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Completion of the vimentin rod domain structure using experimental restraints: a new tool for exploring Intermediate Filament assembly and mutations

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SUMMARY

EPR spectroscopy of full-length vimentin and X-ray crystallography of vimentin peptides has provided concordant structural data for nearly the entire central rod domain of the protein. In this report, we use a combination of EPR spectroscopy and molecular modeling to determine the structure and dynamics of the missing region and unite the separate elements into a single structure. Validation of the L1-2 modeling approach is demonstrated by the close correlation between EPR and X-ray data in the previously solved regions. Importantly, Molecular Dynamics (MD) simulation of the constructed model agrees with spin label motion as determined by EPR. Furthermore, MD simulation shows L1-2 heterogeneity, with a concerted switching of states among the dimer chains. These data provide the first ever experimentally-driven model of a complete IF rod domain, providing research tools for further modeling and assembly studies.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.C.V., J.F.H., D.D.G., and P.G.F.; Methodology, J.C.V., J.F.H., and D.D.G.; Software, D.D.G.; Visualization, J.C.V. and D.D.G.; Validation, D.D.G.; Data Curation, D.D.G., and M.S.B.; Formal Analysis, M.S.B., D.D.G., J.F.H., R.M.M., G.A.L. and J.C.V.; Investigation, M.S.B., D.D.G., J.F.H., R.M.M., G.A.L., and J.C.V.; Writing – Original Draft, J.F.H., D.D.G., and J.C.V.; Writing – Review & Editing, J.F.H., D.D.G., J.C.V. and P.G.F.; Funding Acquisition, P.F.G., J.C.V., and G.A.L.; Resources, J.C.V., P.G.F., and G.A.L.; Project Administration, J.F.H., P.F.G. and J.C.V.; Supervision, J.C.V. and P.F.G.

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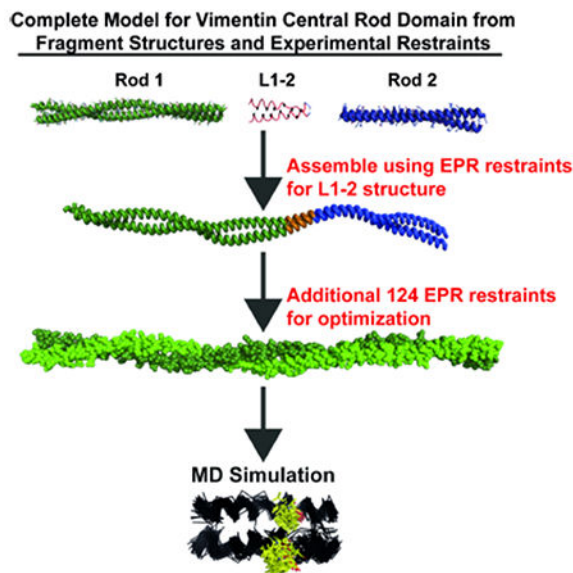
DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes ten figures and six tables can be found with this article online.

Graphical Abstract



eTOC

The linker 1-2 region of the intermediate filament protein has thus far eluded structural determination. Gae et al. use a combination of EPR spectroscopy and molecular modeling to describe linker 1-2 and unite the separate elements of the vimentin rod domain into a single experimentally-based model.

INTRODUCTION

The Intermediate Filament (IF) protein family is a large and diverse group of cytoskeletal proteins found in the cytoplasm and nucleus that have been linked to a multitude of inheritable disorders (Omary, 2009). Although no complete IF protein has been crystallized, analysis of common sequence motif across the IF family have generated a model of cytoplasmic IF proteins that is structurally distinguished by a large central rod domain dominated by coiled-coil structure but interrupted by short “linker” regions that lack a clear structural motif (reviewed in (Herrmann et al., 2007)). The central rod domain is flanked by amino terminal “head” and carboxy terminal “tail” domains (Parry and Steinert, 1999; Steinert et al., 1984; Steinert et al., 1985). Within the central rod domain, analysis identified a common pattern of coiled-coil domains, rod 1A, 1B, 2A and 2B, separated by short “linkers” predicted not to be coiled-coil (Hanukoglu and Fuchs, 1983; Steinert et al., 1984). With a strategy termed “Divide and Conquer”, Strelkov, Herrmann, Aebi and coworkers solved the crystal structure of several peptides derived from human vimentin (Chernyatina and Strelkov, 2012; Herrmann et al., 2000; Nicolet et al., 2010; Strelkov et al., 2001; Strelkov et al., 2002). Strelkov and coworkers showed coiled-coil structure over a large part of vimentin rod 2B, confirming long standing predictions (Strelkov et al., 2000; Strelkov et al., 2002). Other crystal structures have revealed surprises, such as a structure of rod 1A with a single alpha helix, rather than coiled-coil helices (Strelkov et al., 2002). Similarly, the

peptide sequence of rod 2A through linker 2 (L2) adopted a surprising structure: a pair of parallel helices intertwined into a tetrameric antiparallel alignment (Nicolet et al., 2010). Separately, each pair of parallel helices supported the hypothesis made by Parry that rod 2A and L2 adopt a parallel helices structure in intact IFs (Parry, 2006), and were consistent with data from site-directed spin-labeling combined with electron paramagnetic resonance spectroscopy (SDSL-EPR) published at the same time showing straight parallel chains for this region (Hess et al., 2006).

Using human vimentin as a model for IF structure, SDSL-EPR (Aziz et al., 2012; Hess et al., 2006; Hess et al., 2004; Hess et al., 2002) and X-ray crystallography (Aziz et al., 2012; Chernyatina and Strelkov, 2012; Herrmann et al., 2000; Nicolet et al., 2010; Pang et al., 2018; Strelkov et al., 2000; Strelkov et al., 2001; Strelkov et al., 2002) have been highly complementary (Chernyatina et al., 2016), confirming coiled-coil structure for rod 1B, linker 2 and rod 2B. The only remaining uncharacterized region of the central rod domain is termed linker 1-2 (L1-2), located near the middle of the central rod domain. Analysis of amino acid sequence homology across all IF classes led Conway et al. to conclude that L1-2 contained a partially conserved structure that was possibly beta strand (Conway and Parry, 1988). A beta strand fold for L1-2 region has also been predicted by modeling (Guzenko and Strelkov, 2018; Qin et al., 2009). It has been further proposed that L1-2 is more dynamic than other domains, assuming an alpha helical conformation within assembled filaments, with a propensity for beta-like structure in unassembled subunits (Parry and Smith, 2010).

In this study, we use a combination of SDSL-EPR and a novel model building process to analyze the L1-2 region of vimentin, historically defined as the region spanning residues 248-263. We show that the EPR data from this study and previous results (129 positions in total) can be used in conjunction with partial X-ray structural data to construct a molecular model of the complete vimentin rod domain. The resulting model shows a strong correlation with the EPR-derived information on side chain distances and dynamics. The resulting model facilitates the interpretation of experimental data and provides a platform for molecular dynamic (MD) simulation of IF structural elements and disease-associated mutations. Finally, we demonstrate that the atomistic model in combination with inter-strand spin interactions detected by pulsed EPR, provides a tool for probing higher order IF assembly. Together these results show L1-2 is not a random coil joining coiled-coil segments. Instead, our data indicate that L1-2 is an alignment of parallel chains with heterogeneous structure that may play an important role in type III IF filament biology. The data strongly suggest that throughout the central linker region of the vimentin rod domain, the dimer retains mostly helical, parallel strands. By combining SDSL measurements with MD simulations, we have revised the boundary assignments for the L1-2->rod 2A->L2 regions.

RESULTS

EPR analysis of the Linker 1-2 region

Figure 1 shows a schematic of the vimentin rod domain, which is dominated by the heptad sequence motifs along with instances of hendecad insertions. However, no clear motif is evident in the region of 249-273 (Figure 1), which includes the region historically

designated as Linker 1-2 (L1-2). Much of this region also lacks a helical secondary structure prediction (as determined by JPred4 (Drozdetskiy et al., 2015)). To determine the structure and dynamics of L1-2, we used electron paramagnetic resonance spectroscopy of site-directed spin labels (EPR-SDSL) of each position beginning in rod 1B through the predicted L1-2 region of vimentin (residues 224-280).

We have previously shown the ability of SDSL-EPR to delineate coiled-coil regions in rod 1 (Aziz et al., 2012; Hess et al., 2004) and rod 2 (Hess et al., 2006; Hess et al., 2002), as well as the lack of coiled-coil structure in the Linker 2 (L2) region (Hess et al., 2006). These studies demonstrated that for samples in the absence of motion (-100 C), a simple empirical parameter for spectral broadening (d_1/d ; see inset of Figure 2), provides a straightforward method to map sidechain proximity within the vimentin homodimer (Aziz et al., 2009; Aziz et al., 2010; Aziz et al., 2012; Hess et al., 2006; Hess et al., 2004; Hess et al., 2002), as it provides a model-independent assessment for the proximity of spin-labeled side chains (Likhtenshtein, 1993). For evaluation of a large number of spin-labeled sites, the semi-quantitative relationship of d_1/d and spin-label proximity provides a practical method for identifying structural motifs, with close distances (1nm or less) producing values of $d_1/d > 0.5$, and longer distances (2 nm or more) having values of ~ 0.33 .

The EPR spectra for vimentin positions 224-280 were collected at -100 C (see Figure S1), and the resulting d_1/d values plotted in Figure 2. In the region leading up to the predicted L1-2 domain, there is striking agreement between the amplitude of d_1/d and locations of the *a,d* positions of the heptad repeat (highlighted by blue bars; $d_1/d > 0.44$). It should be noted that the sequence motif underlying the coiled-coil structure of rod 1B is distinct from previous regions examined in that it contains a 4-residue extension to the repeat, beginning with position 231. This extended repeat has been designated as a hendecad repeat, with the additional four residues identified as *h-k* (Parry, 2006). However, our EPR analysis (including results described below) suggest the additional four residues structurally resemble the last four residues of the heptad repeat, and it is therefore more useful to designate them as such. Thus, as shown in Figure 1, residues 238-242 are designated *d-g*, but distinguished as hendecad locations by red font. Using this designation, the data in Figure 2 can clearly identify *a,d* positions for residues 224-250 in both the heptad and hendecad repeats, on the basis of their close proximity within the interface of the vimentin homodimer.

The vimentin fragment 3UF1 X-ray structure ends with the predicted onset of the L1-2 region (position 251), and a transition from coiled-coil to parallel helices at the end of the fragment is apparent in the crystal structure (Aziz et al., 2012). The EPR spectra in Figure 2 indeed show a loss of coiled-coil structure in the region of 251-272, where the sites of close proximity (highlighted in violet) do not correlate to a heptad or hendecad pattern. These data confirm the transition from coiled-coil structure at the end of rod 1B and are consistent with the L1-2 region arranged as parallel helices (see below).

Atomic model of the entire vimentin rod domain

Because of the difficulty in crystallizing regions containing linkers, as well as the propensity of such regions to adopt non-native structures when occupying the ends of crystallized fragments, we sought to use EPR constraints to build a full-length model of the vimentin rod

domain, which can be relaxed in a full-scale manner and evaluated for dynamics. The construction of the full-length rod domain was built using two X-ray structural coordinates: 3UF1, which encompasses rod 1B, and 3KLT (Nicolet et al., 2010), which encompasses most of rod 2. The BUILD STRUCTURE module of UCSF CHIMERA was used to build the parallel helices bridging the rod 1 and rod 2 structures. A custom python script enabled the rotation, and the translation of 3UF1 and 3KLT to L1-2, while MODELLER was used to structurally optimize the full-length rod domain. In the first step, each piece of 3UF1, 3KLT and L1-2 coordinates were structurally optimized in MODELLER using 22 structurally-close positions as determined by the measured d_1/d values from this study (residues 224-280). The positions of the model in this initial optimization maintained a close correlation to the X-ray structural distances between $C\alpha-C\alpha$ as well as $C\beta-C\beta$ of the two chains, and served as the physical input distances for the second round of structural optimization in MODELLER. The output from this step resulted in our “unrestrained model”. The full-length “restrained model” underwent an additional optimization using the X-ray structural $C\alpha-C\alpha$ and $C\beta-C\beta$ correlation to EPR-derived d_1/d values from throughout the central rod region (a total of 124 out of 129) from this study and previous work (Aziz et al., 2012; Hess et al., 2006; Hess et al., 2004; Hess et al., 2002). Finally, the optimized full-length rod domain model was checked for geometry and scored using PROSA, and GA341 scoring functions. An illustration of the model building process is shown in Figure 3.

