites along its length may be subject to force-mediated modulation.

Finally, this work has also sought to examine the dynamics of tension-induced unfolding of α -helical proteins. It showed that the simple model of bundle breakage ignores a mechanical intermediate state whose functional impact has yet to be determined. This work is relevant to a large number of proteins that may be exposed to tension, given the abundance of these protein structures within biology. It has already been shown that α -catenin has its interaction with vinculin modulated by force in much the same way as talin.¹⁷⁷ Similar interactions are also being investigated in other α -helical proteins such as α -actinin.²²⁶ The impact of the intermediate state on the binding interactions and thus physiological role of α -helical proteins remains to be investigated. Such investigation would require the development of the single-molecule techniques that have been used so far to study mechanosensitive molecules.

In particular it will be necessary to create more advanced techniques for studying the binding of partners to mechanosensitive proteins in real time as they are exposed to force. Magnetic tweezer devices have been shown to be able to control the forces applied to proteins in the low piconewton regime that would be necessary to study this.^{58, 194} Such a device could be used as the base platform for such an investigation whereby an α -helical protein would be exposed to sufficient force to disrupt the 5-helix

bundle, with the force then quickly lowered to allow the 3-helix intermediate to remain intact. This step-and-hold force was used in the SMD simulations in Chapter 4 (Figure 4.8b). With the 3-helix intermediate exposed, fluorescently-tagged binding partners could be introduced to determine their affinity for this state versus the fully formed 5-helix bundle or the entirely disrupted bundle. This use of fluorescently-tagged binding partners was already demonstrated as being effective in the studies on talin's mechanosensitive interaction with vinculin (Figure 1.3b). Through these experiments it could be determined whether the 3-helix intermediate allows for the binding of partners such as vinculin and thus what functional role it serves.

A combination of these further protein studies with the *in vivo* cancer studies described above would provide a broad, holistic view of the role that mechanosensitive proteins, such as talin, play in cellular process that are vital to both healthy and diseased physiological states.

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APPENDIX 1 – PLASMID SEQUENCES

CHAPTER 3

Bam**HI** and **Kpn****I** cloning sites

Adjusted pFN18A:

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R7-R8

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CACCATCATCATTGCTAA **GGTACC**

R11-R12:

GGATCCATGCTGATCGAGGTGGAAGAAACCTCTGTACGGTGTGGAAGTGTTTGTGGGTGAAACCGCCCACTTCGAGATTGA
ACTGAGCGAGCCGGACGTTACGGTCAATGGAAGTGAAGGGTCAGCCGCTGGCAGCCAGCCCGGACTGTGAGATTAT
CGAAGACGGTAAGAAGCATATCCTGATTCTGCATAATTGCCAGCTGGGTATGACAGGCGAAGTGAGCTTTCAGGCAGCCA
ATACCAAGAGCGCCGCCAACCTGAAAGTGAAAGGAGCTGAGATCCCTGATCGAGGTGGAGAAACCTCTGTATGGCGTTGA
AGTGTGTTGTTGGCGAGACAGCCCACTTCGAGATCGAGCTGAGCGAACC GGATGTTTCATGGTCAGTGGAAGCTGAAGGGC
CAACCTCTGGCCGCCAGCCCTGATTGTGAGATTATTGAAGACGGCAAAAAAGCATATTCTGATTCTGCACAATTGTCAGCTG
GGTATGACCGGTGAGGTGAGTTTCCAGGCCGCCAATACCAAAAGCGCAGCAACCTGAAGGTGAAAGAACTGAGATCCA
ATCGTGGCACCCAAGCCTGCATTACAGCCGCAAGTGCCGTTAGCGGTATTATTGCCGACCTGGATACCACCATTATGTTT
GCCACAGCCGGCACACTGAACCGCGAAGGTACAGAGACCTTTGCCGATCATCGCGAGGGCATCTTAAAAACCGCCAAGG
TGCTGGTTGAAGATACCAAGGTGTTAGTTCAGAATGCAGCCGGCAGCCAAGAAAAATTAGCCCAAGCAGCCAGAGCAG
CGTTGCAACCATTACCCGTCTGGCCGATGTTGTTAAGCTGGGCGCCGCAAGCCTGGGTGCAGAAGACCCTGAGACACAG
GTGGTGCTGATCAATGCCGTGAAAGACGTGGCAAAGGCCCTGGGTGATCTGATCAGTGCAACAAAGGCAGCAGCCGGTA
AGGTGGGTGATGATCCTGCCGTGTGGCAGCTGAAAAACAGCGCCAAGGTGATGTTACCAACGTTACCAGCCTGCTGAA
GACCGTTAAGGCCGTTGAGGACGAAGCAACAAAGGCACACGCGCCCTGGAAGCAACAACCGAACACATCCGTCAGGAA
CTGGCAGTTTTCTGCAGCCCTGAGCCGCCGGCCAAAACAGTACCCCGGAAGACTTCATCCGCATGACCAAAGGCATCA
CCATGGCCACCGCAAAAGCCGTTGCCGCCGGCAATAGTTGTCGCCAAGAGGACGTGATTGCCACCGCAAATCTGAGCCG
TCGCGCAATTGCCGACATGCTGCGCGCATGCAAGAGGGCCGCATATACCCGGAAGTTGCCCGGATGTTCTGCTGCGC
GCACTGCATTACGGTCGCGAGTGTGCAATGGCTACCTGGAAGTGTGGATCACGTTCTGCTGACCCTGCAGAAACCGA
GTCCGGAGCTGAAACAGCAGCTGACCGGCCACAGCAAGCGTGTGGCAGGTAGCGTGACAGAATTAATCCAGGCCGCCG

AGGCCATGAAAGGCACCGAATGGGTTGATAGATCCCTGATCGAAGTTGAGAAGCCGCTGTATGGTGTGAGGTGTTGCTT
GGCGAGACCGCACATTTGAAATTGAGCTGAGTGAGCCGGATGTTACGGCCAGTGGAAGTGAAGGTCAACCTCTGG
CAGCCAGCCCGGATTGCGAAATTATCGAAGATGGTAAAAACACATCCTGATTCTGCATAACTGTCAGCTGGGCATGACA
GGCGAGGTTAGCTTTCAAGCCGCCAACACCAAAAGCGCCGAAACCTGAAAGTTAAGGAGCTGAGATCCCTGATCGAAG
TGGAGAAACCGCTGTACGGTGTGAAGTGTTCGTGGGTGAAACAGCCCATTTTGAAGATCGAGCTGAGCGAGCCGGACGT
GCACGGTCAGTGGAAGTGAAGGTCAACCGTTAGCAGCCAGCCCGGACTGCGAGATCATTGAAGATGGTAAGAAGCAC
ATCCTGATCCTGCACAACCTGCCAACTGGGCATGACCGGCGAAGTTAGCTTCCAGGCAGCAAACACAAAAAGCGCCGCAA
ATCTGAAGGTGAAGGAACTGAGATCTCACCACCATCATCACCATTGCTAA~~GGTACC~~

CHAPTER 4

~~NheI~~ and ~~NcoI~~ cloning sites

Adjusted pFN18A with new cloning sites:

GGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGTATTTAACTTTACTAAGGAGAATTCATCATGGCAGAAATC
GGTACTGGCTTTCCATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTGATGTTGGTCCGCGCG
ATGGCACCCCTGTGCTGTTCTGACGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGCACC
GACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAATCCGACAAACCAGACCTGGGTATTCTTCGACGACC
ACGTCCGCTTCATGGATGCCTTCATCGAAGCCCTGGGTCTGGAAGAGGTCGTCCTGGTCATTACGACTGGGGCTCCGC
TCTGGGTTTCCACTGGGCCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCTATCCCGA
CCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCAGGCCTTCCGCACCACCGACGTGCGCCGCAAGCTGATCAT
CGATCAGAACGTTTTTATCGAGGGTACGCTGCCGATGGGTGTCGTCCGCCCGCTGACTGAAGTCGAGATGGACCATTAC
CGCGAGCCGTTCTGAATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGCCA
GCGAACATCGTCGCGCTGGTCTGAAGAATACATGGACTGGCTGCACCAGTCCCCTGTCCCGAAGCTGCTGTTCTGGGGCA
CCCCAGGCGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGCCTAACTGCAAGGCTGTGGACATCG
GCCCCGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTGACGCTCG
AGATTTCCGGCGAGCCAACCACTGAGGATCTGTACTTTCAGAGCGATAACGCGATCGCCGGATCCATGCTGATTGAAGTG
GAAAAACCTCTGTACGGTGTGGAGGTGTTCTGTGGGTGAAACCGCCCACTTCGAGATCGAACTGAGCGAACCGGATGTGC
ACGGTCAGTGGAAGTGAAGGTGAGCCGCTGGCAGCAAGCCCGGACTGTGAGATTATTGAGGATGGTAAGAAACACAT
TCTGATTCTGCATAATTGTGAGCTGGGTATGACCGGCGAAGTGAGCTTTCAAGCCGCCAACACCAAGAGTGAGCCAACC
TGAAAGTGAAAGAGCTGAGATCCCTGATCGAAGTGGAGAAGCCTCTGTACGGCGTGGAAGTGTGTTGGGGCGAAACAGC
CCATTTTGAGATTGAGCTGAGCGAACCTGATGTGCACGGCCAATGGAAGTGAAGGTCAACCGCTGGCCGCAAGTCCG
GATTGTGAAATTATCGAGGACGGCAAGAAGCACATCCTGATCCTGCATAATTGCCAGCTGGGCATGACAGGTGAGGTGA
GCTTCCAGGCCGCAAATACCAAAAGTGCCGCCAATCTGAAAGTGAAGGAATTA~~GCTAGC~~GATAGCGAGGTGGCAGCCCT
GGTGATCGATAACGGCAGCGGTATGTGCAAAGCCGGCTTTGCCGGCGATGATGCCCTCGCGCCGTTTTTCCGAGCATT
GTTGGTCTGCTCCTCGTCACCAGGGTATTATGGTTGGCATGGGCCAGAAGGACAGCTATGTGGGCGATGAAGCACAGAGTA
AACGCGGCATCCTGACCCTGCGTTACCCGATTGAGCATGGCATTGTGACCAACTGGGACGATATGGAGAAAATTTGGCAT
CATACCTTCTACAACGAAGTGCCTGTTGCACCTGAAGAACATCCGGTGCTGCTGACAGAAGCCCCGATGAATCCGAAGAG
CAACCGCGAGAAAATGACACAAATTATGTTTGAAACCTTCAACGTGCCTGCTTTTATGTGAGCATCCAGGCCGTTCTGAG
CCTGTATAGCAGCGGCCGCACCACAGGCATTGTGCTGGATAGCGGTGATGGCGTGACCCATGTTGTTCCGATCTACGCC
GGCTTCAGTCTGCCGCATGCCATCCTGCGCATTGATCTGGCCGGTCGCGATCTGACCGACTATTTAATGAAGATTCTGAG

