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Keratin 1/10-1B tetramer structures reveal knob-pocket mechanism in intermediate filament assembly

Sherif A. Eldirany, Minh Ho, Alexander J. Hinbest, Ivan B. Lomakin, and Christopher G. Bunick

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 7th Nov 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you can see, both referees express interest in the proposed mechanism of keratin tetramer assembly. However, they also raise concerns that need to be addressed in full before we can consider publication of the manuscript here. Most importantly, referee #2 would like to see whether full-length versions of keratin that carry mutations in the contact interfaces would also display defects in tetramer assembly, similar to the data already presented based on shorter versions of the protein. I find this request reasonable and it will significantly strengthen the manuscript.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS:

Referee #1:

In this contribution, Eldirany and colleagues report the atomic structure of the heterodimeric coiled coil formed by recombinant fragments representing coil 1B of the alpha-helical rods of keratin K1 and keratin K10 within a tetrameric complex. In addition, they follow the in solution complex formation of these fragments by Size Exclusion Chromatography and Multi-Angle Light Scattering. It is a solid piece of work with mutagenesis and detailed analysis.
The two full-length proteins represent two major intermediate filament (IF) components expressed in the upper layers of epidermis. This reports follows a previous publication of the same group on the structure of coil 2B published in the Journal of Investigative Dermatology. Given the fact that

coiled coils of this size are hard to crystalize and that up to now only structures for vimentin (coil 1A, coil 1B and coil 2), keratin K5 and K14 (coil 2B) and the nuclear lamin A (coil 2B) are available, this is an important contribution in order to understand if the various classes of IF proteins follow the same structural principals to form bona fide IF. In addition, the authors present the structure of an octameric complex where one of the partners, K1, carries a missense mutation (S233L) that causes a severe skin disorder, i.e., epidermolysis palmoplantar keratoderma. This is new and gives insight into how such a disease mutation causes aberrant assembly of a K1/K10 IF. Furthermore, the authors have spotted four residues in K10 supposed to form a "stripe" on the alpha-helix and that are further assumed to engage in hydrophobic interactions with K1 needed for tetramer formation.
At first glance, the authors only thoroughly repeat the "divide-and-conquer" strategy as employed previously for vimentin by the groups of Fitzgerald and Strelkov (JBC and PNAS 2012), as well as in a publication the authors, unfortunately, do not cite and discuss (Allen H. Pang et al. (2018) FEBS Journal 285, 2888-2899). In principle, very similar structural arrangements are observed, which is in itself very revealing due to the rather high differences in the amino acid sequences of the three proteins (vimentin, K1. K10). The authors derive models of the octameric complexes of these segments and thus give information how the principal structures, the "protofibrils" (see Aebi et al. 1983 JCB), may arrange. Moreover, they provide models that exhibit how the hydrophobic clusters - or "knobs" engaged in these interaction relate to corresponding segments in keratin K8/K18 and in vimentin. As a side point, it is nice to see the chemical crosslinks found in pioneering work by Peter Steinert nicely fit into the modeled structures.
Having said this, it may be of great interest to overlay and compare the K1 and K10 tetramer structure with that of vimentin to exactly determine if and how the structures differ. Another point that may be worth to be considered in the discussion is the fact that for vimentin both coil 1A and coil 2A segments have been demonstrated by hydrogen-deuterium exchange mass spectrometry to be essentially involved in tetramer formation. Hence, isolated fragments such as coil 1 may indeed exhibit some kind of "eigenleben" as seen for instance with specific lamin A fragments (see L. Kapinos et al. 2011 JMB).

Minor points:
In their IF scheme in Figure 8, the authors entertain a filament with seven protofibrils. However, the STEM data of Alasdair Steven and colleagues (1982, 1983) provided strong evidence that reassembled authentic epidermal keratin IF contain, not considering a certain degree of polymorphism, four protofibrils. See also the data of Herrmann et al. (JMB 1999), which suggest that in vitro assembled K8/K18 IF contain two, three and four protofibrils are present.