A comparison study of the unrestrained model versus the full-restrained model was performed to determine the model quality. For example, without the restraints, the overall RMSD between the unrestrained model and the restrained model was 0.51 nm. The unrestrained model was also compared to 3UF1 (rod 1B), 3KLT (rod 2B), and 3TRT (rod 2B;(Chernyatina and Strelkov, 2012)) X-ray structures respectively with 0.2, 0.67, and 0.34 nm RMSD. While the restrained model to 3UF1, 3KLT, and 3TRT X-ray structures had a RMSD of 0.26, 0.38, and 0.4 nm. Both GA341 and PROSA produced an unrestrained model with a higher deviation from the X-ray structure (Z-score), while the full-restrained model with a lower Z-score. We used the restrained model based on closeness to the X-ray structures and with a reasonable overall energy for all the following analyses below. The significance of the EPR restraints on the resulting structure is shown in Figure S2.

In addition to the RMSD analysis, we also performed solvent accessible surface area (SAS) calculations using ALPHASURF (Edelsbrunner and Koehl, 2003) in order to compare the AB dimer interfaces between X-ray structures. For example, SAS of 3SSU (144-189; (Chernyatina et al., 2012)), 3UF1(144-249), 3SWK (153-238;(Chernyatina et al., 2012)), 5WHF (153-238;(Pang et al., 2018)) between the AB dimer resulted in a value of 2140, 5770, 4530, 4580 \AA^2 , respectively (Table S1). In addition, a value of 5370 \AA^2 which is attained for our modeled structure (144-249) correlates well with the dimer-interfaces of X-ray structures described above. Concerning the ambiguous structure within the L1-2 and rod 1A/L2 region, we analyzed the dimer interface from the end of rod1B through the region containing L1-2 and L2 (250-300). The SAS value for this region of our model is 2370 \AA^2 . Finally, our full modeled structure between 146-333 is calculated to have a value of 9470 \AA^2 at the interface (Table S1).

Consistency of the model with EPR distance analysis

The accuracy of the full-length rod domain model can be evaluated from its agreement with X-ray data from vimentin fragments, the EPR data from this study (57 side chains), as well as EPR results from previous studies. The correlation of experimental d_1/d values spin-spin broadening in the vimentin dimer with the distances between side chains in opposite strands of the dimer is shown in Figure 4. Overall, the model distances between opposing alpha carbons ($p < 10^{-15}$; Figure 4A), as well as beta carbons ($p < 10^{-17}$; Figure 4B), show a strong correlation. For very close Ca-Ca distances (0.8 nm or less), d_1/d is 0.44, while longer Ca-Ca distances have d_1/d values less than 0.44. The stronger correlation between the distances between beta carbons and d_1/d measurements most likely reflects the ability of the more distal side chain position to reflect the dependence of side distances on orientation. To evaluate how the d_1/d value correlates to the model for different classes of residues within the rod domain, data points in Figure 4 are colored according to their position within the heptad motif (red and blue symbols) or their localization to a linker region (green and violet). Similar plots with the residue position of each point labeled are shown in Figure S3. With respect to the overall correlation, the L1-2 region and the *a,d* positions of the coiled-coil regions are evenly distributed. As a group, the d_1/d values for the *b,c,f,g* positions of the heptad distribute below the fit of the data, although this is expected due to the greater orientation dependence of distances measured between the ends of the spin-labeled side chains. In contrast, the d_1/d values for the previously measured L2 region (Hess et al., 2006) are generally higher than predicted from the model, suggesting this tightly constrained region may be more relaxed in the model. The relationship between Ca-Ca versus measured d_1/d values, as well as C β -C β versus d_1/d shows a clear inverse linear correlation and establishes a statistical relationship among physical parameters from two different approaches that can be combined as a general method for structural refinement. In summary, these results support the structural optimization process driven by EPR d_1/d constraints for both X-ray distances and for the modeling of structure in regions that lack a structural template.

Molecular dynamics of the constructed vimentin model reflects unique structural features

To further validate the ability of the full-length rod model to recapitulate structural features, we examined the packing and dynamics of two positions within the Linker 2 region over a 50 nsec time frame. Two positions, 283 and 291, were selected based on our previous studies showing these residues possess unique characteristics, including extremely close packing and high thermal stability (Hess et al., 2006). As shown in Figure S4, both 283 and 291 retain a close proximity to the residue on the opposing strand during the simulated dynamics. Likewise, the narrow RMSD of both positions (Figure S4) also reflects the tightly packed environment evident in EPR analysis (Hess et al., 2006). In addition, the molecular model might be useful to explore the basis of this highly stable region. For example, the model reveals an intra-chain salt-bridge between K282-E286 that can be expected to contribute to the high thermal stability of this region. Similarly, the model also reveals that another pair of intra-chain salt-bridge between E289-K292 is expected to contribute to the high thermal stability as well as restricting the side chain motion of Y291. These restricted dynamics in the model clearly identify the uniqueness of the L2 region that we have previously determined experimentally (Hess et al., 2006).

Correlation of molecular dynamics to spin label motion

To evaluate the dynamics of residues 224-280 in vimentin, we collected EPR spectra at room temperature of individually spin-labeled vimentin, as assembled into protofilaments (Mucke et al., 2004). The line widths of the solution spectrum of a spin-labeled side chain is dependent on both backbone and side chain dynamics (Altenbach et al., 2015; Klare, 2013), and therefore provides a direct method for identifying structural motifs in vimentin (Aziz et al., 2012; Hess et al., 2013; Hess et al., 2006; Hess et al., 2004; Hess et al., 2002). Figure 5A shows the room temperature EPR spectra collected for region representing the final heptad repeat of rod 1 (residues 242-250), with the spectral amplitudes normalized to the same number of spins. Qualitative examination of the spectra reveals three positions (242, 245 and 249) that are extremely broadened. Consistent with previous studies (Aziz et al., 2012; Hess et al., 2013; Hess et al., 2006; Hess et al., 2004; Hess et al., 2002), the broadening at these sites can be attributed to their location at the interface of the coiled-coil (i.e., *a* or *d* positions within the heptad repeat), which imparts both motional restriction and dipolar coupling between side chains in close proximity. A similar correlation between spectral line shape and heptad position (including the hendecad *d* assignment of position 239, see above) is evident for the upstream region of rod 1B (residues 224-241; see Figure S5).

In contrast to the predicted rod 1B region (residues 224-250), the side chain dynamics of residues in the L1-2 region (251-272) do not display a pattern of coiled-coil structure (Figure S5). Rather, multiple positions display spectral broadening, indicative of a rigid and close arrangement of the dimer strands in this region. Notably, the extent of broadening in the room temperature spectra closely follows the broadening seen in the spectra of frozen samples (expressed as d_1/d , see Figure 2). Remarkably, two positions (264 and 265) display two clearly resolved dynamic populations, a feature not present in any of the other 127 residues examined within the vimentin rod region. Whether this unique feature arises from a unique side chain rotamer distribution in each strand, or distinct dynamics within each strand is discussed below.

While multicomponent fitting of solution EPR spectra provides a quantitative assessment of spin label correlation times, the relative dynamics of spin-labeled sites can be determined by (H^{-1}) , a simple model-independent index for the degree of side chain order (Altenbach et al., 2015). The map of side chain dynamics determined by H^{-1} was then compared to the computed B-factors of side chains obtained from a 50 nsec MD simulation of the constructed model. The results are shown in Figure 5B. Similar to the results based on distance between opposite side chains (Figure 4), there is a strong correlation between the EPR-measured dynamics and the side chain order calculated through MD simulation of the model, a finding that further supports the utility of the model in understanding vimentin structure and dynamics. For example, the analysis reveals that the L2 region has greater thermal motion compared to L1-2. Moreover, larger concerted motions can be visualized from the MD simulation of the constructed vimentin model, such as the visualization of the greater flexibility in the rod 2 domain compared to the rod 1 domain (see movie M1).

The comparison of simulated dynamics of native residues to measured side chain dynamics is facilitated by the preferred rotamer of the MTSL spin-labeled side chain on helical structure (Polyhach et al., 2011). To verify this predicted behavior, we performed a single

MD simulation of the spin-labeled side chain at position 224 to evaluate its probable conformer distribution on a vimentin helix. As shown in Figure S6, the spin-labeled side chain populates a densely-compacted space, indicative of a preferred rotamer. Moreover, the spatial distribution of the N-O moiety is largely determined by the χ_1 - χ_5 angle of the MTSL-labeled Cys side chain. This rotamer of the MTSL-labeled Cys has been identified as a favored conformation along helical structure (Polyhach et al., 2011).

Analysis of L1-2 dimensions by pulsed EPR

To differentiate between the 2 hypotheses that L1-2 is largely helical as our EPR and model predict, or dynamic as hypothesized by Parry (Parry and Smith, 2010), we prepared vimentin molecules with a deuterated isoleucine combined with a targeted spin label to detect close interactions within the 256-260 region by ESEEM (Electron Spin Echo Envelope Modulation spectroscopy). This technique utilizes both a specifically deuterated amino acid and a spin labeled position to report unambiguously whether the two labeled sites are within 0.8 nm of each other (Liu et al., 2016). For α -helices, this condition is met for residues 3 or 4 residues removed from the deuterated position. In contrast, for β -strands, this condition is met for residues 2 positions removed from the deuterated residue. To apply this approach to the middle of the L1-2 region, *E. coli* transformed with single-Cys mutations in vimentin were grown in media containing deuterated isoleucine and thus produce proteins isotopically labeled at I256. For each of the three single-Cys mutations (at position 258, 259 or 260) containing the deuterated Ile, the expressed vimentin was purified, and spin-labeled with MTSL. To minimize inter-strand couplings, the labeled protein was diluted with unlabeled vimentin in urea (at a ratio of 1 labeled: 5 unlabeled). The spin-diluted samples were then dialyzed against low ionic strength tris buffer and analyzed by ESEEM. Surprisingly, ESEEM couplings are observed for all three spin-labeled positions (Figure 6A). This suggests either a mixture of beta sheet and alpha helical structure, or a distinct backbone fold where position 256 would maintain a close proximity to each of the three spin-labeled locations (258, 259 and 260).

Although the MD simulations of the constructed model do not show evidence for beta strand formation, the helical distance within this region of L1-2 is distorted relative to a typical α -helix. In fact, over a 50 nsec simulation, the distance of the C β position on 256 to the C β positions in each of the three spin-labeled sites falls within the 0.8 nm threshold for ESEEM (Figure S7). Thus, our model suggests the successive ESEEM signals arise from an unusual helical fold rather than the presence of beta secondary structure.

To test the dimensions of the L1-2 region over a larger scale, we used a second pulsed EPR approach capable of detecting spin-couplings out to 5 nm or more. This method, Double-Electron-Electron-Resonance (DEER) (Jeschke, 2012), we made double-Cys mutants within vimentin and labeled both positions with the MTSL spin label. The double-labeled protein was then spin-diluted (to minimize intermolecular spin couplings) with unlabeled wt vimentin at a ratio of 1:5 (labeled:unlabeled), assembled and measured for inter-spin distances by DEER. We generated three spin-pair combinations for the DEER: (i) positions 225-240 to serve as a reference for a known helical segment in rod 1B; (ii) positions

244-265, encompassing the end of rod 1B and the first 15 residues of L1-2; (iii) positions 250-265, encompassing the first 15 residues of L1-2.