TGAGCGCGGCTACAGCTTTAGCACCACAGCAGAACGCGAGATCGTGCGCGATATCAAAGAGAAGCTGTGCTACGTGGCC
CTGGACTTTGAACAGGAAATGCAGACCGCAGCCCAGAGCAGTAGCATCGAGAAGAGCTATGAACTGCCTGACGGCCAGG
TTATCACCATTGGTAATGAGCGCTTTTCGTGCCCCGGAGGCCCTGTTTCACCCGAGCGTGTTAGGTCTGGAGAGCGCCGG
TATTGACCAAACCACATACAATAGTATTATGAAATGTGATGTTGACGTGCGTAAAGAACTGTATGGCAACATCGTTATGAG
CGGCGGCACCACCATGTTTCCTGGTATCGCCGAACGCATGCAGAAAAGAAATCACCGCCCTGGCCCCCTAGTAGCATGAAG
GTGAAGATCATCGCCCCGCCGAACGCAAGTATAGCGTGTGGATTGGTGGCAGCATTCTGGCAAGCCTGACCACCTTCC
AGCAGATGTGGATTAGCAAGCAGGAGTATGATGAAAGTGGTCCGAGCATCGTGCATCACAAATGTTTC**CCATGG**CTGATC
GAGGTGGAAAAGCCGTTATACGGCGTGGAAGTGTTGTTGGTGAGACCGCCCACTTCGAAATTGAACTGAGTGAACCGG
ATGTGCATGGCCAGTGGAAGCTGAAAGGTCAACCGTTAGCCGCCAGCCCTGATTGCGAGATTATTGAAGACGGCAAGAA
ACATATCCTGATCCTGCATAACTGCCAACTGGGTATGACCGGCGAGGTTAGCTTTCAAGCCGCCAACACCAAGAGTGCCG
CCAATCTGAAGGTGAAGGAACTGAGATCCCTGATTGAGGTGGAAAAACCGCTGTATGGCGTGGAAGTGTTTGTTGGTGAA
ACCGCCCATTTTGAATCGAACTGAGCGAGCCGGACGTGCACGGCCAGTGGAATTAAGGGCCAACCGCTGGCAGCCA
GTCCGGATTGCGAAATTATTGAAGATGGCAAAAAGCATATTCTGATCCTGCACAATTGCCAACTGGGTATGACAGGCGAA
GTGAGTTTCCAGGCCGCCAATACCAAGAGTGCCGCCAATCTGAAGGTTAAGGAGCTGAGATCTCACCATCATCACCATCA
TTGCTAAGGTACCAGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTGATCCGGCTGCTAACAAAGCCCGAAAGGAAG
CTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTG
CTGAAAGGAGGAACTATATCCGGTTCGCTTGCTGTCCATAAAACCGCCAGTCTAGCTATCGCCATGTAAGCCCACTGCA
AGCTACCTGCTTTCTCTTTCGCGTTGCGTTTTCCCTTGTCAGATAGCCAGTAGCTGACATTCATCCGGGGTCAGCACC
GTTTCTGCGGACTGGCTTTCTACGTAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCT
ATTTGTTTATTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAA
AGGAAGAGTATGAGTATTCAACATTTCCGTGTGCCCCATTATCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACC
CAGAAACGCTGGTGAAAGTAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGC
GGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTCAAAGTTCTGCTATGTGGCGCGGTA
TTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGTTGAGTACTCACC
AGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTG
CGGCCAACTTACTTCTGACAACTATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGACAACATGGGGGATCATGTAAC
CGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGG
CAACAACGTTGCGCAAATATTAAGTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCG
GATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGC
GTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAG
TCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAATTCGAAATGAC
CGACCAAGCGACGCCCAACCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAG
GAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCT
CCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCC
TTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGATAGGTCGTTTCGCTCCAAGCTGGGCT
GTGTGCACGAACCCCCCGTTAGCCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACA
CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTG
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AAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGGTTGCAAGCAGCAGATTACGC
GCAGAAAAAAGGATTTCAAGAAGATCCTTTGATCTTTTCTACGGGTCTGACGCTCAGTGAACGAAAACCTCACGTTAAG
GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTATAGTCCGGAAATACAGGAACGCACGCTGGAT

GGCCCTTCGCTGGGATGGTGAAACCATGAAAAATGGCAGCTTCAGTGGATTAAGTGGGGGTAATGTGGCCTGTACCCCTC
TGGTTGCATAGGTATTCATACGGTTAAATTTATCAGGCGCGATTGCGGCAGTTTTTCGGGTGGTTTGTGCCATTTTTAC
CTGTCTGCTGCCGTGATCGCGCTGAACGCGTTTTAGCGGTGCGTACAATTAAGGGATTATGGTAAATCCACTTACTGTCT
GCCCTCGTAGCCATCGAGATAAACCGCAGTACTCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTTTTCAGCCT
GATACAGATTAAATCAGAACGCAGAAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCT
GACCCCATGCCGAACTCAGAAGTGAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAAC
TGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTTGTCCGTGAACGCTCT
CCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCC
CGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGT
TTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT

R9:

GCTAGCATGGGTCAGTTAGAGTGCGAAACCGCCATTGCAGCACTGAATAGTTGCCTGCGCGACCTGGATCAGGCCAGCC
TGGCCGCAGTTAGTCAGCAGCTGGCCCCGCGTGAGGGCATTAGCCAAGAGGCACTGCATACCCAGATGCTGACCGCCG
TGCAGGAAATCAGCCACCTGATTGAGCCGCTGGCCAGCGCAGCACGTGCAGAAGCAAGCCAGCTGGGCCACAAGTTA
GCCAGATGGCCAGTATTTGAGCCGCTGACCTTAGCAGCAGTTGGCGCCGCCAGCAAAACCTTAAGCCATCCGCAACA
GATGGCCCTGCTGGACCAGACCAAAACCTTAGCCGAAAGCGCCCTGCAGCTGCTGTATACAGCCAAAGAAGCCGGCGGT
AACCCGAAACAGGCAGCCACACCCAGGAAGCACTGGAAGAGGCCGTGCAAATGATGACAGAGGCAGTGGAAGATCTG
ACCACCACCCTGAACGAGGCAGCAAGCGCAGCAGGCGTGTTGGT**CCATGG**

R11:

GCTAGCATGACCCAGGCATGCATTACCGCCGCCAGCGCAGTTAGCGGCATCATCGCCGATCTGGATACCACCATCATGT
TTGCCACCGCCGGTACCCTGAATCGCGAAGGTGCCGAAACCTTTGCAGATCACCGCGAAGGCATTCTGAAAACCGCCAA
GGTGCTGGTGGAAGACACCAAAGTGCTGGTGCAAGTGCCGCCGGTAGCCAGGAAAACTGGCCAGGCCGCCAGAG
TAGTGTGGCCACCATACCCGTCTGGCCGATGTTGTGAAACTGGGCGCAGCCAGCCTGGGTGCAGAAGATCCGGAACCC
CAGGTGGTGCTGATCAACGCAGTTAAAGACGTGGCCAAGGCCCTGGGTGATCTGATTAGTGCAACCAAAGCCGCCGCCG
GCAAAGTGGGTGATGATCCGGCCGTTTGGCAGCTGAAGAACAGCGCCAAAGTGATGGTGACCAACGTGACCAGCCTGCT
GAAACCGTGGAAGGCCGTGGAAGATGAGGCCACC**CCATGG**

R9_{WT}-R9_{WT}:

GCTAGCATGGGTCAGCTGGAGTGCGAGACCGCCATTGCCGCCCTGAACAGTTGCCTGCGCGACCTGGATCAGGCAAGC
CTGGCAGCCGTTAGCCAGCAGCTGGCCCCGCGTGAAGGCATTAGTCAAGAGGCCCTGCACACACAGATGTTAACCGCC
GTGCAGGAAATCAGCCACCTGATTGAACCGCTGGCAAGTGCCGCACGTGCAGAGGCCAGTCAGCTGGGTACAAAAGTGA
GTCAAATGGCCAGTACTTCGAGCCGTTAACCTGGCAGCCGTTGGCGCCGCAAGTAAGACCCTGAGCCATCCGCAACA
GATGGCCCTGCTGGACCAACAAAGACCCTGGCCGAAAGCGCACTGCAACTGCTGTATACCGCAAAAGAGGCCGGTGGT
AATCCGAAACAGGCCGCACACACCCAGGAAGCCCTGGAGGAAGCGTTTCAGATGATGACAGAAGCCGTTGAAGACCTGA
CCACAACCCTGAATGAAGCCGCAAGTGCAGCAGGTGTGGTGGTGGTCAGTTAGAGTGTGAGACCGCAATTGCAGCCCT
GAATAGCTGTCTGCGCGATCTGGACCAGGCAAGTCTGGCAGCAGTGAGTCAGCAACTGGCCCCGCGCAAGGCATCAG
CCAAGAAGCCCTGCATACACAAATGCTGACCGCCGTGCAAGAAATCAGTCATCTGATTGAGCCGCTGGCAAGCGCAGCC
CGTGCAGAAGCCAGTCAATTAGGTACAAAGGTGAGCCAGATGGCCCAATACTTTGAACCGTTAACCTGGCCGCCGTGG

GTGCAGCAAGCAAAACCCTGAGCCACCCGACGCAAAATGGCCTTATTAGATCAGACAAAAACCCTGGCCGAGAGCGCCCT
GCAGCTGCTGTACACCGCCAAGGAAGCCGGTGGTAACCCGAAACAGGCAGCCCATACCCAAGAAGCTCTGGAAGAAGC
CGTGCAGATGATGACCGAGGCCGTTGAGGACCTGACCACCACCCTGAATGAGGCCGCCAGTGCAGCAGGTGTGGTGGG
TCCATGG

R9_{N/C} clamps-R9_{WT}:

GCTAGCATGGGCCAGCTGGAATGTGAGACCGCCATTGCCGCCCTGAACAGCTGTCTGCGCGACCTGGATCAGGCAAGC
CTGGCAGCCGTTAGCCAACAACCTGGCCCCGCGCGAAGGTATCAGCCAAGAAGCCTGCCATACCCAGATGCTGACCGCC
GTGCAGGAAATTAGCCACCTGATCGAACCGCTGGCCAGTGCATGTGCGCGAGAAGCCAGCCAACCTGGGCCACAAAGTGA
GCCAGATGGCCCAAGTATTTTGAAGCCGCTGACACTGGCAGCCGTGGGCTGTGCAAGCAAGACACTGAGCCATCCGCAACA
AATGGCACTGCTGGATCAAACCAAAACCCTGGCCGAAAGTGCACTGCAACTGCTGTACACCTGTAAAGAAGCCGGTGGTA
ACCCGAAACAGGCAGCACACACCCAAGAAGCCCTGGAAGAAGCCGTTCAAATGATGACCGAAGCAGTGAGGACCTGAC
CACCACACTGAACGAAGCAGCCAGTGCCGCAGGCGTTGTTGGTGGCCAGTTAGAATGCGAAACCGCCATTGCAGCCCTG
AATAGCTGCCTGCGTGATCTGGACCAGGCCAGCCTGGCCGCCGTTAGCCAGCAGCTGGCACCGCGTGAAGGCATTAGT
CAAGAGGCCCTGCACACCCAAATGCTGACCGCAGTGCAAGGAGATCAGCCATCTGATCGAACCTCTGGCCAGCGCAGCAC
GTGCCGAAGCAAGCCAGCTGGGCCATAAAGTGAGCCAAATGGCCCAATATTTTGAACCGCTGACACTGGCAGCAGTTGG
CGCCGCAAGCAAAACACTGAGCCACCCGCAGCAGATGGCACTGCTGGACCAGACCAAGACCCTGGCAGAAAGCGCCCT
GCAGCTGCTGTACACAGCCAAAGAAGCAGGCGGTAATCCGAAACAGGCCGCCCATACCCAAGAAGCTCTGGAAGAGGC
CGTGCAGATGATGACCGAGGCCGTTGAAGATCTGACCACCACACTGAATGAAGCCGCCAGCGCAGCCGGTGTGTTGGT
CCATGG

CHAPTER 5

Location of mutated cysteine residues

R7-R8_{clamp} (mutation of R7-R8 smAFM construct):

GGATCCATGCTGATCGAGGTGGAAAAACCTCTGTACGGTGTGGAAGTGTGTTGTGGGTGAAACCGCCCATTTTCGAGATTGA
ACTGAGCGAGCCGGACGTTACGGTCAATGGAAACTGAAGGGTCAGCCGCTGGCAGCCAGCCCGGACTGTGAGATTAT
CGAAGACGGTAAGAAGCATATCCTGATTCTGCATAATTGCCAGCTGGGTATGACAGGCGAAGTGAGCTTTCAGGCAGCCA
ATACCAAGAGCGCCGCCAACCTGAAAGTGAAGGAGCTGAGATCCCTGATCGAGGTGGAGAAACCTCTGTATGGCGTTGA
AGTGTGTTGTTGGCGAGACAGCCCACTTCGAGATCGAGCTGAGCGAACCAGGATGTTTCATGGTCAGTGGAAGCTGAAGGGC
CAACCTCTGGCCGCCAGCCCTGATTGTGAGATTATTGAAGACGGCAAAAAGCATATTCTGATTCTGCACAATTGTCAGCTG
GGTATGACCGGTGAGGTGAGTTTCCAGGCCGCCAATACCAAAAGCGCAGCAAACCTGAAGGTGAAAGAACTGAGATCCC
AGCAGGCCCCGGGCCAGAAGGAGTGCGATAACGCACTGCGCGAGCTGGAACCGTGCGTGAGTTACTGGAACCCCGG
TGCAGCCGATTAATGATATGAGCTACTTCGGCTGTCTGGATAGCGTGATGGAGAACAGTAAGGTGCTGGGTGAAGCAATG
ACCGGCATCAGTCAGAACGCAAAGAACGGCAATCTGCCGGAATTTGGCGACGCAATTAGCACCGCAAGCAAAGCCCTGT
GCGGTTTCACCGAAGCCGCCGCACAGGCAGCCTATCTGGTGGGCGTTAGCGATCCTAACAGTCAAGCCGGTCAGTGTG
GCTTAGTGGAACCGACCCAGTTCGCACGCGCAAACCAAGCCATCCAGATGGCATGCCAGAGCCTGGGTGAACCGGGCT
GTACACAGGCCCAAGTTCTGAGCGCAGCCACAATTGTGGCCAAACATACCAGCGCACTGTGCAATAGCTGTGCGCTGGC
CAGTGCACGCACAACCAACCCGACAGCCAAACGTCAGTTCGTTTCAGAGCGCCAAAGAAGTGGCCAACAGTACCGCCAAT

CTGGTGAAGACCATTAAGGCCCTGGATGGTGCATTACAGAAGAAAACCGCGCACAGTGCCGTGCAGCAACAGCACCGT
TACTGGAAGCCGTGGACAATCTGAGCGCATTTCGAAGTAACCCGGAGTTCAGT **TGTATT** CCGGCACAGATCAGTCCTGAA
GGCCGTGCCGCCATGGAACCGATCGTTATCAGCGCCAAGACCATGCTGGAAAGCGCCGGCGGTTTAATCCAAACAGCCC
GTGCACTGGCAGTGAACCCGCGCGATCCGCCGAGCTGGAGTGTCTGGCAGGCCATAGCCGCACAGTGAGCGACAGCA
TTAAGAAGCTGATCACCAGCATGCGTGATAAAGCCCCGGGCAGATCCCTGATCGAAGTTGAGAAGCCGCTGTATGGTGT
GAGGTGTTTCGTTGGCGAGACCGCACATTTTCAAATTGAGCTGAGTGAGCCGGATGTTACGGCCAGTGGAAGTAAAG
GTCAACCTCTGGCAGCCAGCCCGGATTGCGAAATTATCGAAGATGGTAAAAAACACATCCTGATTCTGCATAACTGTCAG
CTGGGCATGACAGGCGAGGTTAGCTTTCAAGCCGCCAACACCAAAAGCGCCGAAACCTGAAAGTTAAGGAGCTGAGAT
CCCTGATCGAAGTTGGAGAAACCGCTGTACGGTGTGAGTGTTCGTGGGTGAAACAGCCATTTTGAAGTTCGAGCTGAG
CGAGCCGGACGTGCACGGTCAGTGGAAGTCAACCGTTAGCAGCCAGCCCGGACTGCGAGATCATTGAAGA
TGGTAAGAAGCACATCCTGATCCTGCACAACTGCCAACTGGGCATGACCGGCGAAGTTAGCTTCCAGGCAGCAAAACA
AAAAGCGCCGCAATCTGAAGGTGAAGGAAGTGAAGTCTCACCACCATCATCACCATTGCTAAGGTACC

GFP-Talin1 (Addgene #26724):

FseI and **ScaI** cloning sites

SnaBI and **EcoRV** cloning sites

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGCGGTTACATAACTTACGGTAAAT
GGCCCGCCTGGCTGACCGCCCAACGACCCCGCCCATGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAG
GGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAA
GTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCT
ACTTGGCAGTAC **ATCTACGTA** ATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAG
CGGTTTGACTACGGGGATTTCAGTCTCCACCCCATGACGTCAATGGGAGTTTGTGTTTGGCACCAAAATCAACGGGA
CTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGACGGTGGGAGGTCTATATAAGCA
GAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGTGCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCC
GGGGTGGTGCCCATCCTGGTTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGG
CGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTG
ACCACCCTGACCTACGGCGTGACGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCA
TGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAA
GCTGGAGTACAACATAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAGTCAAGA
TCCGCCACAACATCGAGGACGGCAGCGTGACGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
TGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGT
CCTGCTGGAGTTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCCGGAATCAGATCTCGAGCT
CAAGCTAGCTTCGAATTCGAGAAGCTGCCACCATGGTTGCGCTTTCGCTGAAGATTAGCATTGGGAATGTGGTGAAGAC
GATGCAATTTGAGCCATCTACCATGGTGTACGACGCCTGCCGCATGATTTCGTGAGCGGATCCCAGAGGCCCTGGCTGGC
CCTCCCAACGACTTTGGGCTCTTTCTGTGATGATGACCCCAAAAAGGCATCTGGCTAGAGGCTGGGAAAGCTTTGGA
CTACTACATGCTCCGAAATGGGGACACCATGGAGTACAGAAAAAGCAGAGACCCCTGAAGATCCGGATGTTAGACGGA
ACGGTGAAGACTATCATGGTGGATGACTCCAAGACCGTCACAGACATGCTCATGACCATTTGTGCCCGAATTGGTATCAC
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GTACT

EGFP-deletion of GFP-Talin1:

GFP deletion region

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APPENDIX 2 – ANALYSIS CODE

CHAPTER 3

```
#pragma rtGlobals=1
#include <Multi-peak fitting 2.0>

Macro myWLC()
    InitializeWLC()
    WLCPanel()
End

Function InitializeWLC()
    NewDataFolder/O root:FX_Analysis:AutoWLC
    NewDataFolder/O root:Data:TmpWaves
    Variable/G root:FX_Analysis:AutoWLC:Sensitivity=50
    Variable/G root:FX_Analysis:AutoWLC:Smoothing=1
    Variable/G root:FX_Analysis:AutoWLC:TSensitivity=50
    Variable/G root:FX_Analysis:AutoWLC:TSmoothing=1
    Variable/G root:FX_Analysis:AutoWLC:WaveAdjust=0
    Variable/G root:FX_Analysis:AutoWLC:DomainLength=100
End

Function WLCPanel()
    PauseUpdate; Silent 1
    DoWindow/F/K thewlcpanel
    NewPanel/K=1/W=(900,60,1300,460) as "WLC Analysis"
    DoWindow/C thewlcpanel

    GroupBox boxWLCPeak,pos={10,10},size={180,185},title="Peaks",font="Times New Roman"
    Slider sliderPeakSens,pos={20,35},size={50,100},proc=SetPeakS
    Slider sliderPeakSens,limits={0,100,5},variable= root:FX_Analysis:AutoWLC:Sensitivity,vert= 1,ticks= 20
    Slider sliderPeakSmooth,pos={20,140},size={160,100},proc=SetPeakS
    Slider sliderPeakSmooth,limits={0,20,1},variable= root:FX_Analysis:AutoWLC:Smoothing,vert= 0,ticks= 20
    Button buttonFindPeak,pos={100,35},size={80,20},proc=procFindPeak,title="Find Peaks"
    Button buttonAddPeak,pos={100,65},size={80,20},proc=procAddPeak,title="Add Peak"
    Button buttonDeletePeak,pos={100,95},size={80,20},proc=procDeletePeak,title="Delete Peak"

    GroupBox boxWLC Trough,pos={10,205},size={180,185},title="Troughs",font="Times New Roman"
    Slider sliderTroughSens,pos={20,230},size={50,100},proc=SetTroughS
    Slider sliderTroughSens,limits={0,100,5},variable= root:FX_Analysis:AutoWLC:TSensitivity,vert= 1,ticks= 20
    Slider sliderTroughSmooth,pos={20,335},size={160,100},proc=SetTroughS
    Slider sliderTroughSmooth,limits={0,20,1},variable= root:FX_Analysis:AutoWLC:TSmoothing,vert= 0,ticks= 20
    Button buttonFindTrough,pos={100,230},size={80,20},proc=procFindTrough,title="Find Troughs"
    Button buttonAddTrough,pos={100,260},size={80,20},proc=procAddTrough,title="Add Trough"
    Button buttonDeleteTrough,pos={100,290},size={80,20},proc=procDeleteTrough,title="Delete Trough"

    GroupBox boxWLCAnalysis,pos={200,10},size={190,130},title="Analysis",font="Times New Roman"
    Button buttonWLCfit,pos={210,35},size={80,30},proc=procWLC,title="Fit WLC"
    Button buttonDelta,pos={210,70},size={80,30},proc=procDelta,title="Delta Hist"
    Button buttonEPDF,pos={300,35},size={80,30},proc=procEPDF,title="EPDF"
    Button buttonForceHist,pos={300,70},size={80,30},proc=procForceHist,title="Force Hist"
    Button buttonAddFit,pos={210,105},size={80,30},proc=procAddFit,title="ShowWLC"
    Button buttonPersHist,pos={300,105},size={80,30},proc=procPersistenceHist,title="Persistence"
```

```

GroupBox boxDomainLength,pos={200,150},size={190,100},title="Domain length",font="Times New Roman"
SetVariable setvarDomLength,pos={210,200},size={150,16},title="Domain length (nm):"
SetVariable setvarDomLength,limits={0,1000,0},value=root:FX_Analysis:AutoWLC:DomainLength

```

```

GroupBox boxWaveAdjust,pos={200,260},size={190,130},title="Adjust Wave",font="Times New Roman"
Slider sliderWaveAdjust,pos={210,285},size={50,100},proc=SetAdjust
Slider sliderWaveAdjust,limits={-50,50,5},variable= root:FX_Analysis:AutoWLC:WaveAdjust,vert= 1,ticks= 20
Button buttonXAdjust,pos={300,290},size={80,40},proc=procFAdjust,title="Wave F-Adjust"
Button buttonReset,pos={300,340},size={80,40},proc=procReset,title="Reset Wave"

```

End

```

Function procFAdjust(ctrlName) : ButtonControl
String ctrlName
ForceAdjustWave()
DoUpdate

```

End

```

Function procAddFit(ctrlName) : ButtonControl
String ctrlName
AddWLC()
DoUpdate

```

End

```

Function procForceHist(ctrlName) : ButtonControl
String ctrlName
ForceHistogram()

```

End

```

Function procPersistenceHist(ctrlName) : ButtonControl
String ctrlName
PersistenceHistogram()

```

End

```

Function procEPDF(ctrlName) : ButtonControl
String ctrlName
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
MyKernel()
TotalGauss()
SetDataFolder dfSav

```

End

```

Function procReset(ctrlName) : ButtonControl
String ctrlName
String tmpwave = "root:Data:TmpWaves:tmp_" + nameofwave(WaveRefIndexed("ForceExtensionAnalysis",1,1))
if (WaveExists($tmpwave))
Duplicate/O/D $tmpwave, $nameofwave(WaveRefIndexed("ForceExtensionAnalysis",1,1))
endif
String tmpxwave = "root:Data:TmpWaves:tmp_" + nameofwave(WaveRefIndexed("ForceExtensionAnalysis",1,2))
if (WaveExists($tmpxwave))
Duplicate/O/D $tmpxwave, $nameofwave(WaveRefIndexed("ForceExtensionAnalysis",1,2))
endif
String tmpwave2 = "root:Data:TmpWaves:tmp_" + nameofwave(WaveRefIndexed("ForceExtensionAnalysis",0,1))
if (WaveExists($tmpwave2))
Duplicate/O/D $tmpwave2, $nameofwave(WaveRefIndexed("ForceExtensionAnalysis",0,1))
endif
Variable/G root:FX_Analysis:AutoWLC:WaveAdjust = 0
DoUpdate

```

End

```

Function SetAdjust(name,value,event)

```

```

        String name
        Variable value,event
        AdjustWave()
        DoUpdate
End

Function procDelta(ctrlName) : ButtonControl
    String ctrlName
    DeltaHistogram()
End

Function procWLC(ctrlName) : ButtonControl
    String ctrlName
    autofit()
    doupdate
End

Function procDeletePeak(ctrlName) : ButtonControl
    String ctrlName
    DeletePeak()
    DoUpdate
End

Function procDeleteTrough(ctrlName) : ButtonControl
    String ctrlName
    DeleteTrough()
    DoUpdate
End

Function procFindTrough(ctrlName) : ButtonControl
    String ctrlName
    RemoveFromGraph/Z WA_TroughCentersX,WA_TroughCentersY
    KillWaves/Z WA_TroughCentersX,WA_TroughCentersY
    MyAutomaticallyFindNegPeaks()
    DoUpdate
End

Function procAddTrough(ctrlName) : ButtonControl
    String ctrlName
    addtroughvalue()
    DoUpdate
End

Function SetTroughS(name,value,event)
    String name
    Variable value, event
    RemoveFromGraph/Z WA_TroughCentersX,WA_TroughCentersY
    KillWaves/Z WA_TroughCentersX,WA_TroughCentersY
    MyAutomaticallyFindNegPeaks()
    DoUpdate
End

Function procAddPeak(ctrlName) : ButtonControl
    String ctrlName
    addpeakvalue()
    DoUpdate
End

Function procFindPeak(ctrlName) : ButtonControl
    String ctrlName
    RemoveFromGraph/Z WA_PeakCentersX,WA_PeakCentersY

```

```

KillWaves/Z WA_PeakCentersX,WA_PeakCentersY
MyAutomaticallyFindPeaks()
DoUpdate
End

Function SetPeakS(name,value,event)
String name
Variable value, event
RemoveFromGraph/Z WA_PeakCentersX,WA_PeakCentersY
KillWaves/Z WA_PeakCentersX,WA_PeakCentersY
MyAutomaticallyFindPeaks()
DoUpdate
End

Function DeletePeak()
Variable Position = hcsr(A)
Variable Height = vcsr(A)
Wave WA_PeakCentersX,WA_PeakCentersY
Variable i
For (i=0;i<numpts(WA_PeakCentersX);i+=1)
    if ( WA_PeakCentersX[i] < Position + 5 && WA_PeakCentersX[i] > Position - 5)
        if( WA_PeakCentersY[i] < Height + 10 && WA_PeakCentersY[i] > Height - 10)
            Deletepoints/m=0 i,1,WA_PeakCentersX,WA_PeakCentersY
            return 1
        endif
    endif
Endfor
End

Function DeleteTrough()
Variable Position = hcsr(A)
Variable Height = vcsr(A)
Wave WA_TroughCentersX,WA_TroughCentersY
Variable i
For (i=0;i<numpts(WA_TroughCentersX);i+=1)
    if ( WA_TroughCentersX[i] < Position + 5 && WA_TroughCentersX[i] > Position - 5)
        if( WA_TroughCentersY[i] < Height +10 && WA_TroughCentersY[i] > Height - 10)
            Deletepoints/m=0 i,1,WA_TroughCentersX,WA_TroughCentersY
            return 1
        endif
    endif
Endfor
End

Function PersistenceHistogram()
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
String coefWaves = Wavelist("coef*",",","")
Variable i
String theWave
for (i=0; i<itemsinlist(coefWaves);i+=1)
    theWave = Stringfromlist(i,coefWaves)
    String persname = "pers_"+NameOfWave($theWave)
    Duplicate/O/D/r=[0,0] $theWave $persname
endfor
String persWaves = Wavelist("pers_*",",","")
concatenate/o/kill persWaves, total_persistence
Make/O/D Persistence_Hist
Variable a=0.04, b=50
Prompt a, "Bin width:"

```



```

Variable c = -0.5 * a
ModifyGraph/w=Force_Histogram offset(Force_Hist)={c,0}
//killwaves total_force
SetDataFolder dfSav

```

End

```

Function DContourHistogram()
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
String dcontourWaves = Wavelist("newDContour_f*", ",", "")
concatenate/o dcontourWaves, total_dcontour
Make/O/D DContour_Hist
Variable a=5, b=40
Prompt a, "Bin width:"
Prompt b, "Number of bins:"
Doprompt "Histogram Values", a, b
if (V_flag)
return -1
endif
Histogram/B={0,a,b}/c total_dcontour DContour_Hist
DoWindow/F DContour_Histogram
if (V_flag == 0)
Display/N=DContour_Histogram DContour_Hist
endif
ModifyGraph/w=DContour_Histogram mode=5
ModifyGraph/w=DContour_Histogram hbFill=2,rgb=(26112,26112,26112),useBarStrokeRGB=1
Variable c = -0.5 * a
ModifyGraph/w=DContour_Histogram offset(DContour_Hist)={c,0}
//killwaves total_dcontour
SetDataFolder dfSav

```

End

```

Function oldDContourHistogram()
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
String dcontourWaves = Wavelist("DContour_f*", ",", "")
concatenate/o dcontourWaves, total_dcontour
Make/O/D DContour_Hist
Variable a=5, b=40
Prompt a, "Bin width:"
Prompt b, "Number of bins:"
Doprompt "Histogram Values", a, b
Variable c = -0.5 * a
if (V_flag)
return -1
endif
Histogram/B={0,a,b}/c total_dcontour DContour_Hist
DoWindow/F DContour_Histogram
if (V_flag == 0)
Display/N=DContour_Histogram DContour_Hist
endif
ModifyGraph/w=DContour_Histogram mode=5
ModifyGraph/w=DContour_Histogram hbFill=2,rgb=(26112,26112,26112),useBarStrokeRGB=1
ModifyGraph/w=DContour_Histogram offset(DContour_Hist)={c,0}
Label left "Number of Events"
//killwaves total_dcontour
SetDataFolder dfSav