Referee #2:

High resolution Information about the assembly and structure of intermediate filaments (IFs) is lacking in general, and for keratins in particular. Here the authors report on the structure (with atomic resolution) of a complex involving the 1B subdomains of K1 and K10, a keratin pairing that is preferentially expressed in the differentiating layers of epidermis and related cornifying epithelia. What makes the study of significant interest is that, given the packing of coiled-coil heterodimers in the crystal lattice generated, the authors are able to infer a substantial amount of new information of significance regarding the formation of tetramers, which represents a key intermediate during IF assembly. They also solved the structure of a K1-K10 complex in which the K1 subunit is mutated a position 233 (S233L), a change that is not only causative for the genetic disorder epidermolytic palmoplantar keratoderma (EPPK) but also inferred to be extremely disruptive to 10 nm filament assembly.

Altogether this is a well-executed, substantive and well-interpreted study that represents a significant step ahead towards our understanding of the assembly of IF polymers, keratin in particular.

Whereas the authors should be commanded for conducting extensive mutagenesis experiments to test the implications of the information inferred from these crystal structures, they do so in the context of the relatively short domains that they used for crystallization trials, and NOT in the context of the full-length K1 and K10 proteins. It would be highly desirable to find out what the

abrogation of key contact interfaces predicted to be important for tetramer formation (given the structural models generated here) would result in if/when tested in the context of full-length proteins through in vitro filament assembly assays with purified proteins, and/or in when transfected in epithelial cells in culture. Such studies would be highly desirable not only for rigorous testing of the WT K1-K10 model but also with the mutant structure associated with EPPK.

Minor points worth addressing:

The authors may wish to better recognize that: 1) The understanding of the early stages of IF assembly goes well beyond being "general" at this stage, based on a variety of biophysical data sets including X-ray crystallography; 2) There is quite a bit of X-ray crystallography-based insight on the structure of IF subunits, particularly for vimentin (type III) and lamin (type V) assemblies; 3) Bernot et al. (2005) were able to predict and formally test for the importance of a "hydrophobic stripe" present within subdomain 1B of many but not all type I keratins (the authors' account of this particular study is superficial and a bit incomplete); 4) the crosslinking data sets generated by Peter Steinert, in particular, are now proving immensely relevant and important. Moreover, the notion that there is no structural insight is available for any of the axial alignments originally proposed by Steinert is incorrect - they is structural insight available at least for the ACN one (check out the lamins).

The authors do not supply information about which buffers were used for their gel filtration studies.

It seems unfortunate that the presence of cadmium in the crystallization buffer for one of the complex solved but not the other seemingly introduces a set of "distractions" that take away from the ability to directly compare the WT K1-K10 1B domains with the mutant one.

1st Revision - authors' response 2nd Feb 2019

Thank you for the opportunity to revise our manuscript entitled, "**Keratin 1/10-1B tetramer crystal structures reveal knob-pocket mechanism in intermediate filament assembly**." We were very encouraged and excited by the positive reviews our manuscript received. As requested, we have addressed all the Editor and Reviewer comments. I present our response to the major request first, then respond in order to all the other suggestions. Our responses are as follows:

Editor Comment:

"Most importantly, referee #2 would like to see whether full-length versions of keratin that carry mutations in the contact interfaces would also display defects in tetramer assembly, similar to the data already presented based on shorter versions of the protein. I find this request reasonable and it will significantly strengthen the manuscript."

Referee #2 Comment:

"It would be highly desirable to find out what the abrogation of key contact interfaces predicted to be important for tetramer formation (given the structural models generated here) would result in if/when tested in the context of full-length proteins through in vitro filament assembly assays with purified proteins, and/or in when transfected in epithelial cells in culture. Such studies would be highly desirable not only for rigorous testing of the WT K1-K10 model but also with the mutant structure associated with EPPK."

Author Response:

We agree with the Editor and Reviewer #2 on the importance of evaluating our knob-pocket mechanism in the context of full-length keratin 1/10 filaments. We are happy to report that we have performed electron microscopy analysis on wild-type and mutant full-length keratin 1/10 filaments and found that the FLAA knob mutation in keratin 1 has a detrimental impact on K1/10 filament formation. To further establish the validity of the knob-pocket mechanism described in our

manuscript, we also repeated the electron microscopy analysis for a different keratin system (keratin 8/18) and a Type III intermediate filament system (vimentin). We found that FLAA knob mutation in K8 had a detrimental impact on K8/K18 filament formation. Similarly, we found that FLAA knob mutation in vimentin negatively impacted vimentin filament formation. Taken together, we believe the data on full-length K1/10, K8/18, and vimentin filaments firmly establishes the knob-pocket mechanism described in this manuscript as a critical player in intermediate filament assembly. We thank the Editor and Reviewer #2 for pushing us to perform these experiments; we feel they add significant value to the manuscript and mark a major step forward for the intermediate filament field.