The results of the DEER measurements are shown in Figure 6B. Due to the spin dilution and the difficulty of concentrating vimentin without inducing aggregation, the signal to noise ratio of the data were not ideal. However as demonstrated by the control rod 1B sample (225-240), the DEER result matches the distance calculated from the 3UFI X-ray structure (Table S2). In contrast, the DEER analysis of the 244-265 pair does not produce a single major peak. In this case, three major distance peaks are obtained, 3.2, 3.7 and 4.5 nm (Table S2). The 3.2 nm DEER distance agrees well with the C β -C β distance measured in our model (3.2 nm). The source of the additional DEER peaks observed for the 244-265 pair is discussed below. The DEER results for the 250-265 pair are also complicated by multiple distances, with three major DEER distance distributions (Figure 6B). Of the three peaks, the distribution centered at 2.5 nm is consistent with the intramolecular distance between the positions in our model (2.3 nm; Table S2), and therefore consistent with a distorted helical structure for the L1-2 region.

The additional DEER peaks for the 250-265 and 244-265 pairs could arise from a mixture of structural conformations in the L1-2 region. However, the presence of 3 distance peaks in the 250-265 pair is better explained by an A12 alignment of assembled vimentin units centered near position 250. In the A12 alignment, adjacent dimers are aligned in an antiparallel manner with overlap in the L1-2 region (Steinert et al., 1993). Thus, it is not surprising that positions in the vicinity of L1-2 experience inter-molecular (inter-strand) interactions with spin labels located on adjacent dimers. In fact, when combined with the full-length molecular model of vimentin, a collection of intermolecular DEER distances provides a method for modeling the assembly of vimentin and other IFs. In this regard, we used the multiple DEER distances for the 250-265 and 244-265 pairs to model an A12 alignment of vimentin dimers (chains *AB* aligned with chains *CD*). The resulting model is shown in Figure S8, with an assignment of the model C β -C β distances to a measured spin-pair DEER distance given in Table S3. The resulting intermolecular C β -C β distances in the modeled A12 alignment are close to the distances reported by DEER. The exception is the C β -C β distance of 2.0 nm between position 250 in chains *B* and *C* in the model. The nearest DEER peak for the 250-265 sample is 2.5 nm. However, the lack of a DEER peak in the 2 nm range is not surprising. Distances on the order of 2 nm or less are difficult to detect with DEER, as the couplings become too intense to be observed within the pulse bandwidth. Alternatively, the 250B-250C species could be contributing to the 2.5 nm DEER peak, implying the C β -C β distance in the model underestimates the distance between the ends of the spin-labels as measured by DEER.

Although the modeled A12 alignment adequately accounts for the additional DEER distributions, the amplitudes of the additional peaks are larger than expected for samples that have been spin-diluted to minimize intermolecular interactions. For example, within a dimer, the probability of intermolecular interaction is 20% given the 1:5 (labeled:unlabeled) spin dilution. However, the probability for intermolecular interaction with labels located in an adjacent dimer in the A12 alignment is higher, since spin-coupling can be achieved with spin labels located in either chain. In addition, for labels located at position 250, the possibility of

coupling to labels located on more than one tetramer exists. The idea is illustrated in Figure S9, which shows a schematic of possible interactions of vimentin tetramers in the A12 alignment (based on the elongated IF cross-section (Sokolova et al., 2006)). Finally, with respect to the relationship of distribution peak amplitudes to the population of spin pairs, the DEER peak assigned to the intramolecular coupling is broader for both the 244-265 and 250-265 spin pairs. As detailed below, the chains of the vimentin dimer show structural heterogeneity in the L1-2 region despite having the identical sequence. Thus, the 244-265 and 250-265 spin pairs may report different distances, depending upon the conformational state of the chain. Indeed, dimensions from the model suggest a significant length difference between the two chains. Measuring the region of residues 250-300, the end to end distances are 7.6 nm and 7.9 nm for chains A and B, respectively.

MD simulation identifies structural heterogeneity among the paired L1-2 strands

Vimentin L1-2 is historically defined as positions 248-263. However, MD simulations define this region as a helical distortion that appears to propagate from positions 251 to 272. This mixed helical distortion was further characterized structurally based on 100 frames of 50 nsec MD trajectory using the DSSP program. The structural coordinates derived from the MD trajectory were entered into a DSSP program, and the α -helical frequency was computed for every position in the vimentin sequence range of 144 to 334 in both chain A and chain B. Interestingly, the α -helical distortion frequency extends out from positions 251 to 272 with the structural heterogeneity predominately for chain A (Figure 7A) compared to chain B (Figure 7B). For the region encompassing L2, chain B shows smaller helical distortions in the sequence range of 282-300. Furthermore, although the average Phi-Psi values (-65 and -40 degrees, respectively) for the L1-2 region resemble a typical α -helix, the uniqueness of this region is also revealed by alterations in ϕ and ψ angle distribution (Figure 7B, inset). According to these parameters, residues D264 and T266 for chain A show the largest dihedral deviation of helical character and appear to have a propensity for β -strand character. Compared to a coiled-coil region, the total energy of the L1-2 region is slightly higher when calculated by a molecular mechanics force field (Maier et al., 2015), with an average value of -182 kcal/mol for chains A and B in the relaxed structure compared to -191 kcal/mol for the coil-coiled region of residues 224-234.

L1-2 is readily identified by its lack of a coherent heptad pattern. Such transitions in coils can manifest as phase shifts, imparting strains that affect the degree and handedness of supercoiling (Brown et al., 1996; Lupas and Gruber, 2005). To evaluate the degree and direction of coiling, the phase yield per residue (ω_n) (Strelkov and Burkhard, 2002) was calculated along the optimized vimentin structure. A canonical left-handed coiled-coil has a $\omega_n = -4^\circ$, whereas a helix without twist has a value of zero, and right-handed supercoiling has positive values of ω_n . As shown in Figure 7C, the unwinding of rod 1B begins with the hendecad insert at position 238 and continues through L1-2 and into rod 2A, where a right-handed twisting force is predicted. The strain at the end of L1-2 (where we also see structural heterogeneity between the chains) is also reflected in the number of backbone H-bonds lost in the optimized structure (Figure 7C, top panel). To obtain a comparative analysis of strain over time, the frequency of H-bonds lost was calculated for the distinct regions over 50 nsec of MD simulation (Figure 7D). Again, L1-2 shows the highest

frequency of H-bond disruption, whereas the coiled-coil segment of rod 1B experiences the lowest frequency.

These results provide a quantitative basis for defining the structural ambiguity of the region spanning L1-2, rod 2A, and L2. Taken together with SDSL measurements, the conformationally unique region of L1-2 is best defined as residues 251-268, positions 269-281 encompassing rod 2A and L2 as residues 282-300. In addition to better delineating the boundaries of these sub-regions within the central region of vimentin, these analyses along with SDSL provide insight onto the relative order within each subdomain. In particular, the subregion of L1-2 distinguished for its chain heterogeneity and L2 for its thermal stability.

As discussed above, we observed a distinct structural diversity of L1-2 despite the sequence of homodimer being identical. To probe this further, we ran 50 nsec of MD simulation for L1-2 dynamics. Throughout this time the heterogeneity of the two chains is maintained, however a switching between alternate states is observed (one chain is helical, the other distorted), showing evidence of concerted dynamics (Movie M2). In addition, the EPR data demonstrate that some sites in L1-2 supports the notion of discrete states for chains A and B (Figure 8).

Using the model for comparative analysis of L1-2 sequences and mutations

We also extended the structural heterogeneity of L1-2 for two other type III IFs: GFAP and DESMIN by performing 50nsecs of L1-2 region MD simulations. Specifically, MD simulation of L1-2 A and B chains in wt vimentin reveals a switching of helical distortion between the chains, revealing a dynamic asymmetry (Figure 9B). This observation was the same for both GFAP and DESMIN. In all three cases (GFAP, DESMIN, VIMENTIN L1-2 region), one backbone chain is well ordered, while the other is distorted.

Using the homodimer model of Vimentin, we generated a homology model of the wildtype Keratin14/5 heterodimer as well as a mutated form of the Epidermolysis bullosa simplex (EBS), and the Alexander Disease into the vimentin homodimer. D257Q was substituted *in silico* for the Vimentin model and simulated for 50 nsecs to mimic the Alexander Disease mutation. We observed a distorted L1-2 in which bent helices were unable to be rescued throughout the 50nsecs. Furthermore, mimics of disease-associated mutation induce global changes in IF structure, suggesting the L1-2 region resides near a conformational threshold. (Figure 9C). Similarly, both D259G and K262C mutations, which mimic the EBS disease mutant form of the Vimentin model, were performed for 10nsecs of MD simulation. Much like the D257Q mutant Vimentin we observed a distorted L1-2 bent helices in the D259G and K262C mutations (Figures 9D–F).

Mean interaction energy (kcal/mol) between chain A and chain B was computed based on the MD simulation runs (Table S4). We observed that wild type Vimentin and Keratin have similar mean interaction energies of -84.9 and -88.7 kcal/mol respectively. Interestingly, D259G has a mean interaction energy of -416.1 kcal/mol. Next, D257Q has mean interaction energy of -332.3 kcal/mol and lastly, K262C has mean interaction energy of -73.5 kcal/mol.

DISCUSSION

Our analysis of L1-2 and modeling of the completed assembly reveals that the middle of the vimentin differs from the historically-predicted structure and provides revised sequence delineations for the structural designations within this region. Our data confirm X-ray crystallography showing the carboxylic end of rod 1B as well as rod 2A through L2 adopt a parallel helices structure instead of a coiled-coil structure (Aziz et al., 2012; Chernyatina and Strelkov, 2012; Nicolet et al., 2010). For the region encompassing rod 2A and L2, the parallel helices feature has been identified as a “paired bundle” (Chernyatina et al., 2015). With the spectroscopic analysis of L1-2 completed, the central rod domain of vimentin can be described in a simpler manner. Instead of a structure composed of 4 coiled-coil domains and 3 linker domains (rod 1A, L1, rod 1B, L1-2, rod 2A, L2, rod 2B), our data are consistent with a tripartite central rod domain: a central parallel helices domain flanked by left-handed coiled-coil domains. It should be noted that within this central linker region, there are distinct levels of order roughly corresponding to the L1-2, rod 2A and L2 designations (especially the chain heterogeneity of the L1-2 portion, and the highly stable nature of the L2 portion). Based on these findings, we present a revised schematic map of the vimentin structural domains in Figure 10, which includes the locations of structural motifs common to IF proteins.

Our designation of rod domains is based upon direct evidence for left-handed coiled-coil structure established by crystal structures or detection of the heptad repeat by SDSL-EPR. However, insertions and deletions into the heptad can either loosen or tighten super-coiling within coils (Lupas et al., 2017), such as the hendecad repeat near the end rod 1B (Fig. 1) that serves to unwind the helices. SDSL-EPR data detect the hendecad repeats at the end of rod 1B and the region encompassing L2. No coil pattern is evident for the L1-2 region, where SDSL-EPR, pulsed EPR and simulations are consistent with a metastable structure. In addition, SOCKET (Walshaw and Woolfson, 2001) analysis for “knobs-into-holes” over the assembled structure assigns coiled-coil geometry for residues 154-245 and 305-330, but not in the region spanning L1-2 through L2. Although *a/d* proximity for positions 273 and 276 in rod 2A is detected by SDSL-EPR, this segment may be too short to impart the twist commensurate with knobs-into-holes packing.

Although X-ray crystallography has been extremely useful for obtaining structural information on IF proteins, fragmented and modified vimentin peptides can adopt non-native or disordered structure within the unit cell. For example, 3KLT (Nicolet et al., 2010) displays high thermal motions at its N-terminal end (residues 263 through 268), resulting in a mean crystallographic B-factor of 58. The same structure also contains uncertainty at position 334. Another example of structural uncertainty is observed in 3SSU (Chernyatina et al., 2012), which lacks reliable information for positions 144 to 148 (a mean crystallographic B-factor value of 131). In the 3SWK (Chernyatina et al., 2012) structure for the 153-238 vimentin region, the N-terminal position has a mean B-factor of 66. Finally, in the overlapping region beyond position 221 in the 5WHF, 3UF1 and 3SWK vimentin crystal fragments deviate significantly (RMSD greater than 3.0). Within this region, the coordinates of our final model reside near the average positions of these three structures. SDSL-EPR is

therefore very complementary to crystallographic efforts, in that it can clarify local ambiguities in the structure.