```

End

```

Function DContourAdj()
NVAR a = root:FX_Analysis:AutoWLC:DomainLength

```

```

String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
String dcontourWaves = Wavelist("DContour_f*", "", "")
variable i
String theWavename
For (i=0; i<itemsinlist(dcontourwaves); i+=1)
    thewavename = stringfromlist(i,dcontourwaves)
    string newname = "new"+thewavename
    Wave theWave = $thewavename
    Duplicate/O thewave $newname
    Wave newWave = $newname
    Variable j
    For (j=0; j<numpts(theWave); j+=1)
        newWave[j] = a - newwave[j]
    Endfor
Endfor
SetDataFolder dfSav
End

```

```

Function AutoFit()
    Wave WA_PeakCentersX
    Wave WA_TroughCentersX
    sort WA_PeakCentersX,WA_PeakCentersX,WA_PeakCentersY
    sort WA_TroughCentersX,WA_TroughCentersX,WA_TroughCentersY
    Make/D/O/N=100 ContourLengths
    Make/D/O/N=99 Delta
    Make/D/O/N=99 DContour
    Variable j = 0
    for (j=0;j<numpts(WA_PeakCentersX);j+=1)
        Variable clength_guess = WA_PeakCentersX[j] + 10
        Variable ending = WA_PeakCentersX[j]
        Variable beginning = WA_TroughCentersX[j]
        ContourLengths[j] = newQuickFit(0.5,clength_guess,j,ending,beginning)
    endfor
    WaveCoef()
    WaveSigma()
    Sort/R ContourLengths,ContourLengths
    Variable i = 0
    for (i=0;i<numpts(delta);i+=1)
        Delta[i] = ContourLengths[i] - ContourLengths[i+1]
    endfor
    Variable k = 0
    for (k=0;k<numpts(dcontour);k+=1)
        if (ContourLengths[k+1] != 0)
            DContour[k] = ContourLengths[0] - ContourLengths[k+1]
        endif
    endfor
    TrimWave(ContourLengths)
    CleanDelta(Delta)
    TrimWave(DContour)
    FindIntercept()
    //Duplicate/O/D WA_PeakCentersY $PeakForceName
    //DeletePoints/M=0 (numpts($PeakForceName)-1),1,$PeakForceName
End

```

```

Function WormLikeChain(w,x) : FitFunc
    Wave w
    Variable x

```

//CurveFitDialog/ These comments were created by the Curve Fitting dialog. Altering them will
 //CurveFitDialog/ make the function less convenient to work with in the Curve Fitting dialog.

```

//CurveFitDialog/ Equation:
//CurveFitDialog/ f(x) = (4.11/p)*((0.25*(1.00-x/c)^-2.00) - 0.25 + x/c)
//CurveFitDialog/ End of Equation
//CurveFitDialog/ Independent Variables 1
//CurveFitDialog/ x
//CurveFitDialog/ Coefficients 2
//CurveFitDialog/ w[0] = p
//CurveFitDialog/ w[1] = c

```

```

return (4.11/w[0])*((0.25*(1.00-x/w[1])^-2.00) - 0.25 + x/w[1])

```

End

```

Function newQuickFit(a,b,c,ending,beginning)
Variable a,b,c,ending,beginning
Wave yWave = WaveRefIndexed("ForceExtensionAnalysis",1,1)
Wave xWave = WaveRefIndexed("ForceExtensionAnalysis",1,2)
Variable start = FindPoint(xWave,beginning)
Variable endp = FindPoint(xWave,ending)
NVAR N = root:FX_Analysis:DisplayStartNumber
//String scsr,ecsr
String newWavename = "root:FX_Analysis:AutoWLC:fit"+num2str(c)
String newcoefname = "root:FX_Analysis:AutoWLC:coef"+num2str(c)+"_"+num2str(N)
String newsigname = "root:FX_Analysis:AutoWLC:sigma"+num2str(c)+"_"+num2str(N)
String newresname = "root:FX_Analysis:AutoWLC:res"+num2str(c)+"_"+num2str(N)
Duplicate/O/D yWave $newWavename
Duplicate/O/D yWave W_Res
Make/D/N=2/O W_coef
Make/D/n=2/o W_sigma
W_coef[0] = {a,b}
//Make/O/T/N=2 T_Constraints
//T_Constraints[0] = {"K0 > .2","K0 < 1"}
FuncFit/H="10"/NTHR=0 WormLikeChain W_coef yWave[start,endp]/X=xWave/D=$newWavename/R=W_Res/A=0
Killwaves $newWavename
Duplicate/O/D w_coef $newcoefname
Duplicate/O/D w_sigma $newsigname
Duplicate/O/D w_res $newresname
extendwlc(yWave,$newcoefname,c)
return w_coef[1]

```

End

```

function extendwlc(yWave,coef,number)
Wave yWave,coef
Variable number
String wlcWavename = "root:FX_Analysis:AutoWLC:wlc"+num2str(number)+"_"+NameOfWave(yWave)
String wlcWaverealname = "wlc"+num2str(number)+"_"+NameOfWave(yWave)
String tagname = "tag"+num2str(number)
Make/D/O/N=4000 theWave
Variable i
for (i=0;i<numpts(theWave);i+=1)
    theWave[i] = (4.11/coef[0])*((0.25*(1.00-i/coef[1])^-2.00) - 0.25 + i/coef[1])
endfor
Variable t
for (t=0;t<numpts(theWave);t+=1)
    if (theWave[t] > 300)
        break
    endif
endfor
duplicate/d/o/r=[0,t] theWave $wlcWavename
killWaves theWave
appendtograph/b/c=(0,0,0) $wlcWavename
ModifyGraph lstyle($wlcWaverealname)=2

```



```

//CheckDisplayed/A W_AutoPeakInfo
//Edit W_AutoPeakInfo.Id

//DoWindow ShowPeaks
//if( WaveExists(wx) )
//Display/N=ShowPeaks w vs wx
//else
//Display/N=ShowPeaks w
//endif
AppendToGraph WA_PeakCentersY vs WA_PeakCentersX
ModifyGraph rgb(WA_PeakCentersY)=(65535,0,0)
ModifyGraph mode(WA_PeakCentersY)=3
ModifyGraph marker(WA_PeakCentersY)=19
endif
endif
if( peaksFound < 1 )
DoAlert 0, "No Peaks found!"
endif
return peaksFound
End

Function MyAutomaticallyFindNegPeaks()
Variable maxPeaks=100
NVAR minPeak = root:FX_Analysis:AutoWLC:TSensitivity
NVAR negsmoothf = root:FX_Analysis:AutoWLC:Tsmoothing
Wave WA_PeakCentersY
Variable pBegin,pEnd
if (waveexists(csrwaveref(A)))
Wave thew=csrWaveref(A)
Wave wx=CsrXWaveRef(A)
pBegin= pcsr(A)
pEnd= pcsr(B)
else
Wave thew=WaveRefIndexed("ForceExtensionAnalysis",1,1)
Wave wx=WaveRefIndexed("ForceExtensionAnalysis",1,2)
pBegin = 0
pEnd = FindPoint(thew,WA_PeakCentersY[0])
endif
Wave wtemp=thew
Duplicate/O/D wtemp w
Variable i
For (i=0;i<numpts(wtemp);i+=1)
w[i] = -1*wtemp[i]
Endfor
Variable/C estimates= EstPeakNoiseAndSmfact(w,pBegin, pEnd)
Variable noiselevel=real(estimates)
Variable smoothingFactor=negsmoothf

Variable peaksFound= AutoFindPeaks(w,pBegin,pEnd,noiseLevel,smoothingFactor,maxPeaks)
if( peaksFound > 0 )
Wave W_AutoPeakInfo

peaksFound= MyTrimAmpAutoPeakInfo(W_AutoPeakInfo,minPeak)
if( peaksFound > 0 )

Make/O/N=(peaksFound) WA_TroughCentersY = w[W_AutoPeakInfo[p][0]]
invertWave(WA_TroughCentersY)
AdjustAutoPeakInfoForX(W_AutoPeakInfo,w,wx)
Make/O/N=(peaksFound) WA_TroughCentersX = W_AutoPeakInfo[p][0]

```

```

        AppendToGraph WA_TroughCentersY vs WA_TroughCentersX
        ModifyGraph rgb(WA_TroughCentersY)=(0,60000,0)
        ModifyGraph mode(WA_TroughCentersY)=3
        ModifyGraph marker(WA_TroughCentersY)=19
    endif
endif
if( peaksFound < 1 )
DoAlert 0, "No Trough found!"
endif
if (!waveexists(csrwaveref(A)))
    make/o/d/n=1 temptroughx
    make/o/d/n=1 temptroughy
    temptroughx = wx[findpoint(thew,0)]
    temptroughy = thew[findpoint(thew,0)]
    concatenate/o {WA_TroughCentersX,temptroughx}, alltroughx
    concatenate/o {WA_TroughCentersY,temptroughy}, alltroughy
    duplicate/o alltroughx, WA_TroughCentersX
    duplicate/o alltroughy, WA_TroughCentersY
    killWaves temptroughx,temptroughy,alltroughx,alltroughy
endif
return peaksFound
End

Function invertWave(theWave)
    Wave theWave
    Variable i
    for (i=0;i<numpts(theWave);i+=1)
        theWave[i]=-1*theWave[i]
    endfor
End

Function MyTrimAmpAutoPeakInfo(wpi,ymin)
    Wave wpi
    Variable ymin
    Variable i= DimSize(wpi,0)-1 // index of last row

    do // user want peaks
to be bigger than this
        if( wpi[i][2] < ymin )
            DeletePoints i,i,wpi
        endif
        i -= 1
    while(i>0)
    return DimSize(wpi,0)
end

Function FindPoint(xWave,value)
    Variable value
    Wave xWave
    Variable i
    for (i=0;i<numpts(xWave);i+=1)
        if (xWave[i] >= value)
            return i
        endif
    endfor
end

Function FindPointfromabove(xWave,value)
    Variable value