This work has been incorporated into the main text as a new, final section under **Results**:

"Electron microscopy demonstrates knob mutation is detrimental to IF assembly in three IF systems

To take our mutation analysis further, we determined whether the K1^{FLAA} mutation could also affect intermediate filament assembly of full-length proteins (recombinantly produced and purified). To assess intermediate filament assembly, negative-stain electron microscopy was used (Fig. 6). Full-length wild-type K1 and K10 were assembled into K1/K10 filaments under identical parameters and conditions (e.g. 10 minutes assembly time) as full-length K1^{FLAA} mutant with K10. The K1^{FLAA} mutation caused a significant reduction in the number and length of filaments formed (Fig. 6a). The fewer, shorter filamentous structures visible for K1^{FLAA} suggest that loss of the anchoring knob in K1 generates instability at the tetramer and unit-length-filament level that precludes formation of normal wild-type filaments.

Since multiple sequence alignment and homology modeling suggested the anchoring knob is conserved among type II and type III IFs (see Figs. 7 and 8 and Discussion), we additionally examined whether FLAA mutation altered filament assembly in two other IF systems (K8/K18 and vimentin). Full-length recombinant wild-type K8 and K18 were assembled into K8/K18 filaments under identical parameters and conditions as full-length K8^{FLAA} mutant (F223A+L227A) with K18. K8^{FLAA} had similar effect as K1^{FLAA} on filament formation, causing a reduction in number and length of filaments formed (Fig. 6a). Full-length recombinant vimentin (which forms homodimers) was assembled into vimentin filaments under identical parameters and conditions as full-length vimentin^{FLAA} mutant (F233A+L237A). Vimentin^{FLAA} was unable to form short or long filamentous structures, and appeared arrested at the unit-length-filament stage of IF assembly. In summary, EM studies demonstrate that loss of knob structure has a damaging impact on IF assembly (the rate of and/or the length of) across IF types (type II vs type III), for keratins with long heads and tails (K1/K10), for keratins with short heads and tails (K8/18), and for heterodimeric and homodimeric (vimentin) IF proteins (Fig. 6c)."

A new Figure 6 has been added to show the electron microscopy data, with the previous Figures 6-8 now denoted Figures 7-9. The **new Figure 6 legend** reads:

"Fig. 6. Anchoring knob mutation disrupts IF assembly for three IF systems. (a) Negative-stain electron microscopy (EM) images comparing wild-type (WT) and anchoring knob mutant (FLAA) filament formation for full-length K1/K10, K8/K18, and vimentin. The duration of filament assembly was 10 minutes for all three IF systems. Double mutation (FLAA) of F314A+L318A in K1, F223A+L227A in K8, and F233A+L237A in vimentin causes detrimental effects on K1/K10, K8/K18, and vimentin IF assembly, respectively. The FLAA filaments are fewer in number and shorter in length despite IF assembly and EM protocols identical to the WT. These data strongly indicate the anchoring knob interaction with the hydrophobic pocket plays an important structural role in stabilizing the IF tetramer, unit-length-filament, and intact filament. The experiments were independently replicated twice. (b) Coomassie-stained SDS-PAGE demonstrating purified, recombinant wild-type (WT) and mutant (FLAA) full-length proteins for K1/K10 (left), K8/K18 (center), and vimentin (right) used in this EM analysis. (c) Illustration of the domain organization for the IF proteins in this EM experiment in order to highlight the differences in overall protein length, the length of the heads and tails, and the dimerization state (hetero- vs homo-). The location of the hydrophobic

pocket and anchoring knob at the N- and C-termini of helix 1B for K1, K8, and vimentin is noted."