X-ray crystallography of a vimentin fragment including the rod 2A-L2 region revealed two pairs of widely spaced, parallel helices interlaced into tetrameric structure with apparently normal coiled-coil domains forming downstream (Nicolet et al., 2010). Based on this result, rod 2A through L2 was suggested to adopt a continuous region of straight helical chains running in parallel, consistent with prior EPR data of L2 (Hess et al., 2006). In the case of the L1-2 region, SDSL-EPR shows the 3TRT (Chernyatina et al., 2012) structure is influenced by the engineered disulfide at the fragment's N-terminal end (residues 261-269). After position 270, the disulfide shows no further perturbation on the vimentin structure, and there is good agreement between SDSL and the 3TRT structure. Finally, there is a significant divergence from structure 3UF1 (Aziz et al., 2012) to structure 5WHF (Pang et al., 2018) in the sequence range of 221 to 238, suggesting dynamics within this region produces multiple states. We propose an alternative approach in comparing these two structures by superimposing it on our dynamic model which possesses both dynamic spin-label restraints at particular positions as well as using the X-ray structural distances with energy minimization.

Although the initial building of our model was based upon the 3UF1 (Aziz et al., 2012) and 3KLT (Nicolet et al., 2010) structures, our final model complements information reported by other crystallography efforts on vimentin fragments. As noted above, ambiguity in aspects of these structures, such as the significant divergence in the region of residues 221-235 between the recently published 5WHF (Pang et al., 2018) and 3UF1 X-ray structures. Our results demonstrate the utility of EPR-derived constraints in model optimization to clarify structures and provide dynamic information that is absent in crystal structures. When excluding ambiguous regions of X-ray structure (due to missing residues in the X-ray structures or having high B-factor values, such as 3KLT and 3SSU) to our model, we find a strong agreement with other vimentin crystal structures. For example, superimposition of 3UF1 (residues 146-220) and 3KLT (residues 269-333) via CEALIGN on chains A and B of our final model results in a RMSD of 0.24 and 0.35 nm, respectively. In addition, our modeled structure for the vimentin rod 1A region resembles the 5WHF structure of this region by Pang et al. (Pang et al., 2018), where a superimposition of positions of 154-220 in chains A and B via CEALIGN on our final model results in a RMSD of 0.3 nm. Other structures such as 3SSU (Chernyatina et al., 2012) (residues 149-187), 3SWK (Chernyatina et al., 2012) (residues 154-237), 3S4R (Chernyatina et al., 2012) (residues 149-189), and 3TRT (Chernyatina et al., 2012) (residues 270-333) superimpose on our final model results in a RMSD of 0.13, 0.3, 0.18, 0.37 nm, respectively.

EPR data and MD of L1-2 suggests distinct conformations among the chains in the dimer (helical and distorted helix). Intriguingly, the two protein strands have the ability to “flip” in a concerted mechanism from one conformation to another. We view this dynamic as a plausible explanation for the hypothesis of Parry et al (Parry and Smith, 2010) suggesting that this region first adopts a beta structure and that a beta to alpha switch occurs during assembly of L1-2 helix. Modeling also suggests that paired H-bond networks around the vimentin L1-2 may stabilize the mixed-helical nature of the region. In particular, 255 OE1 to

255 NE2 as well as 255 NE2 to 259 OD1 remain extremely stable through the MD simulation trajectory. This alternate conformation of identical protein sequences in vimentin was also seen in the structure of rod 1B assembled into an A11 like tetramer (Aziz et al., 2012). The chain distinction within homopolymeric vimentin may be an inherent property of IFs, given this feature is expected among heteropolymeric IFs. Furthermore, the strain within the L1-2 structure suggests this region of vimentin may be structurally sensitive to mutation. As such, a D257Q substitution to mimic a mutation in GFAP that leads to Alexander disease was probed by MD simulation. The resulting bent structure of L1-2 remains stable throughout the 50 nsec simulation. It is therefore reasonable to postulate that the stability of the native L1-2 region resides near a conformational threshold, with the D257Q point mutation switching the region into a relaxed, bent structure that would impede filament assembly.

The determination of the structure of the central rod domain of vimentin using spectroscopic techniques, followed by the creation of a valid molecular model opens the door for several future studies. We previously published spectroscopic structural data for the vimentin head and tail domains, regions for which no x-ray crystal structures have been solved. Using the modeling techniques described here, it is possible molecular structures for these domains could be created. With or without models of the head and/or tail domains, the existence of a molecular model of the central rod domain opens up the study of higher order vimentin assembly through the *in silico* modeling of A11, A22 and A12 interactions as vimentin molecules pack in different ways to generate a 10 nm filament. As exemplified in this study, spin labels in the L1-2 vicinity support the existence of the A12 tetramer structure.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John C. Voss (jcvoss@ucdavis.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For protein expression, *E. coli* BL21(AI) was used.

METHOD DETAILS

Site-directed mutagenesis and purification for spin labeling—Vimentin mutants were constructed using site-directed mutagenesis, and recombinant proteins were produced in *E. coli*. Vimentin readily forms inclusion bodies; these are isolated and the recombinant vimentin purified and spin-labeled as described previously (Aziz et al., 2009; Aziz et al., 2010; Hess et al., 2006; Hess et al., 2002). In short, site-directed mutagenesis was used to introduce cysteine residues at specific sites in a vimentin expression construct (originally provided by Roy Quinlan, University of Durham, Durham, UK) using Bio-Rad iProof DNA polymerase (Bio-Rad, Hercules, CA) and mutagenic oligonucleotides (Invitrogen, Carlsbad, CA). Coding sequence changes were confirmed by automated DNA sequencing (Davis Sequencing, Davis, CA). Mutant vimentin protein was produced by bacterial overexpression using a pT7 vector (Studier et al., 1990) and *E. coli* BL21AI (Invitrogen, Carlsbad, CA). 1 L

of culture in LB medium containing 0.1 mg/ml ampicillin was grown at 37 °C to a cell density of 0.6 (according to OD₆₀₀). Thereafter protein expression was induced by adding 1 mM IPTG, followed by 3 additional hr of cultivation at 37 °C. Cells were then pelleted by centrifugation and washed in phosphate-buffered saline. Inclusion bodies were purified using lysozyme/DNase, high/low salt washes (Nagai and Thøgersen, 1987), followed by gel filtration and ion-exchange chromatography (AKTA FPLC, GE Healthcare, Piscataway, NJ) as described in (Chernyatina et al., 2016). Site-directed spin labeling was performed by treating the purified protein with 100 μM TCEP (Tris-(2-carboxyethyl-phosphine hydrochloride, Invitrogen) followed by spin labeling with 500 μM thio-specific nitroxide spin label 1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl methanethiosulfonate (MTSL; Toronto Research Chemicals, Canada). Unincorporated spin label was removed from spin-labeled vimentin by chromatography over a Source S or Source Q column (both work well (AKTA FPLC, GE Healthcare, Piscataway, NJ)). Protein concentrations were measured by the BCA method (Pierce, Rockford, IL). Purified spin-labeled proteins were stored at -80°C.

In vitro filament assembly and electron microscopy—Filament assembly was conducted by dialyzing the spin labeled protein (~500 μg/ml) overnight in 8 M urea, 50 mM Tris, pH 7.5 against filament assembly buffer (10mM Tris, pH 7.5, 160mM NaCl). Following dialysis, 10 μl of the sample was removed, placed on formvar-coated carbon grids and subsequently stained with 1% uranyl acetate. Grids were examined using a Phillips CM-120 electron microscope, with a Biotwin Lens, (FEI, Hillsboro, OR) operated at 80 kV acceleration voltage. Images were acquired with a Gatan MegaScan 794/20 digital camera (2K X 2K) or a Gatan BioScan 792 (Gatan, Pleasanton, CA).

EPR Spectroscopy of site-directed spin labels—EPR measurements of the spin-labeled proteins were conducted on a JEOL X-band spectrometer fitted with a loop-gap resonator (Hess et al., 2002). Spectra were collected from ~6 μl of purified, spin labeled, dialyzed protein, at a final protein concentration of 25-100 μM, loaded in a sealed, quartz capillary tube. Spectra were obtained at room temperature (unless otherwise specified) by a single 2 min scan with 100 Gauss sweep width at a micro-wave power of 4 mW Modulation amplitude (0.125 mT) was optimized to the natural line width of the attached nitroxide as previously described (Aziz et al., 2009; Aziz et al., 2010; Hess et al., 2006; Hess et al., 2002). Normalization of the spectra to the same number of spins was done by normalizing each spectrum to the same integrated intensity/amplitude and concentration. To improve the fidelity of the calculation, each sample was double-integrated after solubilization in 2% SDS. Low temperature spectra were collected from samples flash frozen and maintained at -100°C. Protein samples in 5mM Tris, pH 7.5 were mixed with the appropriate volume of 10X IF assembly buffer (100mM Tris, pH 7.5, 1.6M NaCl) in an Eppendorf tube. 25 μl of the mixture was quickly pipetted into a capillary and placed into a low-speed bench top centrifuge to collect the assembling filaments at the bottom. Mixing, pipetting and centrifugation was repeated to generate 2 capillaries for each sample. Both capillaries were placed in the low temperature cavity for low temperature data collection. Distance proximity from frozen spectra was estimated using the semi-empirical broadening parameter d_1/d

(Hess et al., 2002; Likhtenshtein, 1993), which was calculated from the intensity of the hyperfine extrema divided by the intensity of the central resonance line.

Model building of L1-2 linker 250-262 (chain A) and 250-264 (chain B)—The initial model building of L1-2 linker for chain A and chain B was carried out using BUILD STRUCTURE module of CHIMERA1.11 (Pettersen et al., 2004) using the following input sequence:

$^{230}\text{EIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTAALR}^{270}$ where the phi and psi angle were set at -60 , -45 degrees to maintain a right handed alpha-helical geometry with two chains separated by 0.7 nm distance. Next, initial 3UF1 coordinates of chain A and chain B (Aziz et al., 2012) and 3KLT coordinates of chain C and chain D (Nicolet et al., 2010) were utilized. For 3UF1, 34 Selenomethionine (MSE) were mutated to 34 methionine (MET) using a custom python script, while the 3KLT was unaltered.

Structural alignment of 3UF1 and 3KLT PDB coordinates to the L1-2 linker—3UF1 sequences ($^{230}\text{EIAFLKKLHEEEI}^{242}$) was structurally aligned to L1-2 model via the CEALIGN program (Shindyalov and Bourne, 1998). Next the 3KLT coordinates were aligned to the C-terminal L1-2 sequence ($^{263}\text{PDLTAALR}^{270}$) using the rotation and translation function in CHIMERA1.11 (Pettersen et al., 2004). Following this procedure, flanking 230 to 242 model coordinates were deleted and replaced with $^{230}\text{EIAFLKKLHEEEI}^{242}$ 3UF1 coordinates, which leads to the L1-2. Similar to findings in the L2 region (Hess et al., 2006), the backbone for sequence positions 263-270 were suggested to be parallel based upon the pattern of EPR-derived d_1/d values, and validated upon the linear correlation between d_1/d values and the $\text{C}\alpha$ - $\text{C}\alpha$ and the $\text{C}\beta$ - $\text{C}\beta$ distances of X-ray structural data. Therefore, the L1-2 region was initially set as parallel right-handed helices prior to refinement in the MODELLER program.

$\text{C}\alpha$ - $\text{C}\alpha$ spatial restraints of chain A to chain B for parallel helix refinement via MODELLER.—In the first step of model refinement, EPR spectra that define d_1/d values of 0.42 or greater served as defining positions for the 3UF1, Linker 1-2 and 3KLT coordinates. d_1/d positions that correspond to the $\text{C}\alpha$ - $\text{C}\alpha$ distance of 0.6 nm between the two chains were defined in the MODELLER (Sali and Blundell, 1993) python script (Table S5). Next, the positions within the region (250-263) for which we do not have the structural $\text{C}\alpha$ - $\text{C}\alpha$ and $\text{C}\beta$ - $\text{C}\beta$ values were extrapolated from the closest value of X-ray data. For example, the d_1/d value for position 250 resembles the values for positions 148, 155, 162, 169, 176, 183, 190, 197, 204, 211, 218, 225, 232, and 243 (having d_1/d values of 1.48, 1.35, 1.25, 1.19, 1.19, 1.29, 1.27, 1.11, 1.29, 1.25, 1.25, 1.29, 1.36, and 1.38 nm). Taken together, the average 1.28 nm value serves as a good estimated value for position 250. Subsequently, positions 251 through 263 were extrapolated via the closest previous X-ray structural values and reported in Table S6 and used for the secondary refinement. The third and final refinement was performed using EPR constraints from 124 unique spin-labeled positions corresponding to the structural $\text{C}\alpha$ - $\text{C}\alpha$ and $\text{C}\beta$ - $\text{C}\beta$ distances, including the extrapolated distances.

