

Sequence divergence of coiled coils—structural rods, myosin filament packing, and the extraordinary conservation of cohesins

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Abstract

The amino acid sequences of the long, anti-parallel coiled coils of the cohesin subunits SMC1 and SMC3 are almost totally conserved in mammals. To understand this exceptional conservation more broadly, we analyzed amino acid sequence variation for several groups of coiled-coil proteins. Some long coiled coils, including giantin, NuMA, and Ndc80p/Nuf2p diverge ~20% from humans to rodents, suggesting they function as spacer rods, whose sequence divergence is constrained only by the need to maintain the coiled-coil structure. Other coiled coils such as skeletal muscle myosin, intermediate filaments, and the lamins diverge only 1–3%. We suggest that this sequence divergence is constrained by the extensive packing contacts over the entire surface of the coiled-coil. The coiled coils of SMC5/6 and SMC2/4 (condensin) are slightly more constrained than the presumed spacer rods, diverging 10–15%. Conversely, the coiled coils of SMC1/3 (cohesin) diverge only 0.0–1.0%. This extreme constraint suggests that the entire surface of the coiled coil is intimately involved in the mechanism of sister chromatid cohesion. Direct binding of the coiled coils to chromatin, or perhaps the need to avoid such binding, are two possible mechanisms. Finally, analysis of the heptad repeat shows that the *a* and *d* positions are more constrained in spacer rods, and the *bcefg* positions more constrained in skeletal muscle myosin.

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1. Introduction

The α -helical coiled-coil was one of the first protein structural motifs known to modern day biochemists, and the elucidation of its structure involved some of the greatest scientific minds of the 20th century, including Pauling, Perutz, and Crick (Gruber and Lupas, 2003; Lupas and Gruber, 2005). Many coiled-coil proteins serving a wide variety of functions have now been discovered. Furthermore, mutations of coiled-coil domains are associated with a number of disease states in humans (Rose and Meier, 2004).

Coiled-coil formation requires hydrophobic amino acids at positions *a* and *d* of the heptad repeat while the amino acids at positions *b*, *c*, *e*, *f*, and *g* face the outside of the rod

and are generally hydrophilic (Lupas, 1998; Rose and Meier, 2004). The simplest function of a coiled-coil is to serve as a rod, providing a spacer separating two functional domains. If a coiled-coil is functioning solely as a rod, one could expect substantial tolerance towards amino acid substitution in the divergence of species, especially in the outward facing residues at positions *bcefg*.

In many proteins, however, the function of the coiled-coil extends beyond the formation of an inert rod. An extreme example is the tail of striated muscle myosin. These coiled coils pack together in a mixed parallel and anti-parallel array to assemble the bipolar thick filament. The packing is quite complex, and involves contacts with adjacent tails on all sides and over most of the surface tail (Au, 2004). It is well established that protein–protein interfaces are highly specific and complementary (Chothia and Janin, 1975). As such, changing an amino acid on one side

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of the contact interface will often require a complementary change on the other side. Once the myosin tail evolved the optimal contacts necessary to form the thick filament, it is reasonable to expect that preserving these contacts would impose strong constraints on sequence divergence. Similar constraints should apply to other coiled coils with extensive packing, such as intermediate filaments (Herrmann and Aebi, 2004).

The SMC (structural maintenance of chromosomes) proteins are a family of proteins involved in condensing, connecting or repairing DNA. The proteins are all dimers with a similar architecture (Fig. 1, (Cobbe and Heck, 2004; Haering and Nasmyth, 2003; Jessberger, 2002; Melby et al., 1998)). Each subunit of the dimer has globular N- and C-terminal domains, separated by long coiled-coil segments, with a globular hinge in the middle (Fig. 1). The molecule folds at the hinge to form a long, anti-parallel coiled-coil. This brings the N- and C-terminal domains together to form a functional “head,” with the coiled-coil forming a rod separating the head from the hinge (Haering et al., 2002; Melby et al., 1998). Specific pairs of SMC proteins (1 with 3, 2 with 4, 5 with 6) form dimers by binding at their hinge domains (Haering et al., 2002). In most SMC proteins, the two heads also bind to each other and to smaller accessory proteins. In the case of SMC2/4 (condensin), the coiled coils bind to each other along the majority of their length, producing a rod shaped dimer with the two hinges at one end and the two heads at the other (Anderson et al., 2002). For SMC1/3 (cohesin), the coiled coils do not associate, so the binding of the heads to each other produces an open circular structure (Anderson et al., 2002; Haering et al., 2002). Cohesin will be a major focus of our analysis. Its biology and molecular mechanisms have been discussed extensively in two recent reviews (Hirano, 2005; Nasmyth, 2005).