The protocols for this work have been added into the **Materials and Methods** as:

"Electron Microscopy Analysis of Intermediate Filaments. pET-21a(+) based plasmids of human full-length wild-type K1, K1 containing F314A+L318A mutations, wild-type K8, K8 containing F223A+L227A mutations, wild-type vimentin, and vimentin containing F233A+L237A mutations were purchased from GenScript (Piscataway, NJ); wild-type K10 and K18 were similarly purchased in pET-24a(+) plasmid. K10 was expressed in *E. coli* BL21(DE3)pLysS cells (Invitrogen, Waltham, MA) at 20°C for 72hrs using an autoinduction method (Studier, 2005). Expression of all other keratins and vimentins occurred in *E. coli* BL21(DE3) cells using lysogeny broth at 37°C for 3hrs with 1mM IPTG for induction. An inclusion body pellet was purified from the cells using a previous protocol (Nagai & Thøgersen, 1987) modified to include sonication at each step of pellet resuspension. Inclusion bodies were resuspended in 6M urea solution and purified by anion exchange chromatography (Q/SP sepharose, GE Healthcare, Marlborough, MA) as described (Coulombe & Fuchs, 1990, Paladini, Takahashi et al., 1996) using a 200mM guanidine-HCl gradient, followed by size exclusion chromatography (Superdex 75, GE) using 6M urea solution. Heterodimeric complexes of K1/K10 and K8/K18, and homodimeric complex of vimentin, were made by mixing individual protein in a 1:1 molar ratio; the complexes subsequently were purified with Q sepharose using a 200mM guanidine-HCl gradient, and then dialyzed into 50mM Tris-HCl buffer (pH 8.5) containing 6M urea and 2mM DTT. Before initiating filament assembly, all IF complexes were concentrated to 0.49µg/µL and dialyzed into 25mM Tris-HCl buffer (pH 8.5) containing 9M urea and 2mM DTT at room temperature for 4hrs. K1/K10 filament formation followed established "Assembly method 4", whereas K8/18 and vimentin filaments were assembled from established "Assembly method 1" (Herrmann, Wedig et al., 2002). Filament assembly was terminated after 10min by adding stop buffer (0.2% glutaraldehyde, 20mM KCl, 0.7 mM Na_2HPO_4). Filament samples were immediately applied to a Carbon type B-400 mesh-Copper grid charged with Pelco easiGlow (Ted Pella, Redding, CA) at 25mA for 30s, and negatively stained using 2% aqueous uranyl acetate. Images were captured with a Talos L120C Electron Microscope from FEI (Hillsboro, OR)."

Referee #1 Comment:

At first glance, the authors only thoroughly repeat the "divide-and-conquer" strategy as employed previously for vimentin by the groups of Fitzgerald and Strelkov (JBC and PNAS 2012), as well as in a publication the authors, unfortunately, do not cite and discuss (Allen H. Pang et al 2018 FEBS Journal 285, 2888-2899).

Author Response:

We agree with Reviewer #1 on the importance of the "divide-and-conquer" strategy for studying intermediate filament structure by x-ray crystallography. This approach is in fact the only effective strategy to study IFs by x-ray crystallography at the current time. We previously discussed this for our K1/10-2B structure (Bunick and Milstone, 2017, *JID* 137:142-150). Both the "Fitzgerald 2012 JBC" and the "Strelkov PNAS 2012" publications were already cited in our manuscript, and these citations remain. We have added, however, a citation to the original 2001 Strelkov et al. paper describing the "divide-and-conquer" approach in the **Results/Wild-type K1/K10-1B structure** section as follows:

"Using the divide-and-conquer approach (Strelkov, Herrmann et al., 2001), the x-ray crystal structure of the human K1/K10 helix 1B heterotetrameric complex was determined to 3.0 Å resolution."