The output of the refined model was manually checked for clashes and distances, and the TWISTER (Strelkov and Burkhard, 2002) program was utilized to determine heptad repeats

and coiled-coil statistics. TWISTER version 2006 was kindly provided by Professor Sergei V. Stelkov. The full vimentin model was assessed for surface energy using the GA341 energy function (Melo et al., 2002). In addition, the RMSD difference between the models and X-ray structures on the basis of the Z-score was carried out using PROSA (Wiederstein and Sippl, 2007). Finally, vimentin model was assessed using PROSA (Wiederstein and Sippl, 2007) webserver and compared against the MODELLER's energy score function.

Supercoiling of helical strands was evaluated according to the coiled-coil phase yield per residue (ω_n) of the restrained vimentin model. ω_n was calculated using TWISTER and is determined from the average (over both chains) of the mean of dihedral angles formed about the helix and superhelix axes.

Molecular Dynamics Studies.—Molecular dynamics (MD) simulation was performed for the wild-type vimentin and for the 224 MTSL variant vimentin using the hybrid X-ray structures (3UF1 and 3KLT) and EPR restraint L1-2 linker model. All simulations were designed to mimic physiological conditions (e.g. neutral pH). AMBER ff14SB parameters (Maier et al., 2015) were used for amino acids and parm10.dat in AMBERTOOLS16 (Case and D.S. Cerutti, 2017) was used for cysteine moiety of MTSL and the nitroxide moiety parameters taken from the work of Stendardo et al (Stendardo et al., 2010). MTSL topology was created using the ANTECHAMBER. The model was solvated in a box of TIP3P water (183,879 water molecules in the wild type system and 183,650 water molecules in the 224 MTSL variant system). Appropriate counter ions (Na⁺ or Cl⁻) were placed to obtain a neutral system. The system was step-wise minimized. Vimentin was fixed for 3000 steps of Conjugate Gradient (CG) minimization to relax the surrounding water molecules. Next the protein was minimized with 1000 steps of CG, while fixing the water. A final minimization with 1500 steps of CG was carried out to relax the whole system. The CUDA version of NAMD2.12 program (Phillips et al., 2005) was used for the minimization process.

The system was heated incrementally by increasing the temperature from 10K to 298K over 30 picoseconds. It was further equilibrated for 120 picoseconds at a constant pressure of 1 atm using Berendsen coupling method (Berendsen, 1984) and constant temperature of 298K using the reassign-hold method (Phillips et al., 2005). During the entire equilibration procedure, the vimentin was restrained with a harmonic potential using a force constant of 2 kcal/mol/Å² in order to maintain the model. This was done to maintain side chain conformation of the heptad repeat patterns as observed in the X-ray protein crystallography. At the production phase, wild type vimentin restraints were released, temperature was maintained at 298K and 1 atm pressure was held constant using a Berendsen coupling method (Berendsen, 1984). In addition, two simulations were carried out using a wild type vimentin model or a 224 MTSL variant model with the addition of harmonic restraint at the *a,d* heptad positions using a force constant of 10 kcal/mol/Å², as well as geometric restraints on the N-terminal and C-terminal ends using a force constant of 0.01 kcal/mol/Å² and an 0 Å displacement (RMSD and rotation). The restraints were implemented using COLVAR module (Fiorin et al., 2013) integrated in NAMD2.12. Periodic boundary conditions were applied throughout the simulations. Long range electrostatic interactions were treated with particle mesh Ewald [PME] method (Darden et al., 1993) and a switching function was employed between 9 to 12 Å to gradually reduce the electrostatics and van der Waals

interactions (Phillips et al., 2005) to zero. The *verlet algorithm* was used for time integration with a time step of one femtosecond. Three independent simulations, each of fifty nanoseconds, were performed for each of the three proteins (wild type without restraints, wild type with restraints, and 224 MSTL variant with restraint). Snapshots were collected every ten picoseconds during these simulations for later analysis.

D257Q, D259G, K262C vimentin mutant variants and a homology model of keratin 14/5 were also created *in silico* using the same procedure described for the vimentin Molecular Dynamics study. Computed Bfactor over the simulations were computed using the following equation:

$$Bfactor(i) = \frac{8}{3}\pi^2 \sqrt{\frac{1}{N} \sum_{j=1}^N (r_i^j - \langle r_i \rangle)^2}$$

where r_i^j refers to the position of atom i at timeframe j , $\langle r_i \rangle$ is the mean position of the same atom i over the whole simulation, and the summation extends over all N timeframe considered.

The frequency of backbone (N to O) hydrogen bond lost was determined using the following approach. A TCL script is used to produce PDB snapshots from the MD simulation trajectory. Then each PDB snapshot is ran through the find-pairs PYMOL function to generate all backbone hydrogen bonds. Lastly, all backbone hydrogen bonds are filtered using a custom bash script calculated over a 10-residue window with the Hydrogen bond lost frequency computed as $HB_{lost} = (H/20) - 1$. H is identified by the number of actual backbone hydrogen bonds using a cutoff distance of 3.2 Å with an angle of 45 ° in each snapshot normalized to the 20 possible backbone hydrogen bonds for 10 residues within both chains of the vimentin dimer.

For the above equations, TCL scripts were written and analyzed in the VMD1.9.3 (Humphrey et al., 1996) interface. Visualization and rendering are done using either PYMOL (Schrödinger, LLC) or Blender (<http://www.blender.org>) program. Plots are generated using MATLAB (The MathWorks, Inc.) platform.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification and statistical analysis of electron paramagnetic resonance data and molecular modeling and the determination of structures by electron paramagnetic resonance are integral parts of existing algorithms and software used which are described in Method Details.

DATA AND CODE AVAILABILITY

Coordinate files for the vimentin models and scripts written for this study are readily available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- EPR-based restraints for backbone structure and optimizing models of protein assemblies
- L1-2 is mostly helical with the two chains forming a heterogenous structure
- L1-2 through rod 2A contains distinct levels of order and lacks supercoiling
- Assembled model provides basis for exploring IF dynamics and assembly

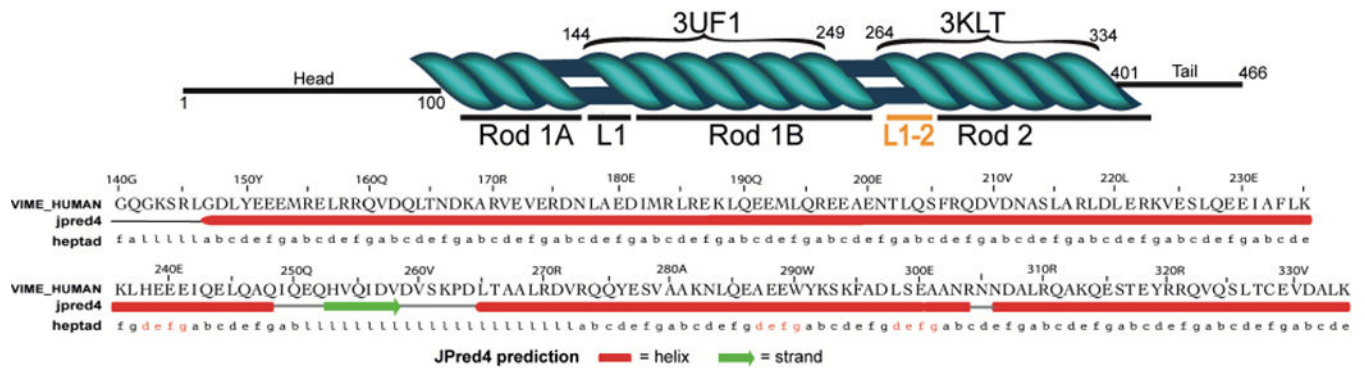


Figure 1.

Schematic diagram of Vimentin sequence, secondary structural prediction, and EPR/X-ray structural analysis. The top diagram provides an overview of the structural regions within the rod domain, along with the fragments that have been defined by X-ray crystallography. The orange color highlights the region analyzed by EPR spectroscopy for this study. The bottom portion provides the primary sequence of Human Vimentin within the rod domain presented in the top line. The Jpred4 secondary structure prediction is provided in the second line. The third line provides the heptad assignment of residues by the TWISTER program, with the L1-2 positions designated as (l), and residues part of a hendecad repeat shown in red.

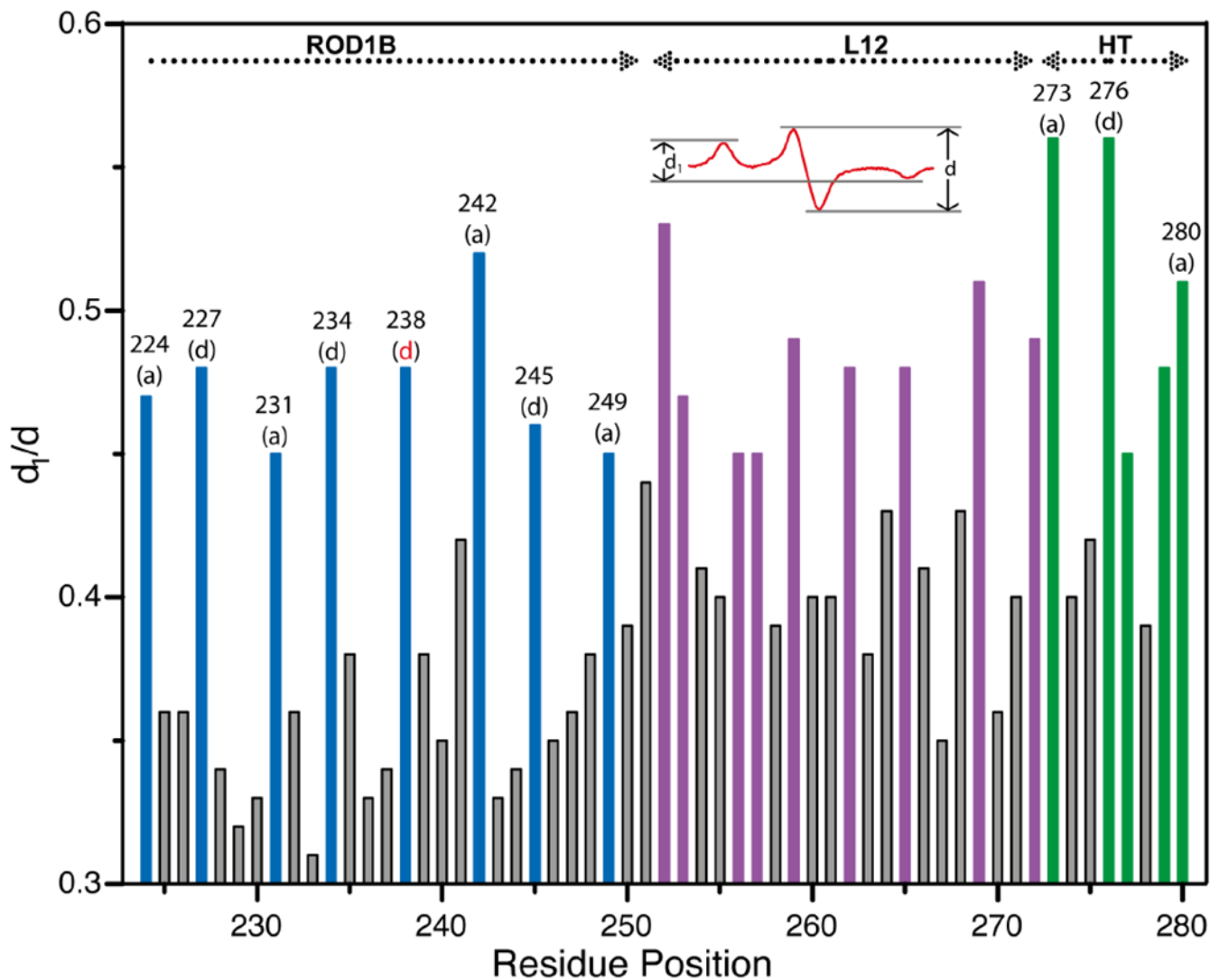


Figure 2.