In the following analysis, we have determined the divergence of amino acid sequence for a wide range of coiled-coil proteins. We relate the divergence to the role of the coiled-coil, either as a rod or as involved in extensive packing contacts. This analysis provides the context to discuss the extraordinary conservation of the coiled coils of cohesin.

2. Materials and methods

2.1. Protein sequences

Protein sequences were downloaded from the Protein Database at the NCBI's web site (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=protein>). Typically, the human sequence was obtained first via an Entrez search. If a reference sequence was available, it was always used; otherwise, the most current sequence in the database was downloaded. A BLAST (Altschul et al., 1997) search of the *Mammalia* database was then used to identify sequences from other mammals. The majority of sequences used are Reference sequences.

The following list of proteins analyzed gives the species names, GenBank accession number, followed by the beginning and ending of the coiled-coil domains; or when there are three or more coiled-coil segments, the total number of amino acids (AAs) in the coils (for the *Homo sapiens* proteins only). **Desmin:** *H. sapiens* desmin (NP_001918.3/human/N-coil 103–267/C-coil 294–413), *Mus musculus* desmin (NP_034173.1/mouse). **Giantin (Golgin B):** *H. sapiens* giantin (Q14789/2712 AAs total coil), *Gallus gallus* giantin (XP_424476.1/chicken), *Rattus norvegicus* giantin (BAA05026.1/rat). **Kinectins:** *H. sapiens* kinectin (NP_891556.1/994 AAs total coil), *M. musculus* kinectin (NP_032503.1). **Kinesin (kar3):** *H. sapiens* KIF11 (NP_004514.2/361–523), *M. musculus* KIF11 (NP_034745.1). **Kinesin 4 (kif4):** *H. sapiens* (NP_036442.2/518

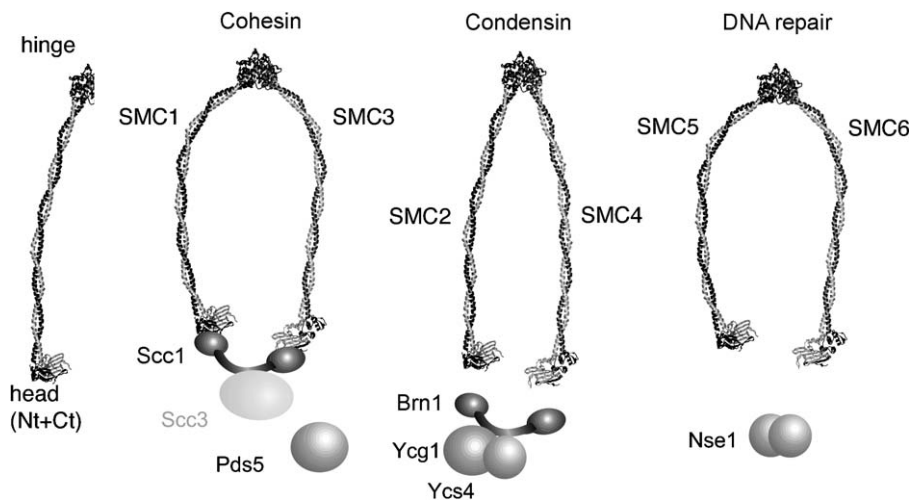


Fig. 1. The structure of SMC proteins. The left panel shows a single SMC subunit, with the hinge at the top and the combined Nt and Ct domains forming the active head, at the bottom. The coiled coils typically are 350 amino acids on each side, for a length of 50 nm. Eukaryotic SMC proteins form heterodimers by binding at the hinge, and accessory proteins typically bind to the heads. Binding of accessory proteins to SMC5/6 has recently been shown to involve Nse2 binding to the coiled-coil of SMC5 (Sergeant et al., 2005) the only known protein binding to an SMC coiled-coil. The figure is reprinted from Haering and Nasmyth, *BioEssays* 25:1178–91, 2003 with permission of the publisher.