We thank Reviewer #1 for bringing the Pang *et al*. 2018 manuscript to our attention. The reason we did not cite this manuscript initially was because our submitted manuscript was actually written

prior to the publication date of Pang *et al*. 2018. We agree with Reviewer #1 that Pang *et al*. 2018 warrants citation and we rectify this; however, we have significant concerns over the methodology and conclusions in that manuscript. First, it is extremely important to separate the experimentally derived data from the hypothetical data in that study. Pang *et al*. crystallized vimentin-1B and therefore the experimental crystal lattice is defined by the molecular parameters/surface features of vimentin 1B. There is no guarantee that the vimentin-1B crystal lattice packing will faithfully recapitulate the tetrameric packing arrangements present in full-length vimentin filaments. Pang *et al.* even state in their own article "These staggered interactions of coil 1B with itself may not be present in a full-length vimentin filament." We agree with Pang et al. on this statement, because, from our own experience working with 1B and 2B coils from K1/K10, it is very difficult to discern what is crystal lattice artifact or biologically relevant filament packing. Conclusions on filamentous assembly, therefore, require a degree of biochemical, biophysical, and/or cell biology validation. Pang *et al*. do not acquire any de novo experimental data to validate their filamentous assembly conclusions. Moreover, our prior work on the K1/10-2B structure (Bunick and Milstone, 2017, *JID* 137:142-150) demonstrated that small sequence differences between IF types (e.g., K1/10 vs K5/14) can cause very different molecular surface chemistries structurally. Therefore, it is very unlikely that vimentin-2B will actually pack against vimentin-1B in the identical lattice configuration for 1B alone, because the molecular surface of 1B is not equal to 2B. Lastly, the model proposed by Pang *et al*. does not seem to incorporate or address the concept of pitch/spiraling which is in both KIF and vimentin models proposed by Peter Steinert (JMB, 1993; JBC, 1993).

We do not feel that our current manuscript is the appropriate location to include the details of the above discussion. While we may respectfully disagree with Pang *et al*. on some of the methodology and conclusions, we do appreciate and value their hard work and the difficulty of trying to understand intermediate filament assembly mechanisms. Importantly, we do agree with their assessment that the A_{12} mode of assembly is orthogonal to the A_{11} and A_{22} modes, which we arrived at independently and demonstrate in our model. Therefore, we have added a citation to Pang *et al* 2018 in the **second to last paragraph of the Discussion** as follows:

"The orthogonal relationship between the A_{12} mode and the A_{11}/A_{22} modes is supported by a recent vimentin-1B structure (Pang, Obiero et al., 2018). Our model also illustrates how the A_{12} interactions may cause the pitch or spiraling behavior observed in prior electron microscopy (Aebi, Fowler et al., 1983) and cross-linking studies (Steinert, Marekov et al., 1993a, Steinert, Marekov et al., 1993c)."

Referee #1 Comment:

It may be of great interest to overlay and compare the K1 and K10 tetramer structure with that of vimentin to exactly determine if and how the structures differ.

Author Response:

We have added a new Expanded View figure (Fig. EV4 in the revised manuscript) that compares the K1/K10-1B and vimentin-1B tetramer structures. We highlight some of the molecular surface differences between the structures. The figure legend for Fig. EV4 is:

"Figure EV4. Comparison of K1/K10-1B and vimentin-1B tetramer structures. (a) Sequence alignments of K1-1B and K10-1B each with vimentin 1B. There is 36% sequence identity between K1-1B and vimentin 1B (yellow), and 33% sequence identity between K10-1B and vimentin 1B (green). (b) The wild-type K1/K10-1B and vimentin-1B (PDB Code 3UF1) tetramer structures were superimposed and have a RMSD of 1.3 Å. (c) The keratin residues that are identical to vimentin-1B were mapped onto a molecular surface representation of the K1/K10-1B tetramer structure and colored yellow (K1-1B) or green (K10-1B). All K1/K10-1B residues not identical to vimentin-1B were mapped onto the molecular surface of the K1/K10-1B tetramer structure and colored red, demonstrating a significant proportion of the K1/K10-1B molecular surface will have differences in chemistry compared to vimentin-1B. (d) To

further illustrate how the molecular surface of the K1/K10-1B tetramer differs from vimentin-1B, both identical residues (lighter colors) and residues unique to K1 or K10 (darker colors) were divided into hydrophobic, positively charged, and negatively charged groups and mapped onto the K1/K10-1B tetramer molecular surface. The majority of the unique hydrophobic residues are located in the central region of the 1B domain, whereas the unique charged residues appear evenly distributed. Definitions of residues were acidic (D, E), basic (K, R), and hydrophobic (A, F, I, L, M, P, V, W)."