Plot of the d_1/d values vs sequence position for residues 224-280 in human vimentin. The heptad residues in rod 1 domain (224-250) with $d_1/d > 0.44$ are highlighted in blue, with the a,d heptad position indicated (the d position from the hendecad instance shown in red). Linker 1-2 (L1-2) residues (251-272) with $d_1/d > 0.44$ are shown in magenta. Sites within the intervening residues ("rod 2A"; 273-280) between L1-2 and L2 with $d_1/d > 0.44$ are shown in green, with the predicted a and d positions of the heptad indicated.

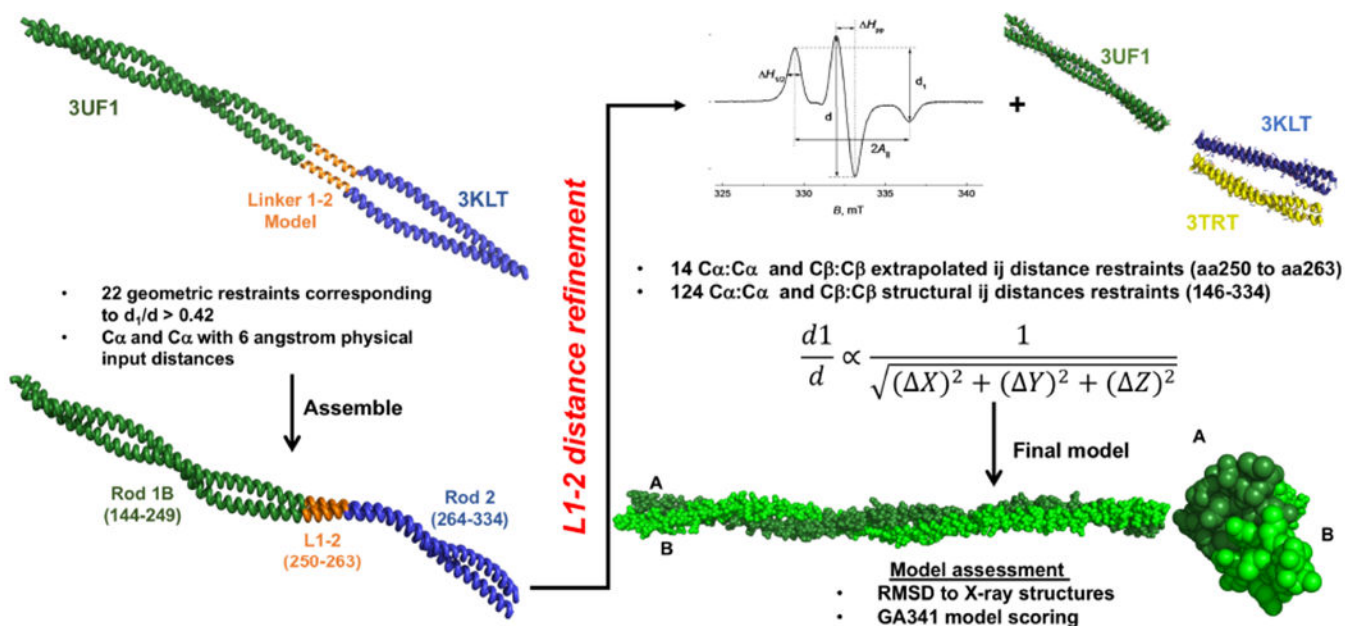


Figure 3. Model building of Vimentin Rod1B (3UF1:146-249), L1-2 (250-263), and Rod2B (3KLT: 264-334). The construction of the full-length rod domain was assembled using the two protein X-ray structural coordinates: 3UF1 and 3KLT. This includes the Vimentin Linker 12, using UCSF CHIMERA with rotate and translate of 3UF1 and 3KLT to Vimentin Linker 1-2, while MODELLER optimized the full-length rod domains. The full-length “restrained model” underwent an additional optimization using the X-ray structural $C\alpha$ - $C\alpha$ and $C\beta$ - $C\beta$ correlation to EPR-derived d_1/d values from throughout the central rod region (a total of 124 out of 129) from this study and previous work.

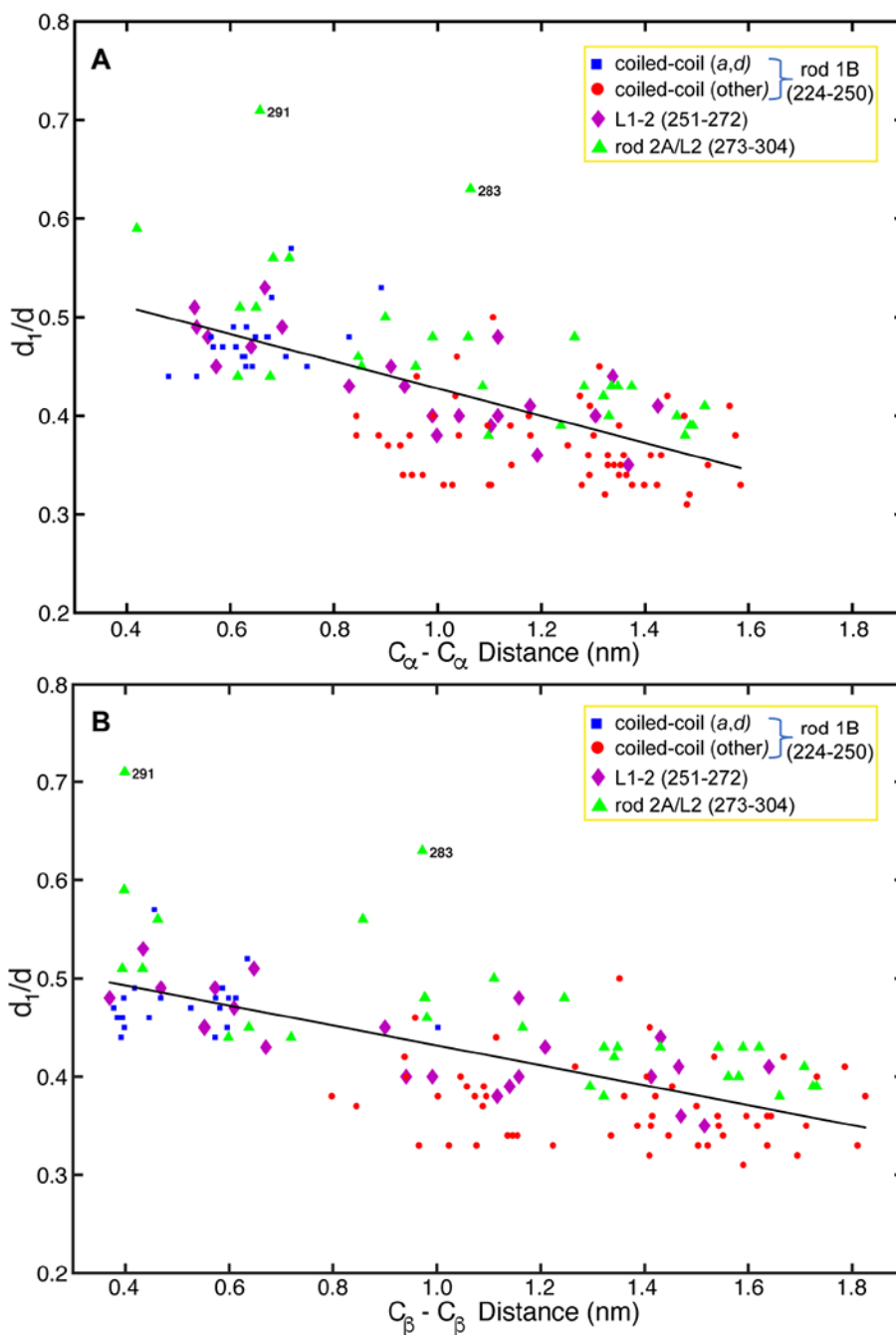


Figure 4. Correlation between distances in the model and EPR results. For each sequence position in the vimentin dimer, the distance between the A and B chains in the model are plotted according to its EPR-measured d_1/d value. The correlation of d_1/d to both the C_α - C_α distance (A) and the C_β - C_β distance (B) is shown. In each graph, the d_1/d values for 129 spin labeled positions are plotted against side chain distances with symbols indicating structural domain assignment. For coiled-coil regions, distances are defined by the X-ray structures 3UF1 and 3KLT. The distances for the residues in linker regions are obtained from

the optimized model. The Pearson correlation coefficient indicates a strong linear relationship between $C\alpha-C\alpha$ and d_1/d ($p < 10^{-15}$), and the correlation is even stronger when using the $C\beta-C\beta$ distance ($p < 10^{-17}$), which can better account for sidechain projections. Positions 283 and 291 are highlighted due to their unique stability properties (Hess et al., 2006).

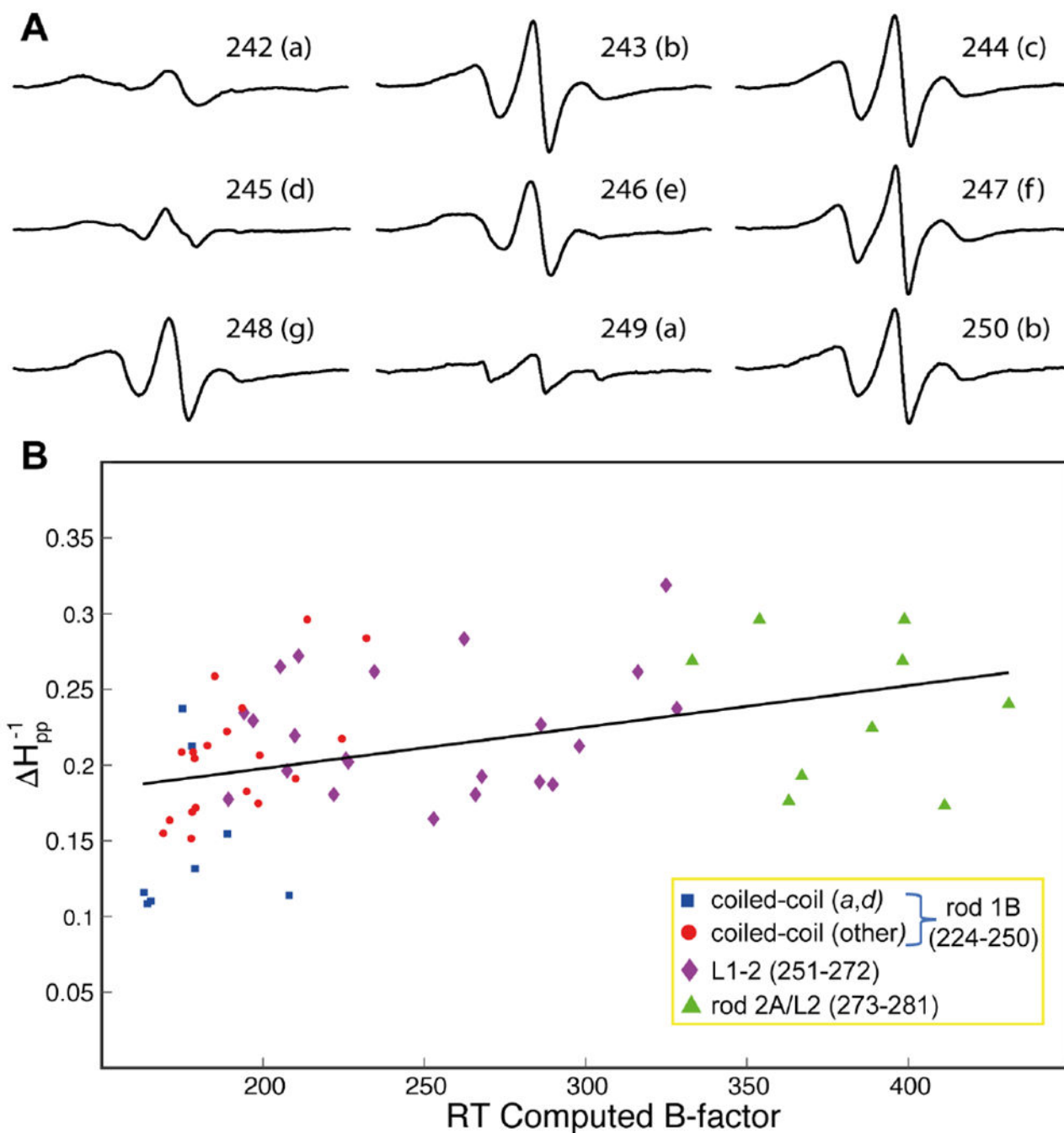


Figure 5.