AAs total coil), *M. musculus* kif4 (NP_032472.1), *R. norvegicus* kif4 (XP_343798.2). **Kinesin 5b** (kif5b): *H. sapiens* kinesin 5b (NP_004512.1/325–564), *M. musculus* kinesin 5b (NP_032474.2), *R. norvegicus* kinesin 5b (XP_341539.2). **Lamins**: *H. sapiens* lamin A/C (P02545/24–224), *H. sapiens* lamin B1 (NP_005564.1/26–224), *M. musculus* lamin A (NP_001002011.1), *M. musculus* lamin B1 (P14733), *R. norvegicus* lamin A/C (NP_001002016.1), *R. norvegicus* lamin B1 (NP_446357.1). **Myosins**: *H. sapiens* α -cardiac myosin II (P13533/Head 1–768/Neck 769–840/Coil 841–1935), *H. sapiens* β -cardiac myosin II (P12883/Head 1–766/Neck 767–838/Coil 839–1936), *H. sapiens* embryonic fast skeletal myosin II (P11055/Head 1–767/Neck 768–838/Coil839–1935), *H. sapiens* non-muscle myosin IIA (NP_002464.1/Head 1–764/Neck Hs 1–835/Coil 836–1961), *H. sapiens* Myh12 Myosin Va (CAA69035.1/902–1105), *H. sapiens* smooth muscle myosin (P35749/Head 1–771/Neck 772–843/Coil 844–1937), *M. musculus* α -cardiac myosin II (NP_034986.1), *M. musculus* smooth muscle myosin (O08638), *M. musculus* myosin Va (Q99104), *M. musculus* smooth muscle myosin II (O08638), *Oryctolagus cuniculus* smooth muscle myosin (P35748/rabbit), *R. norvegicus* α -cardiac myosin II (NP_058935.1), *R. norvegicus* β -cardiac myosin II (P02564), *R. norvegicus* non-muscle myosin IIA (Q62812). **Ndc80p**: *H. sapiens* Ndc80p (NP_006092.1/N-coil 252–425/C-coil 455–642), *M. musculus* Ndc80p (NP_075783.1), *R. norvegicus* Ndc80p (XP_217489.3). **Nuf2p**: *H. sapiens* Nuf2p (NP_663735.1/145–458), *M. musculus* Nuf2p (NP_075773.1), *R. norvegicus* Nuf2p (NP_001012028.1). **NuMA**: *H. sapiens* NuMA (CAA77669.1/1462 AAs total coil), *M. musculus* NuMA (NP_598708.2). **SMC1**: *Anopheles gambiae* SMC1 (CAD59403.1/mosquito), *Bos taurus* SMC1 (NP_777039.1/cow), *Drosophila melanogaster* SMC1 (CAB76376.1/fruit fly/N-coil: 183–526/C-coil: 683–1083), *Drosophila pseudoobscura* SMC1 (EAL27947.1/fruit fly), *G. gallus* SMC1 (NP989847.1), *H. sapiens* SMC1 (CAI42647.1/N-coil 178–503/C-coil 658–936), *M. musculus* SMC1 (NP_062684.1), *R. norvegicus* SMC1 (NP_113871.1). **SMC2**: *A. gambiae* SMC2 (CAD59404.1), *D. melanogaster* (NP_610995.1/N-coil: 172–507/C-coil: 669–1020), *H. sapiens* SMC2 (CAD89875.1/N-coil 173–507/C-coil 672–1031), *M. musculus* SMC2 (NP_032043.2), *R. norvegicus* SMC2 (XP_342838.1), *Xenopus laevis* SMC2 (P50533/frog). **SMC3**: *A. gambiae* SMC3 (CAD59405.1), *B. taurus* SMC3 (NP_776720.1), *D. melanogaster* SMC3 (NP_523374.2/N-coil: 176–502/C-coil: 671–1106), *D. pseudoobscura* (EAL31729.1), *G. gallus* SMC3 (NP_989848.1), *H. sapiens* SMC3 (NP_005436.1/N-coil 173–504/C-coil 668–1022), *M. musculus* SMC3 (NP_031816.2), *R. norvegicus* SMC3 (NP_113771.1), *X. laevis* SMC3 (CAD59446.1). **SMC4**: *A. gambiae* SMC4 (XP_317674.2), *D. melanogaster* (NP_723996.1/N-coil: 272–603/C-coil: 777–1054), *D. pseudoobscura* (EAL33605.1), *H. sapiens* SMC4 (NP_001002800.1/N-coil 272–588/C-coil 767–1020), *M. musculus* SMC4 (NP_598547.1), *R. norvegicus* SMC4 (XP_215573.2), *X. laevis* SMC4 (P50532). **SMC5**: *H. sapiens* SMC5 (NP_055925.1/N-coil 207–458/C-coil 649–930), *M. musculus* SMC5 (NP_722503.1), *R. norvegicus* SMC5 (XP_215254.2), *X. laevis* SMC5 (BAC56936.1). **SMC6**: *G. gal-*