```

```

Wave xWave
Variable i
for (i=0;i<numpts(xWave);i+=1)
    if (xWave[i] <= value)
        return i
    endif
endfor
end

Function CleanDelta(Waved)
Wave Waved
NVAR N = root:FX_Analysis:DisplayStartNumber
String newWavename = "root:FX_Analysis:AutoWLC:"+NameOfWave(Waved)+"_FX_"+num2str(N)
Variable j
for (j=1;j<=numpts(Waved);j+=1)
    if (Waved[j]==0)
        deletepoints j-1, numpts(Waved)-(j-1), Waved
    endif
endfor
Duplicate/O/D Waved $newWavename
End

Function TotalForce()
String dfSave = GetDataFolder(1)
SetDataFolder "root:FX_analysis:AutoWLC"
String forcewaves=wavelist("peakforce_*",";",",")
Make/O/D PeakForceTotal
Concatenate/O/NP=0 forcewaves, PeakForceTotal
SetDataFolder dfSave
End

Function WaveCoef()
NVAR N = root:FX_Analysis:DisplayStartNumber
String dfSave = GetDataFolder(1)
SetDataFolder "root:FX_analysis:AutoWLC"
String coefwave = "Coef_FX_"+NUM2STR(N)
Killwaves/Z $coefwave
String coefwaves=wavelist("coef*_"+num2str(N),";",",")
Concatenate/DL/O/KILL coefwaves, $coefwave
SetDataFolder dfSave
End

Function WaveSigma()
NVAR N = root:FX_Analysis:DisplayStartNumber
String dfSave = GetDataFolder(1)
SetDataFolder "root:FX_analysis:AutoWLC"
String sigmawave = "Sigma_FX_"+num2str(N)
Killwaves/Z $sigmawave
String sigmawaves=wavelist("sigma*_"+num2str(N),";",",")
Concatenate/DL/O/KILL sigmawaves, $sigmawave
wave thewave = $sigmawave
variable size = dimsize(thewave,1) - 1
string newwavename = "DeltaSig_FX_"+num2str(N)
make/o/d/n=(size) newwave
variable j
for (j=0;j<numpts(newwave);j+=1)
    newwave[j] = thewave[1][j+1] + thewave[1][j]
endfor
Duplicate/O newwave $newwavename
SetDataFolder dfSave
End

```

```

Function TrimWave(Waved)
    Wave Waved
    NVAR N = root:FX_Analysis:DisplayStartNumber
    String newWavename = "root:FX_Analysis:AutoWLC:"+NameOfWave(Waved)+"_FX_"+num2str(N)
    Variable j
    for (j=0;j<=numpts(Waved);j+=1)
        if (Waved[j] == 0)
            deletepoints j, numpts(Waved)-j, Waved
        endif
    endfor
    Duplicate/O/D Waved $newWavename
    Killwaves waved
End

```

```

Function addpeakvalue()
    Wave xWave = CsrXWaveRef(A)
    Wave yWave = csrWaveref(A)
    Wave WA_PeakCentersX
    Wave WA_PeakCentersY
    make/o/d/n=1 temppeakx
    make/o/d/n=1 temppeaky
    temppeakx = xWave[pcsr(A)]
    temppeaky = yWave[pcsr(A)]
    concatenate/o {WA_PeakCentersX,temppeakx}, allpeaksx
    concatenate/o {WA_PeakCentersY,temppeaky}, allpeaksy
    duplicate/o allpeaksx, WA_PeakCentersX
    duplicate/o allpeaksy, WA_PeakCentersY
    killWaves temppeakx,temppeaky,allpeaksx,allpeaksy
end

```

```

Function AddTroughValue()
    Wave xWave = CsrXWaveRef(A)
    Wave yWave = csrWaveref(A)
    Wave WA_TroughCentersX
    Wave WA_TroughCentersY
    make/o/d/n=1 temptroughx
    make/o/d/n=1 temptroughy
    temptroughx = xWave[pcsr(A)]
    temptroughy = yWave[pcsr(A)]
    concatenate/o {WA_TroughCentersX,temptroughx}, alltroughx
    concatenate/o {WA_TroughCentersY,temptroughy}, alltroughy
    duplicate/o alltroughx, WA_TroughCentersX
    duplicate/o alltroughy, WA_TroughCentersY
    killWaves temptroughx,temptroughy,alltroughx,alltroughy
end

```

```

Function AdjustWave()
    NVAR adjust = root:FX_Analysis:AutoWLC:WaveAdjust
    //GetWindow ForceExtensionAnalysis, wavelist
    Wave thewave = WaveRefIndexed("ForceExtensionAnalysis", 1, 1)
    String tmpwavename = "root:Data:TmpWaves:tmp_"+nameofwave(thewave)
    If (!Waveexists($tmpwavename))
        Duplicate/D thewave, $tmpwavename
    Endif
    variable i
    Wave original = $tmpwavename
    for (i=0;i<numpts(thewave);i+=1)
        thewave[i] = original[i] + adjust
    endfor

```

End

```
Function xAdjustWave()  
    Wave ywave = WaveRefIndexed("ForceExtensionAnalysis",1,1)  
    Wave xwave = WaveRefIndexed("ForceExtensionAnalysis",1,2)  
    Variable point = findpoint(ywave,-200)  
    String tmpwavename = "root:Data:TmpWaves:tmp_"+nameofwave(xwave)  
    If (!Waveexists($tmpwavename))  
        Duplicate/D xwave, $tmpwavename  
    Endif  
    Variable xadjust = xwave[point]  
    Variable i  
    for (i=0;i<numpts(xwave);i+=1)  
        xwave[i] = xwave[i] - xadjust  
    endfor  
End
```

```
Function MyKernel()  
    Variable p  
    String deltawaves = wavelist("Delta_FX_","",",")  
    variable i  
    for (i=0;i<itemsinlist(deltawaves);i+=1)  
        String thewave = stringfromlist(i,deltawaves)  
        Wave deltawave = $thewave  
        sscanf thewave,"Delta_FX_%.f",p  
        String thesigmawave = "DeltaSig_FX_" + num2str(p)  
        Wave sigmawave = $thesigmawave  
        Make/O/D/n=1000 xwave  
        variable o  
        for (o=0;o<1000;o+=1)  
            xwave[o] = o/10  
        endfor  
        variable k  
        for (k=0;k<numpts(deltawave);k+=1)  
            variable delta = deltawave[k]  
            variable sigma = sigmawave[k]  
            Make/O/D/n=1000 gausswave  
            variable j  
            for (j=0;j<1000;j+=1)  
                gausswave[j] = (1/(sigma*sqrt(2*pi)))*exp((-1)*(1/2)*(((xwave[j]-delta)/sigma)^2))  
            endfor  
            String gausswavename = "Gauss_B" + num2str(p) + "_" + num2str(k)  
            Duplicate/O gausswave $gausswavename  
            Killwaves gausswave  
        endfor  
    endfor  
end
```

```
Function totalgauss()  
    DoWindow/K EPDF  
    Killwaves/Z Gauss_Total  
    Wave xwave  
    string gausswaves = wavelist("gauss_","",",")  
    Concatenate/DL/O/Kill gausswaves, gausstotal  
    Make/o/d/n=1000 Gauss_Total  
    variable i  
    for (i=0;i<1000;i+=1)  
        variable j  
        for (j=0;j<dimsize(gausstotal,1);j+=1)  
            Gauss_Total[i] += gausstotal[i][j]  
        endfor  
    endfor
```

```

    endfor
    Display/N=EPDF Gauss_total vs xwave
    Killwaves gausstotal
End

Function Findintercept()
    Removefromgraph/Z forcewave
    KillWaves/z forcewave, forcexwave
    NVAR N = root:FX_Analysis:DisplayStartNumber
    String coefwavename = "root:FX_Analysis:AutoWLC:Coef_FX_" + num2str(N)
    Wave coefwave = $coefwavename
    Variable g = dimsize(coefwave, 1) - 1
    Make/o/d/n=(g) forcewave
    Make/o/d/n=(g) forcexwave
    Variable h
    For (h=0;h<g;h+=1)
        Variable m,c
        Wave WA_PeakCentersX,WA_PeakCentersY,WA_TroughCentersX,WA_TroughCentersY
        m = (WA_PeakCentersY[h] - WA_TroughCentersY[h+1]) / (WA_PeakCentersX[h] -
WA_TroughCentersX[h+1])
        c = (WA_PeakCentersX[h] * WA_TroughCentersY[h+1] - WA_TroughCentersX[h+1] * WA_PeakCentersY[h])
/ (WA_PeakCentersX[h] - WA_TroughCentersX[h+1])
        Make/o/d/n=5000 straightline
        Variable i
        For (i=0;i<numpts(straightline);i+=1)
            straightline[i] = m * (i/10) + c
        Endfor
        Variable a = coefwave[0][h]
        Variable b = coefwave[1][h]
        Make/o/d/n=5000 wlcwave
        Variable j
        for (j=0;j<numpts(wlcwave);j+=1)
            wlcwave[j] = (4.11/a)*((0.25*(1.00-(j/10)/b)^-2.00) - 0.25 + (j/10)/b)
        endfor
        Variable x,k
        For (k=1;k<numpts(straightline);k+=1)
            If (abs(straightline[k] - wlcwave[k]) < abs(straightline[k-1] - wlcwave[k-1]) && wlcwave[k] > wlcwave[k-
1])
                x = k/10
            Endif
        Endfor
        Print x
        forcexwave[h] = x
        forcewave[h] = wlcwave[x*10]
        //Appendtograph straightline, wlcwave
    Endfor
    Killwaves straightline, wlcwave
    Appendtograph forcewave vs forcexwave
    Modifygraph mode(forcewave)=3
    ModifyGraph rgb(forcewave)=(0,0,0)
    ModifyGraph marker(forcewave)=19
    String PeakForceName = "root:FX_Analysis:AutoWLC:PeakForce_FX_" + num2str(N)
    Duplicate/O forcewave $PeakForceName
End

Function Autoforceadjust()
    Wave ywave=WaveRefIndexed("ForceExtensionAnalysis",0,1)
    Variable thestart = findpointfromabove(WaveRefIndexed("ForceExtensionAnalysis",0,2),50)
    Make/O/D ForceAdjustHist
    Histogram/B=3/C/R=[0,thestart] ywave, ForceAdjustHist
    Make/D/O/N=4 ForceAdjustCoef
    Duplicate/D ForceAdjustHist,ForceAdjustDestWave