Relationship of computed dynamics to the dynamics of spin-labeled side chains. Panel (A) shows solution EPR spectra of vimentin acquired at room temperature. Scan width is 100 G. The letters in parenthesis represent the location of the residue within the heptad. Panel (B) shows the relationship between the H_{pp}^{-1} (see Fig. 1) values of the solution EPR data to the room temperature (RT) computed B-factor parameters of the model. Fifty-eight data points of H_{pp}^{-1} were compared against the computed B-factor of the restrained model over a 50 nanoseconds Molecular Dynamics simulation to determine a relationship between the

two physical parameters. The symbols for each position are distinguished according to structural domain assignment.

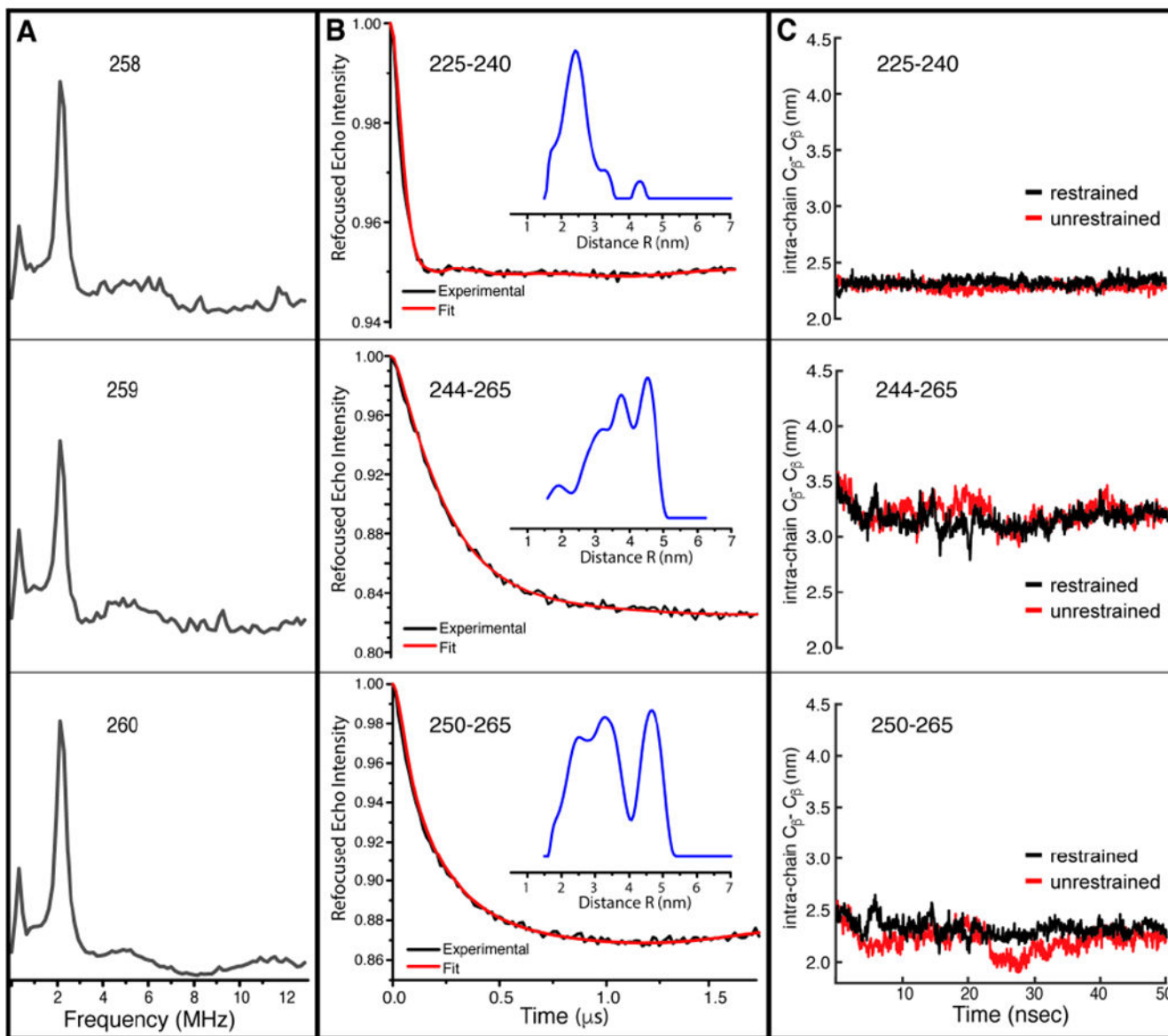


Figure 6.

Pulsed EPR data of vimentin L1-2 region. Panel (A) shows the three-pulse ESEEM frequency domain data of vimentin containing a deuterated (d_{10}) Ile256 paired with nitroxide spin labels placed at positions 258, 259 or 260. Weak dipolar couplings between the ^2H nuclear spin and the nitroxide are detected for all three spin label locations ($i+2$, $i+3$, $i+4$). Time domain data and $C\beta$ - $C\beta$ distance calculations from MD simulation are shown in Fig. S7. Panel (B) shows the 4-pulse Q-band DEER data for the indicated spin pairs introduced into vimentin. Decays represent the background-subtracted dipolar evolutions of the spins, with the distance probability distributions from Tikhonov regularization given in the inset. To limit intermolecular interaction of spin labels, each double-labeled protein was spin-diluted with unlabeled wt vimentin at a ratio of 1:5 (labeled:unlabeled). Panel (C) shows the average distance between $C\beta$ positions in model over 50 nsecs of MD simulation.

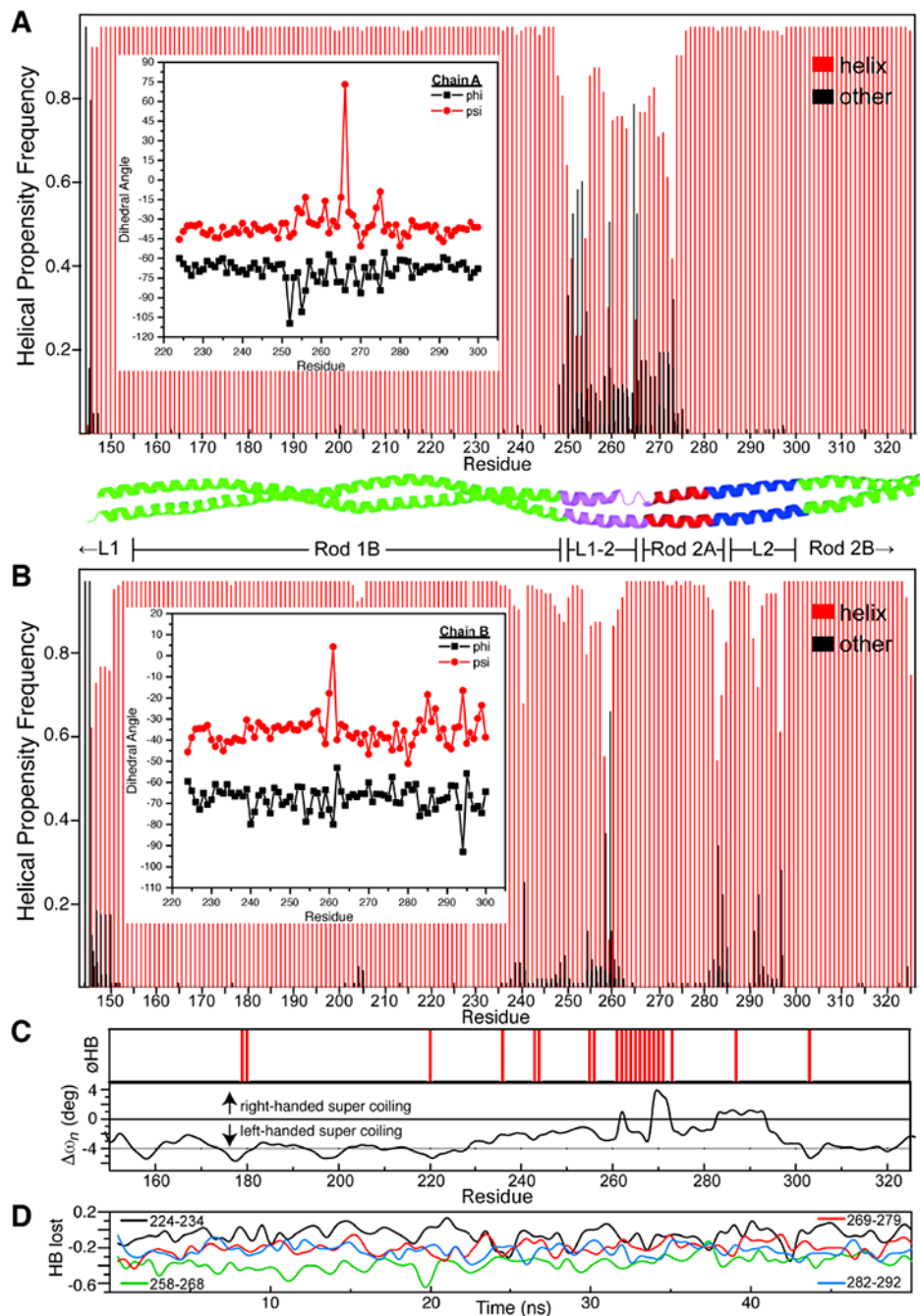


Figure 7.

A quantitative definition of structural domains within vimentin. The mixed α -helical distortion was structurally characterized based on 100 frames of 50 nsecs MD trajectory, and coordinates entered into the DSSP program. The α -helical frequency was computed for every position in the vimentin sequence range of 144 to 325 and plotted for chains A and chain B (panels **A** and **B**, respectively). Chain A shows greater helical distortion in the L1-2 region, whereas chain B shows greater helical distortion in the rod 2A/L2 region. The uniqueness of L1-2 is further identified by dihedral angle variation (insets of panels **A** and

B). Panel **C** reports the coiled-coil phase yield (ω_n) per residue as a function of residue number for the restrained vimentin model calculated by TWISTER. The phase yield value of an ideal left-handed coiled-coil (-4) is indicated by the gray line. The location of residues lacking backbone H-bonds (\emptyset HB) is shown in the upper sub-panel. A plot comparing the frequency of H-bonds lost for the distinct regions is shown in panel **D**. Plotted are the frequencies for the rod 1B (224-234), L1-2 (258-268), rod 2A (269-279) and L2 (282-292) regions over 50 nsec of MD simulation.