Referee #1 Comment:

Another point that may be worth considering in the discussion is the fact that for vimentin both coil 1A and coil 2A segments have been demonstrated by hydrogen-deuterium exchange mass spectrometry to be essentially involved in tetramer formation. Hence isolated fragments such as coil 1 may indeed exhibit some kind of "eigenleben" as seen for instance with specific lamin A fragments (see L. Kapinos et al. 2011 JMB).

Author Response:

We thank Reviewer #1 for bringing to our attention the excellent manuscript on vimentin structural dynamics from hydrogen-deuterium exchange by Premchandar *et al*. (JBC 2016). The most critical aspect of their paper, with respect to our manuscript, is their observation that "We observed strong protection [from H/D exchange] for nearly the entire coil 1B. The regions close to the N and C terminus of coil 1B barely began to register some exchange after 20 mins of incubation. These two regions are the most stable segments of the vimentin tetramer." (page 24934 of their paper).

We believe this finding from Premchandar *et al.* strongly supports and helps validate the data and conclusions in our manuscript. Our structural and mutational analysis on the knob-pocket mechanism now fully explains why the ends of coil 1B are so stable in the vimentin and keratin tetramers. Premchandar *et al*. referred to these ends of coil 1B as "structural anchors," but did not describe the atomic resolution mechanism behind the structural anchors. Our manuscript does just that. Moreover, the full-length K1/10, K8/K18, and vimentin filament electron microscopy studies we performed as part of the revision process validate that the knob-pocket mechanism is key for proper tetramer and filament assembly.

With respect to coils 1A and 2A, we agree with Reviewer #1. Our model suggests that coils 1A and 2A are involved in both tetramer assembly and filament assembly. Our interpretation of Premchandar *et al*. is coils 1A and 2A become stabilized the most during the transition from the tetramer to filament stage (Figure 2D in their paper). We do believe that future crystallographic studies should aim to understand the role of 1A and 2A coils in tetramer and filament formation. We also understand the importance of "eigenleben" raised by Reviewer #1. It is this very concept that led us to include in our Discussion comments about four vimentin-1B structures, all of which displayed some unusual characteristics: Two did not contain the pocket region, and two did not contain the knob region. In our case of 1B, we believe there is significant evidence that our observations are not artifact due to 1B being isolated from the rest of the filament. This includes the data within our initial submission, the H/D exchange data on coil 1B from Premchandar *et al*, and most importantly, the electron microscopy validation of the knob-pocket mechanism we performed at the request of Reviewer #2 and the Editor.

We incorporate the above discourse into the end of the **Discussion section** in our manuscript, as follows:

It is evident from our model, as well as in cross-linking studies (Steinert et al., 1993a, Steinert, Marekov et al., 1993b, Steinert et al., 1993c), that coils 1A and 2A also play a role in A_{11} tetramer stabilization and intermediate filament formation. This was confirmed by hydrogen-deuterium exchange experiments on vimentin filament assembly; the stability of coils 1A and 2A increased during filament formation (Premchandar, Mücke et al., 2016). Importantly,

hydrogen-deuterium exchange also identified the N and C termini of vimentin coil 1B as the most stable segments of the entire vimentin tetramer. The biochemical, structural, and electron microscopy data we present here provide a molecular basis for why this occurs in multiple IF systems: symmetrical knob-pocket interactions at the termini of coil 1B enhance tetramer formation and filament stability.

Referee #1 Comment:

In their IF scheme in Figure 8, the authors entertain a filament with seven protofibrils. However, the STEM data of Alasdair Steven and colleagues (1982, 1983) provided strong evidence that reassembled authentic epidermal keratin IF contain, not considering a certain degree of polymorphism, four protofibrils. See also the data of Herrmann et al. (JMB 1999), which suggest that in vitro assembled K8/K18 IF contain two, three and four protofibrils.

Author Response:

We agree with Reviewer #1. The model figure (now Figure 9) has been updated to reflect four protofibrils in an IF. In addition, we have modified the end of the **Figure 9 legend** to read as follows:

Together, the A_{11} and A_{12} modes of alignment are used by A_{11} tetramers to generate a protofibril; four protofibrils then assemble to form a 10-nm intermediate filament (Aebi et al., 1983, Herrmann & Aebi, 1999, Steven, Hainfeld et al., 1983, Steven, Wall et al., 1982).