```

```

ForceAdjustCoeff[0] = 0;
CurveFit/Q/H="1000"/NTHR=0 gauss kwCWave=ForceAdjustCoef, ForceAdjustHist /D=ForceAdjustDestWave
Killwaves/Z ForceAdjustDestWave
return ForceAdjustCoef[2]

```

End

```

Function ForceAdjustWave()
Variable a = Autoforceadjust()
Wave thefwave = WaveRefIndexed("ForceExtensionAnalysis",0,1)
String tmpfwavename = "root:Data:TmpWaves:tmp_"+nameofwave(thefwave)
If (!Waveexists($tmpfwavename))
    Duplicate/D thefwave, $tmpfwavename
Endif
Wave originalf = $tmpfwavename
thefwave = originalf - a
Wave thebwave = WaveRefIndexed("ForceExtensionAnalysis",1,1)
String tmpbwavename = "root:Data:TmpWaves:tmp_"+nameofwave(thebwave)
If (!Waveexists($tmpbwavename))
    Duplicate/D thebwave, $tmpbwavename
Endif
Wave originalb = $tmpbwavename
thebwave = originalb - a
End //add no csr functionality and button for now

```

```

Function DeleteData()
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
NVAR N = root:FX_Analysis:DisplayStartNumber
string matchstring = "*" + num2str(N)
string wavenames=wavelist(";",",","")
string name
variable i
for (i=0;i<itemsinlist(wavenames);i+=1)
    name = stringfromlist(i,wavenames)
    if (stringmatch(name,matchstring))
        removefromgraph/z $name
        killwaves/z $name
    endif
endfor
SetDataFolder dfSav

```

End

```

Function SaveDCWaves()
String experimentname = igorinfo(1)
String savename = "waves_" + experimentname
Save/T ::FX_Analysis:AutoWLC:total_dcontour,::FX_Analysis:AutoWLC:total_force as savename

```

End

```

Function DeleteTraces()
String dfSav = GetDataFolder(1)
SetDataFolder "root:FX_Analysis:AutoWLC"
Make/O/D/N=999 AnalysedWaveNo
Variable i
For (i=0; i<999; i+=1)
    String theWave = "coef_fx_" + num2str(i)
    if (waveexists($theWave))
        AnalysedWaveNo[i] = 1
    endif
endfor
SetDataFolder dfSav
Variable j
For (j=0; j<999; j+=1)

```

```

        Wave tempwave = root:FX_Analysis:AutoWLC:AnalysedWaveNo
        if (tempwave[j] != 1)
            String extwave = "ExtensionRaw_B" + num2str(j)
            String forwave = "ForceRaw_B" + num2str(j)
            killwaves/z $extwave, $forwave
        endif
    endfor
End

```

```

Function SaveTraces()
    String experimentname = igorinfo(1)
    String savename = "extension_" + experimentname
    String savenameb = "traces_" + experimentname
    Save/T/B wavelist("*_b*", ",", "") as savename
End

```

```

Function ForcePeaks()
    String dfSav = GetDataFolder(1)
    SetDataFolder "root:FX_Analysis:AutoWLC"
    String forcewaves = wavelist("PeakForce_*", ",", "")
    Make/O/D/N=0 allforce
    Variable i
    For (i=0;i<itemsinlist(forcewaves);i+=1)
        String thewavename = stringfromlist(i,forcewaves)
        Wave thewave = $thewavename
        If (numpts(thewave) == 3)
            Concatenate {thewave}, allforce
        Endif
    Endfor
    SetDataFolder dfSav
End

```

```

Function SeparateDC()
    String dfSav = GetDataFolder(1)
    SetDataFolder "root:FX_Analysis:AutoWLC"
    String dcwaves = wavelist("DContour_FX*", ",", "")
    Make/O/D/N=0 sepdC
    Variable i
    For (i=0;i<itemsinlist(dcwaves);i+=1)
        String thewavename = stringfromlist(i,dcwaves)
        Wave thewave = $thewavename
        If (numpts(thewave) < 4)
            Redimension/N=3 thewave
            Concatenate {thewave}, sepdC
        Endif
    Endfor
    String experimentname = igorinfo(1)
    String savename = "sepdC_" + experimentname
    Save/T sepdC as savename
    SetDataFolder dfSav
End

```

```

Function SeparateDelta()
    String dfSav = GetDataFolder(1)
    SetDataFolder "root:FX_Analysis:AutoWLC"
    String deltawaves = wavelist("Delta_FX*", ",", "")
    Make/O/D/N=0 sepdelta
    Variable i
    For (i=0;i<itemsinlist(deltawaves);i+=1)
        String thewavename = stringfromlist(i,deltawaves)
        Wave thewave = $thewavename
        If (numpts(thewave) < 4)

```

```

        Redimension/N=3 thewave
        Concatenate {thewave}, sepdelta
    Endif
Endfor
String experimentname = igorinfo(1)
String savename = "sepdelta_" + experimentname
Save/T sepdelta as savename
SetDataFolder dfSav
End

Function Separateforce()
    String dfSav = GetDataFolder(1)
    SetDataFolder "root:FX_Analysis:AutoWLC"
    String forcewaves = wavelist("PeakForce_*", ",", "")
    Make/O/D/N=0 sepforce
    Variable i
    For (i=0;i<itemsinlist(forcewaves);i+=1)
        String thewavename = stringfromlist(i,forcewaves)
        Wave thewave = $thewavename
        If (numpnts(thewave) == 3)
            Concatenate {thewave}, sepforce
        elseif (numpnts(thewave) == 2)
            Insertpoints 0, 1, thewave
            Concatenate {thewave}, sepforce
        Elseif (numpnts(thewave) == 1)
            Insertpoints 0, 2, thewave
            Concatenate {thewave}, sepforce
        Endif
    Endfor
    String experimentname = igorinfo(1)
    String savename = "sepforce_" + experimentname
    Save/T sepforce as savename
    SetDataFolder dfSav
End

Function AllForceAdjustWave()
    Variable i = 0
    For (i=0;i<351;i+=1)
        String bwavename = "ForceRaw_B" + num2str(i)
        String fwavename = "ForceRaw_F" + num2str(i)
        String fxwavename = "ExtensionRaw_F" + num2str(i)
        Wave ywave=$fwavename
        Wave fxwave = $fxwavename
        Variable thestart = findpointfromabove(fxwave, 50)
        Make/O/D ForceAdjustHist
        Histogram/B=3/C/R=[0,thestart] ywave, ForceAdjustHist
        Make/D/O/N=4 ForceAdjustCoef
        Duplicate/D ForceAdjustHist,ForceAdjustDestWave
        ForceAdjustCoef[0] = 0;
        CurveFit/Q/H="1000"/NTHR=0      gauss      kwCWave=ForceAdjustCoef,      ForceAdjustHist
/D=ForceAdjustDestWave
        Killwaves/Z ForceAdjustDestWave
        Variable a = ForceAdjustCoef[2]
        Wave thefwave = $fwavename
        String tmpfwavename = "root:Data:TmpWaves:tmp_" + nameofwave(thefwave)
        If (!Waveexists($tmpfwavename))
            Duplicate/D thefwave, $tmpfwavename
        Endif
        Wave originalf = $tmpfwavename
        thefwave = originalf - a
        Wave thebwave = $bwavename
        String tmpbwavename = "root:Data:TmpWaves:tmp_" + nameofwave(thebwave)
    Endfor

```

```

        If (!Waveexists($tmpbwavename))
            Duplicate/D thebwave, $tmpbwavename
        Endif
        Wave originalb = $tmpbwavename
        thebwave = originalb - a
    Endfor
End //add no csr functionality and button for now

Function CombWaves()
    String dcwaves = wavelist("total_dcontour*", "", "")
    concatenate/o dcwaves, total_dc
    String fwaves = wavelist("total_force*", "", "")
    concatenate/o fwaves, total_f
End

Function DrawGauss()
    Variable a,b,c
    Prompt a, "Location"
    Prompt b, "Width"
    Prompt c, "Amplitude"
    Doprompt "Gaus Values", a, b, c
    if (V_flag)
        return -1
    endif
    Make/o/d/n=200 GaussWave
    Variable i = 0
    For (i=0;i<200;i+=1)
        GaussWave[i] = c * exp( -1 * ((i - a)/(b))^2)
    Endfor
    Appendtograph GaussWave
End

Function DrawGauss1()
    Variable a,b,c
    Prompt a, "Location"
    Prompt b, "Width"
    Prompt c, "Amplitude"
    Doprompt "Gaus Values", a, b, c
    if (V_flag)
        return -1
    endif
    Make/o/d/n=200 GaussWave1
    Variable i = 0
    For (i=0;i<200;i+=1)
        GaussWave1[i] = c * exp( -1 * ((i - a)/(b))^2)
    Endfor
    Appendtograph GaussWave1
End

Function DrawGauss2()
    Variable a,b,c
    Prompt a, "Location"
    Prompt b, "Width"
    Prompt c, "Amplitude"
    Doprompt "Gaus Values", a, b, c
    if (V_flag)
        return -1
    endif
    Make/o/d/n=200 GaussWave2
    Variable i = 0
    For (i=0;i<200;i+=1)
        GaussWave2[i] = c * exp( -1 * ((i - a)/(b))^2)
    Endfor

```

```

    Endfor
    Appendtograph GaussWave2
End

```

```

Function CombDCHistogram()
    Wave total_dc
    Make/O/D DContour_Hist
    Variable a=5, b=40
    Prompt a, "Bin width:"
    Prompt b, "Number of bins:"
    Doprompt "Histogram Values", a, b
    if (V_flag)
        return -1
    endif
    Histogram/B={0,a,b}/c total_dc DContour_Hist
    DoWindow/F DContour_Histogram
    if (V_flag == 0)
        Display/N=DContour_Histogram DContour_Hist
    endif
    ModifyGraph/w=DContour_Histogram mode=5
    ModifyGraph/w=DContour_Histogram hbFill=2,rgb=(26112,26112,26112),useBarStrokeRGB=1
    Variable c = -0.5 * a
    ModifyGraph/w=DContour_Histogram offset(DContour_Hist)={c,0}
End