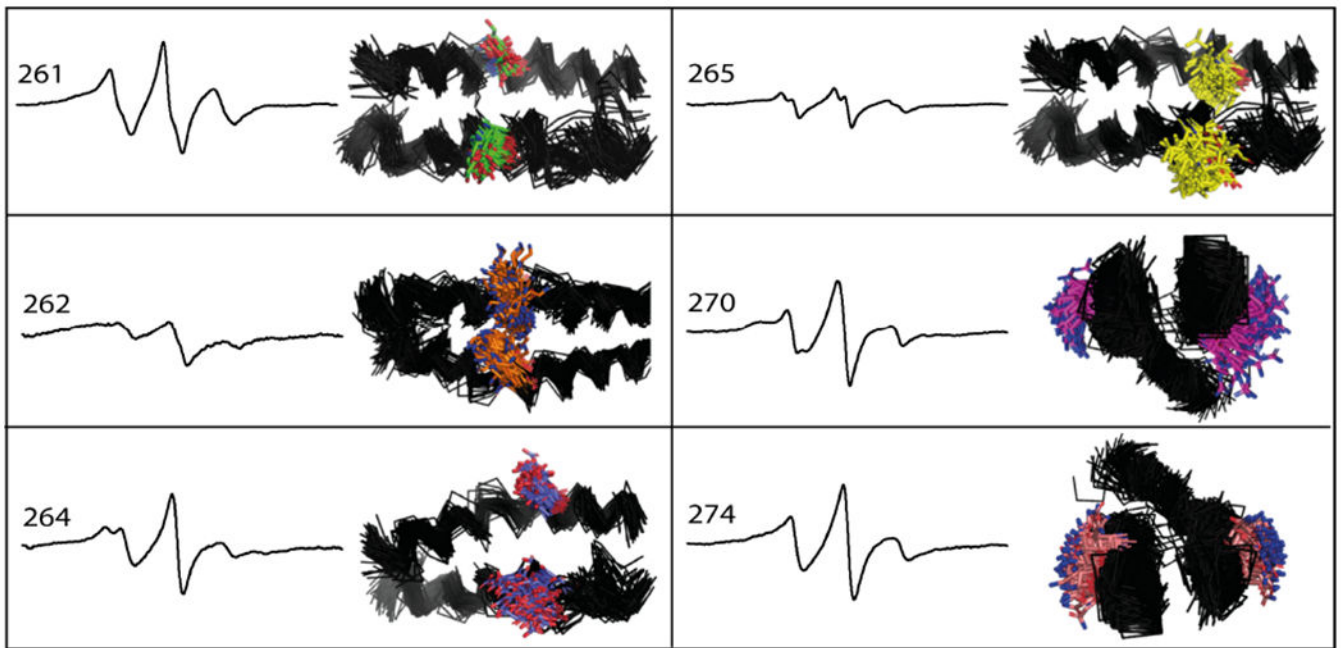


Figure 8.

Side chain motions show distinct two conformational motions that correlate with two-component EPR spectra. Side chain dynamics were structurally characterized based on 100 frames of 50 nsecs wild type vimentin MD trajectory.

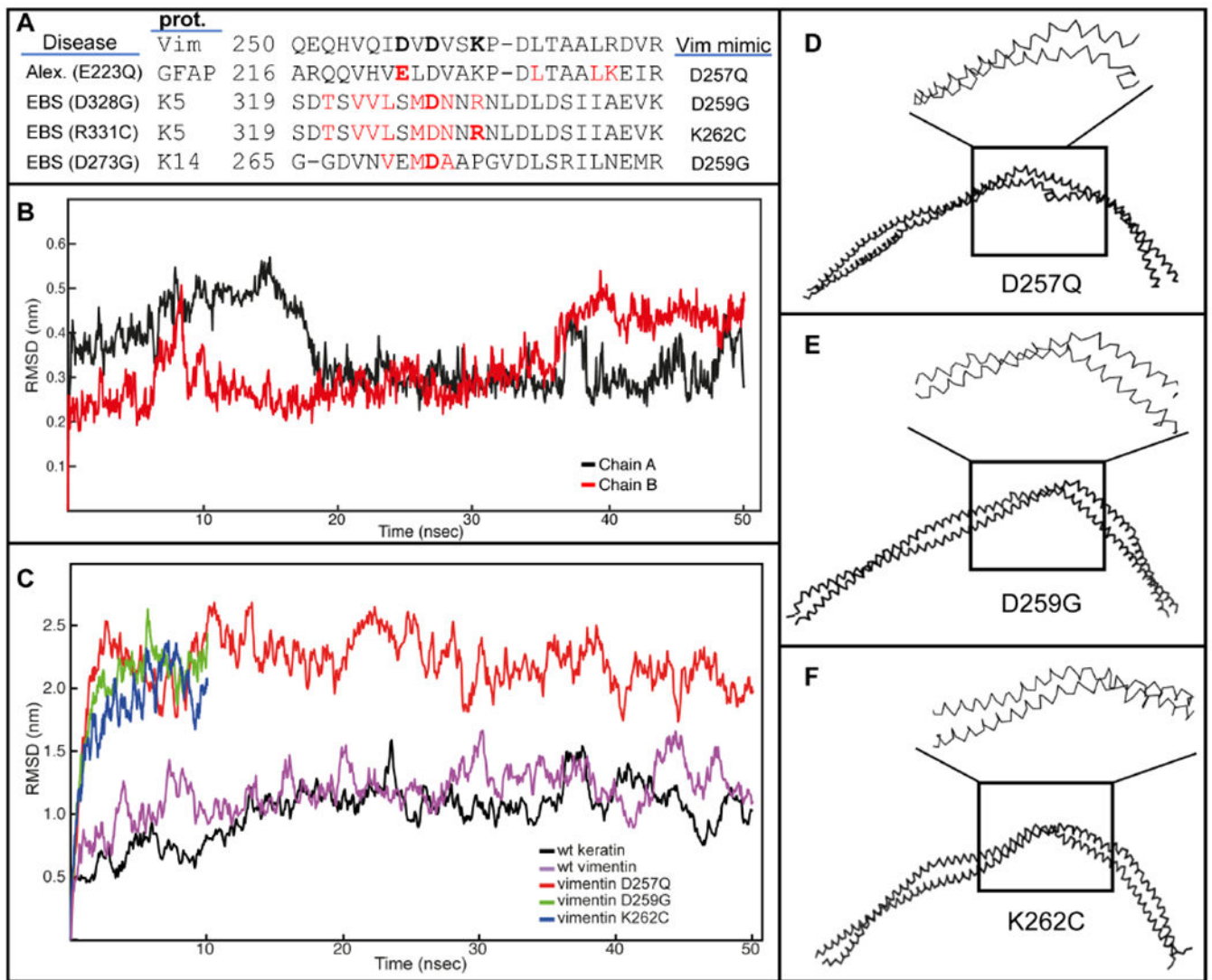


Figure 9. Consequences of mutations in the L1-2 region of IF proteins explored by MD simulation. (A) Sequence alignment of L1-2 region of human IF proteins. Mutations within L1-2 in GFAP and K5/14 that have been linked to human disease are shown in red. Positions used for disease mimic in vimentin are shown in bold. (B) MD simulation of L1-2 A and B chains in wt vimentin reveals a switching of helical distortion between the chains, revealing a dynamic asymmetry. (C) Mimics of disease-associated mutations induce global changes in IF structure, suggesting the L1-2 region resides near a conformational threshold. RMSD calculations were performed for the D257Q, D259G, K262C mutations and compared to wild type keratin 14/5 and vimentin. (D-F) Snapshots of the MD simulations show the L1-2 region becomes distorted and bent upon introduction of the disease-causing mutations.

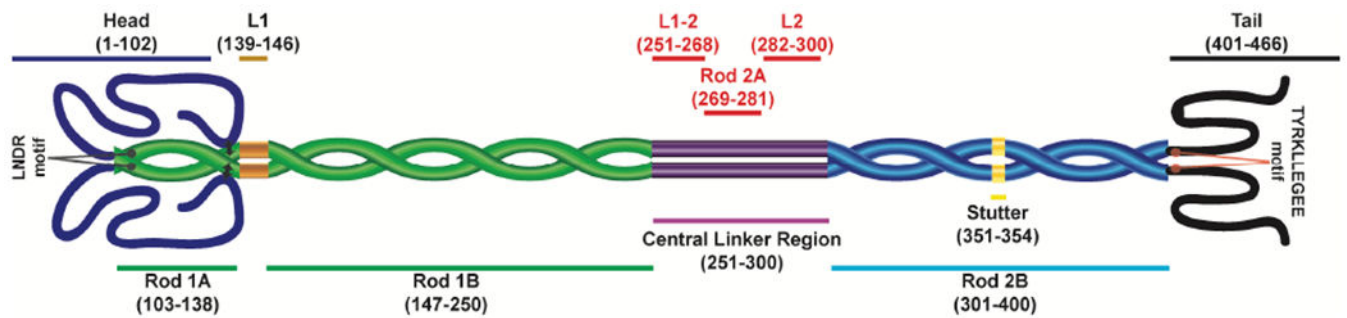


Figure 10.

Revised structural map of the central linker region in vimentin. The revised boundaries of Linker 1-2 (L1-2), rod 2A and Linker 2 (L2) are indicated. These compare to the historical designations (c.f. (Herrmann and Aebi, 2004)) of 248-263 for L1-2, 264-282 for rod 2A and 283-290 for L2. EPR restraints provided in this study span from the middle of rod 1B through Rod 2A. Previous EPR studies revealed L2 is comprised of ridged straight helices (Hess et al., 2006). The coiled-coil structure of the rod 1A/B and rod 2B segments is indicated, with definitions established by previous EPR and X-ray crystallography studies: rod 1A (Aziz et al., 2009; Meier et al., 2009), Rod 1B (Aziz et al., 2012; Chernyatina et al., 2012) and rod 2B (Hess et al., 2006; Hess et al., 2002; Nicolet et al., 2010; Strelkov et al., 2002). Also noted is the LINDR motif at the beginning of rod 1A, a site in IF proteins associated with congenital skin disease (Hess et al., 2005; Omary, 2009), as well as the stutter sequence in rod 2B (Brown et al., 1996; Strelkov et al., 2002). The structures of the head and tail domains remain unknown, however aspects such as the localization of the head to rod 1A ((Aziz et al., 2009); black double arrows) and the level of structural order in the tail (Hess et al., 2013) have been determined. The YTRKLLLEGEE motif at the beginning of the tail is also noted, as it is conserved among IF proteins (Herrmann et al., 2000; Parry and Steinert, 1999) and shows a close proximity among the acidic residues in each strand (Hess et al., 2013).

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>E. coli</i> BL21 (AI)	Invitrogen	1665055EDU
pT7	Invitrogen	720-0021
Chemicals, Peptides, and Recombinant Proteins		
iProof DNA polymerase	Bio-Rad	1725301
1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl Methanethiosulfonate (MTSL)	Toronto Research Chemicals	A167900
Source S	GE Healthcare	17117801
Source Q	GE Healthcare	17117701
Oligonucleotides		
Oligonucleotides for site-directed mutagenesis (typically 25-35 nucleotides long) were synthesized by Invitrogen. Sequences were identical the vimentin DNA sequence with the exception of the mutated position, at which position the codon was changed to TGC.	Invitrogen	NA
Equipment		
CM-120 electron microscope, biotwin Lens	FEI	Philips CM120
Gatan MegaScan 794/20 digital camera	2K X 2K	Model 794/20
Gatan Bioscan 792	Gatan	Model 792
JEOL X-band spectrometer	JEOL-USA	FA-100 and TE100
AKTA FPLC	Amersham Biosciences	UPC-900
Software and Algorithms		
AMBERTOOLS16	Case et al.	https://ambermd.org/
Modeller	Sali et al.	https://salilab.org/modeller/
Matlab	Mathworks, Inc.	https://www.mathworks.com
NAMD (CUDA)	Phillips et al.	https://www.ks.uiuc.edu/Research/namd/
Pymol	Schrodinger, Inc.	https://pymol.org/2/
PROSA	Wiederstein et al.	https://prosa.services.came.sbg.ac.at/prosa.php
TWISTER	Strelkov et al.	https://gbiomed.kuleuven.be/english/research/50000715/50000719
UCSF Chimera	Pettersen et al.	https://www.cgl.ucsf.edu/chimera
VMD	Humphrey et al.	https://www.ks.uiuc.edu/Research/vmd/current/
ALPHASURF	Edelsbrunner et al.	http://web.cs.ucdavis.edu/~koehl/
Model building and refinement scripts	This paper	jcvoss@ucdavis.edu
MD analysis scripts	This paper	jcvoss@ucdavis.edu
Deposited Data		
Structure coordinate source	Aziz et al., 2012	PDB ID: 3UF1
Structure coordinate source	Nicolet et al., 2010	PDB ID: 3KLT
Structure coordinate source	Pang et al., 2018	PDB ID: 5WHF
Structure coordinate source	(Chernyatina and Strelkov, 2012)	PDB ID: 3TRT

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Structure coordinate source	(Chernyatina et al., 2012)	PDB ID: 3SSU
Structure coordinate source	(Chernyatina et al., 2012)	PDB ID: 3SWK

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