Referee #2 Comment:

The authors may wish to better recognize that: 1) The understanding of the early stages of IF assembly goes well beyond being "general" at this stage, based on a variety of biophysical data sets including X-ray crystallography. 2) There is quite a bit of X-ray crystallography-based insight on the structure of IF subunits, particularly for vimentin (type III) and lamin (type V) assemblies. 4) the crosslinking data sets generated by Peter Steinert, in particular, are now proving immensely relevant and important. Moreover, the notion that there is no structural insight available for any of the axial alignments originally proposed by Steinert is incorrect – there is structural insight available at least for the ACN one (check out the lamins).

Author Response:

For point #1, we have removed the word "general" in the second sentence of the **Introduction** and re-written it to read:

"Multiple biophysical studies have defined the stages of IF assembly as: one type I keratin and one type II keratin pair to form a parallel heterodimer; heterodimers then bind to form an anti-parallel tetramer; tetramers then merge to form a protofibril/unit-length filament; and finally protofibrils assemble into the complete KIF (Aebi et al., 1983, Herrmann & Aebi, 2016, Parry, Marekov et al., 2001)."

With respect to points #2 and #4, we agree with Reviewer #2 that the vimentin and lamin structures have provided key insights into IF structure and assembly. Our original point was that there are no experimentally determined structures of human keratins to validate the proposed axial alignments. In order to provide more clarity, we have expanded the second paragraph of the **Introduction** to recognize the role of vimentin and lamin structures as follows:

"To date, there have been no crystal structures of human keratins that elucidate the molecular mechanisms of any of these axial alignments. For other types of IF proteins, however, crystal structures of vimentin (type III IF) and lamin A (type V IF) domains have provided molecular

insights into the A_{11} and A_{CN} modes of tetramer assembly, respectively (Aziz, Hess et al., 2012, Chernyatina, Guzenko et al., 2015, Chernyatina, Nicolet et al., 2012, Strelkov, Schumacher et al., 2004). In the case of lamin A, it was proposed that head-to-tail association occurs because clusters of positively-charged arginine residues in the head and tail domains interact with negatively-charged residues in the ends of the helical rod domain (Strelkov et al., 2004). The arginine clusters, however, are not conserved among keratin heads and tails. This difference highlights why it is necessary to study further the structural mechanisms governing higher-order IF assemblies, especially for keratins."

With respect to #4 and Peter Steinert's cross-linking data, we agree. The crosslinking data sets are an invaluable tool to help understand the biological relevance of IF subdomain crystal lattice packing. However, we recognize they may not always be the only means to validate or interpret a structure. We have added the following to the **second paragraph of the Discussion**:

"The cross-linking data by Steinert and colleagues provides an important means to correlate and validate IF domain packing in crystal lattices with the packing observed in filaments."

Referee #2 Comment:

The authors may wish to better recognize that: 3) Bernot *et al*. (2005) were able to predict and formally test for the importance of a "hydrophobic stripe" present within subdomain 1B of many but not all type I keratins (the authors' account of this particular study is superficial and a bit incomplete).

Author Response:

For point #3 - To more thoroughly discuss the key findings of the very important Bernot *et al*. (2005) manuscript, we have expanded the beginning of the 3rd paragraph of **Results** / "**Hydrophobic interactions drive 1B tetramer formation**" to read:

"Adjacent to the hydrophobic pocket, and aligned along the outer aspect of the α-helical ridge, are several K10 residues constituting a predominantly hydrophobic stripe (Fig. 2a,b). A type I keratin "hydrophobic stripe" was identified from modeling analyses of K6/K16/K17 dimers (Bernot, Lee et al., 2005); this work showed that most, but not all (e.g. K10), type I keratins contained a consensus hydrophobic sequence at alternating b- and f- positions of the heptad repeat (L-x-x-x-(I/V)-x-x-A-x-x-x-L) contributing to tetramer stability. However, K10 has threonine in the second position of this motif."