```

```

Function CombForceHistogram()
    Wave total_f
    Make/O/D Force_Hist
    Variable a=2, b=30
    Prompt a, "Bin width:"
    Prompt b, "Number of bins:"
    Doprompt "Histogram Values", a, b
    if (V_flag)
        return -1
    endif
    Histogram/B={0,a,b}/c total_f Force_Hist
    DoWindow/F Force_Histogram
    if (V_flag == 0)
        Display/N=Force_Histogram Force_Hist
    endif
    ModifyGraph/w=Force_Histogram mode=5
    ModifyGraph/w=Force_Histogram hbFill=2,rgb=(26112,26112,26112),useBarStrokeRGB=1
    Variable c = -0.5 * a
    ModifyGraph/w=Force_Histogram offset(Force_Hist)={c,0}
End

```

```

Function CombDCAdj()
    Wave adj_total_dc
    Variable b = 120.8
    Variable i
    For (i=0;i<numpts(adj_total_dc);i+=1)
        adj_total_dc[i] = b - adj_total_dc[i]
    EndFor
End

```

```

Function CombAdjDCHistogram()
    Wave adj_total_dc
    Make/O/D Adj_DContour_Hist
    Variable a=5, b=40
    Prompt a, "Bin width:"
    Prompt b, "Number of bins:"
    Doprompt "Histogram Values", a, b

```

```

    if (V_flag)
        return -1
    endif
    Histogram/B={0,a,b}/c adj_total_dc Adj_DContour_Hist
    DoWindow/F Adj_DContour_Histogram
    if (V_flag == 0)
        Display/N=Adj_DContour_Histogram adj_DContour_Hist
    endif
    ModifyGraph/w=Adj_DContour_Histogram mode=5
    ModifyGraph/w=Adj_DContour_Histogram hbFill=2,rgb=(26112,26112,26112),useBarStrokeRGB=1
    Variable c = -0.5 * a
    ModifyGraph/w=Adj_DContour_Histogram offset(Adj_DContour_Hist)={c,0}
End

```

```

Macro TraceComb()
    RenameWaves()
    AlignVariables()
    TraceAlignPanel()
End

```

```

Function AlignVariables()
    Variable/G root:TraceNo=0
    Variable/G root:Adjustment=0
End

```

```

Function WLCvariables()
    Variable/G root:HaloLen=150
    Variable/G root:HaloPers=0.5
    Variable/G root:EventNo=0
    Variable/G root:OneLen=10
    Variable/G root:TwoLen=20
    Variable/G root:ThreeLen=30
End

```

```

Function TraceAlignPanel()
    Wave F0
    Wave E0

    PauseUpdate; Silent 1
    DoWindow/F/K thetracealignpanel
    NewPanel/K=1/W=(900,60,1300,160) as "Trace Alignment"
    DoWindow/C thetracealignpanel

    SetVariable setvarFXstart,pos={20,15},size={75,16},proc=AppendTrace,title="Trace"
    SetVariable setvarFXstart,limits={0,10000,1},value= root:TraceNo

    Slider sliderWaveAdjust,pos={20,40},size={350,50},proc=TraceAdjust
    Slider sliderWaveAdjust,limits={-100,100,2},variable= root:Adjustment,vert= 0,ticks= 80

    Display F0 vs E0
    Modifygraph IStyle = 1
End

```

```

Function WLCPanel()
    PauseUpdate; Silent 1
    DoWindow/F/K thewlcpanel
    NewPanel/K=1/W=(900,60,1100,360) as "WLC Fitting"

    Slider sliderWLClen,pos={20,10},size={150,50},proc=DrawWLC
    Slider sliderWLClen,limits={100,300,2},variable= root:HaloLen,vert=0,ticks=50

```

```
Slider sliderWLCpers,pos={20,70},size={150,50},proc=DrawWLC
Slider sliderWLCpers,limits={0,1,0.05},variable= root:HaloPers,vert=0,ticks=50
```

```
SetVariable setOneLen,pos={20,130},size={100,16},proc=setWLC,title="Event 1"
SetVariable setOneLen,limits={0,300,1},value= root:OneLen
```

```
SetVariable setTwoLen,pos={20,150},size={100,16},proc=setWLC,title="Event 2"
SetVariable setTwoLen,limits={0,300,1},value= root:TwoLen
```

```
SetVariable setThreeLen,pos={20,170},size={100,16},proc=setWLC,title="Event 3"
SetVariable setThreeLen,limits={0,300,1},value= root:ThreeLen
```

```
SetVariable setEventNo,pos={20,200},size={100,16},proc=setWLC,title="Event No"
SetVariable setEventNo,limits={0,3,1},value= root:EventNo
```

End

Function DrawWLC(name,value,event)

```
String name
Variable value,event
WLCDraw()
DoUpdate
```

End

Function setWLC(ctrlName,varNum,varStr,varName) : SetVariableControl

```
String ctrlName
Variable varNum
String varStr, varName
WLCDraw()
```

End

Function WLCDraw()

```
NVAR HL = root:HaloLen
NVAR HP = root:HaloPers
NVAR EN = root:EventNo
NVAR Li = root:OneLen
NVAR Lii = root:TwoLen
NVAR Liii = root:ThreeLen
Variable iL = HL - Li
Variable iiL = HL - Lii
Variable iiiL = HL - Liii
Make/D/O/N=4000 HaloWLC
Make/D/O/N=4000 iWLC
Make/D/O/N=4000 iiWLC
Make/D/O/N=4000 iiiWLC
Removefromgraph/Z HaloWLC
Removefromgraph/Z iWLC
Removefromgraph/Z iiWLC
Removefromgraph/Z iiiWLC
Variable i
for (i=0;i<4000;i+=1)
    HaloWLC[i] = (4.11/HP)*((0.25*(1.00-i/HL)^-2.00)-0.25+i/HL)
endfor
Variable t
For (t=0;t<4000;t+=1)
    if (HaloWLC[t] > 300)
        break
    endif
Endfor
Variable Endp = 4000 - t
DeletePoints t, Endp, HaloWLC
t = 0
```

```

appendtograph/b/c=(0,0,0) HaloWLC
i=0
if (EN > 0)
    for (i=0;i<4000;i+=1)
        iWLC[i] = (4.11/HP)*((0.25*(1.00-i/iL)^-2.00)-0.25+i/iL)
    endfor
    For (t=0;t<4000;t+=1)
        if (iWLC[t] >300)
            break
        endif
    Endfor
    Endp = 4000 - t
    DeletePoints t, Endp, iWLC
    t = 0
    appendtograph/b/c=(0,0,0) iWLC
endif
i=0
if (EN > 1)
    for (i=0;i<4000;i+=1)
        iiWLC[i] = (4.11/HP)*((0.25*(1.00-i/iiL)^-2.00)-0.25+i/iiL)
    endfor
    For (t=0;t<4000;t+=1)
        if (iiWLC[t] >300)
            break
        endif
    Endfor
    Endp = 4000 - t
    DeletePoints t, Endp, iiWLC
    t = 0
    appendtograph/b/c=(0,0,0) iiWLC
endif
if (EN > 2)
    for (i=0;i<4000;i+=1)
        iiiWLC[i] = (4.11/HP)*((0.25*(1.00-i/iiiL)^-2.00)-0.25+i/iiiL)
    endfor
    For (t=0;t<4000;t+=1)
        if (iiiWLC[t] >300)
            break
        endif
    Endfor
    Endp = 4000 - t
    DeletePoints t, Endp, iiiWLC
    t = 0
    appendtograph/b/c=(0,0,0) iiiWLC
endif
End

```

```

Function AppendTrace(ctrlName,varNum,varStr,varName) : SetVariableControl
String ctrlName
Variable varNum
String varStr, varName
AddTrace()
End

```

```

Function Addtrace()
NVAR adj = root:Adjustment
NVAR no = root:TraceNo
String theextwave = "E" + num2str(no)
String theforwave = "F" + num2str(no)
Wave extwave = $theextwave

```

```

Wave forwave = $theforwave
RemovefromGraph/Z $theforwave
Modifygraph rgb = (65535,0,0)
AppendToGraph forwave vs extwave
Modifygraph IStyle = 1
ModifyGraph Isize=2
Modifygraph rgb($theforwave)=(0,0,0)
adj=0
End

Function TraceAdjust(name,value,event)
String name
Variable value,event
AdjustTrace()
DoUpdate
End

Function AdjustTrace()
NVAR adj = root:Adjustment
NVAR no = root:TraceNo
String thewavename = "E" + num2str(no)
Wave thewave = $thewavename
String tempwavename = "temp_E" + num2str(no)
If (!Waveexists($tempwavename))
    Duplicate/D thewave $tempwavename
Endif
Wave original = $tempwavename
thewave = original + adj
End

Function RenameWaves()
String ExtWaves = wavelist("E*",",",",")
Variable i
For (i=0; i<itemsinlist(ExtWaves); i+=1)
    String newextwave = "E" + num2str(i)
    String theextwave = stringfromlist(i,ExtWaves)
    Rename $theextwave $newextwave
Endfor
String ForWaves = wavelist("F*",",",",")
Variable j
For (j=0; j<itemsinlist(ForWaves); j+=1)
    String newforwave = "F" + num2str(j)
    String theforwave = stringfromlist(j,ForWaves)
    Rename $theforwave $newforwave
Endfor
End

Function Shifter()
String thewaves = wavelist("E*",",",",")
Variable i
For (i=0; i<itemsinlist(thewaves); i+=1)
    String thewavename = stringfromlist(i,thewaves)
    Wave thewave = $thewavename
    thewave = thewave + 60
Endfor
End

```

CHAPTER 5

```
Function simpleStore()  
    Wave WA_PeakCentersX  
    Wave WA_PeakCentersY  
    Wave yWave = WaveRefIndexed("ForceExtensionAnalysis", 1, 1)  
    NVAR N = root:FX_Analysis:DisplayStartNumber  
    sort WA_PeakCentersX, WA_PeakCentersX, WA_PeakCentersY  
    String PeakForceName = "root:FX_Analysis:AutoWLC:PeakForce_FX_" + num2str(N)  
    Duplicate/R=[0, (numpnts(WA_PeakCentersY) - 2)]/O WA_PeakCentersY $PeakForceName  
    Variable a = numpnts(WA_PeakCentersX) - 1  
    Make/D/O/N=(a) Length  
    Variable i = 0  
    For (i=0; i<a; i+=1)  
        Length[i] = WA_PeakCentersX[a] - WA_PeakCentersX[i]  
    EndFor  
    String PeakLocName = "root:FX_Analysis:AutoWLC:Length_FX_" + num2str(N)  
    Duplicate/O Length $PeakLocName  
End
```

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