We also added a sentence in the **Discussion** to address the differences in *in vitro* filament assembly between knob and hydrophobic stripe mutants:

"Second, Coulombe and colleagues identified a hydrophobic stripe on type I keratins during K6/K16/K17 modeling (Bernot et al., 2005); as hypothesized, the K10 hydrophobic stripe participates in K1/K10-1B tetramer formation. However, the stripe's role in A_{11} tetramer formation proved more complex than anticipated: it was one of four key regions defining tetramer assembly, it did not self-associate, and its main interactions occurred with K1 residues. Importantly, prior mutation of hydrophobic stripe residues in mouse K16 and K17 did not significantly affect mature filament formation *in vitro* (Bernot et al., 2005); this is in contrast to anchoring knob mutants of human K1, K8, and vimentin which impaired *in vitro* filament formation for K1/K10, K8/K18, and vimentin (Fig. 6)."

Referee #2 Comment:

The authors do not supply information about which buffers were used for their gel filtration studies.

Author Response:

In **Methods** / **Protein Production and Purification**, we state "Both wild-type and mutant heterodimers were purified using the same procedure…The clarified solution containing untagged heterocomplex was applied to a Superdex75 (26/60) gel filtration column in 100mM Tris-HCl buffer (pH 7.4) containing 0.2M NaCl."

To add clarity to our K1/K10-1B knob mutation studies, we edited **Methods / Multi-angle light scattering** to read:

"Analysis of $K1^{F314A}$/K10-1B, $K1^{L318A}$/K10-1B, and $K1^{F314A+L318A}$/K10-1B was performed similarly, using 100mM Tris-HCl buffer (pH 7.4) containing 0.2M NaCl."

Referee #2 Comment:

It seems unfortunate that the presence of cadmium in the crystallization buffer for one of the complexes solved but not the other seemingly introduces a set of "distractions" that take away from the ability to directly compare the WT K1-K10 1B domains with the mutant one.

Author Response:

While the presence of cadmium in the WT K1/K10-1B structure did locally perturb the N-terminus of that structure, the rest of the 1B WT structure was comparable to the mutant. The fact that the two structures were so similar at the heterodimer and tetramer level, despite two different crystallization conditions, provides credibility that we captured the biologically relevant K1/K10-1B A_{11}-tetramer. I see the two structures as validating each other. Also, since proteins are dynamic molecules, two different crystallization conditions can sometimes capture differences in protein structure due to dynamics. Since the mutant K1/K10-1B diffracted to much higher resolution, this also will generate differences in side chain placement with the expectation of more accuracy in the higher resolution structure.

In conclusion, we sincerely thank the Editor and both Reviewers for their helpful critique and recommendations. We feel the recommendations significantly improved our manuscript, and we hope it is now suitable for publication at *The EMBO Journal*.

2nd Editorial Decision 1st Mar 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees whose comments are shown below.

As you will see, the referee finds that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before I can send the official acceptance letter, there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS:

Referee #1:

Christopher Bunick and colleagues have handed in an extensively revised version of their original manuscript. They correspond in a convincing manner to all major points made by the reviewers, and this reviewer thinks that the paper will now proof to enhance future research on the complex biology of epidermal keratin IF. In addition, their treatment and in particular their new experiments and modeling of simple epithelial keratins as well as vimentin provides interesting clues to the question of how similar these sequence-divergent members of an evolutionarily old multigene family are in thier assembly and filament structure.

2nd Revision - authors' response 6th Mar 2019

The authors performed all requested editorial changes.

3rd Editorial Decision 14th Mar 2019

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in The EMBO Journal.

Congratulations on the very nice work!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christopher G. Bunick
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-100741

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA (non-applicable).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA (non-applicable).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA (non-applicable).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA (non-applicable).
For animal studies, include a statement about randomization even if no randomization was used.	NA (non-applicable).
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA (non-applicable).
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA (non-applicable).
5. For every figure, are statistical tests justified as appropriate?	NA (non-applicable).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA (non-applicable).

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Is there an estimate of variation within each group of data?	NA (non-applicable).
Is the variance similar between the groups that are being statistically compared?	NA (non-applicable).

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA (non-applicable).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Escherichia coli BL21(DE3) and BL21(DE3)pLysS cells were purchased from Agilent Technologies and Invitrogen, respectively

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA (non-applicable).
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E- Human Subjects

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 6EC0 (wild-type) and 6E2J (mutant).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 6EC0 (wild-type) and 6E2J (mutant).
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