lus SMC6 (XP_419962.1), *H. sapiens* SMC6 (NP_078900.1/N-coil Hs 215–456/C-coil 660–914), *M. musculus* SMC6 (NP_079971.2), *R. norvegicus* SMC6 (XP_233970.2), *X. laevis* SMC6 (BAC56937.1).

2.2. Amino acid sequence divergence

In each instance, the human sequence was used as the reference sequence for the analysis of mammalian proteins. Each human sequence was entered into the program COILS (Lupas et al., 1991) (http://www.ch.embnet.org/software/COILS_form.html) to identify the coiled-coil segments. All non-coil segments, generally those with a score below 0.5 on the 28-residue window, were then removed from sequences prior to final alignment. A preliminary alignment of the proteins was used to identify sequence gaps between the proteins being compared. These gap segments were removed from the other sequence prior to our determination of sequence divergence. Large proteins with multiple coil segments had their non-coil segments and gaps removed, and the coil was considered as a single entity. For those proteins where a large coil was present on the N-terminal or C-terminus “half” of the protein, amino acid numbers from the human sequence are indicated for each segment in the Tables and the two segments were compared separately. Each pair of sequences was aligned using the “BLAST 2 sequences” tool accessed through the Biology WorkBench of the San Diego Super Computer (<http://seqtool.sdsc.edu/CGI/BW.cgi>). The % amino acid sequence divergence was calculated from the output of the paired alignment. For this analysis, we used the simplest measure of sequence divergence: simply counting the number of amino acid changes between the two sequences, without any consideration of physical properties such as charge, hydrophobicity, etc. This is the same measure used by Makalowski and Boguski (1998) in their extensive comparison of human and rodent sequences.

Analysis of the amino acid sequence divergence for each position of the heptad repeats were conducted in the following manner. The *a*, *b*, *c*, *d*, *e*, *f*, and *g* positions of the human coiled-coiled sequence were assigned by COILS. The human coiled-coil protein was then aligned with the appropriate non-human ortholog and the number of amino acid changes for *a*, *d*, and the combined *bcefg* positions of the heptad repeat was then determined for each protein pair.

3. Results and discussion

3.1. General comments on sequence variation in proteins

In this study, we found that coiled coils can be grouped into two extreme classes, based on amino acid sequence variation. One group shows substitutions from human to rodent at ~20% of the amino acids. We suggest that these coiled coils are serving primarily as rods, and they have little interaction with other proteins on their surface.

The other group shows amino acid variation of only 1–3%. This group contains coiled coils known to involve extensive packing interactions over their surface, and we suggest that the constraint on sequence variation is imposed by these packing interactions.

It is important to put this analysis into the more general context of protein sequence divergence among mammals. Makalowski and Boguski (1998) analyzed the sequence divergence between 1200 pairs of orthologous sequences in human and rat, and found that the average difference was 12%. 10% of sequences showed a divergence greater than 37%, and 4.3% were 100% identical.

Examples of totally conserved proteins include ribosomal subunits, histones, actins, and actin interacting proteins. The majority of these proteins are small (100–200 amino acids in length) while approximately 10% are in the range of 400 amino acids. β -adaplin, with 950 amino acids, was the largest totally conserved protein. A common property of these highly conserved proteins is that they form binding interfaces with other proteins over much of their surface. For example, actin makes three contacts with other actin subunits to form the filaments, and the external surfaces of actin filaments make contact with myosins and a number of actin binding proteins (Millman, 1998; Squire and Morris, 1998). β -Adaplin contacts other subunits of the AP-2 adaptor complex as well as clathrin (Ferguson, 2001). Ribosomal proteins and histones contact other protein subunits and also make extensive contacts with RNA or DNA (Chakravarthy et al., 2005; Khan and Krishnamurthy, 2005). Since protein–protein interactions involve highly complementary interfaces (Chothia and Janin, 1975), a mutation on one side may weaken the interaction unless there is a complementary mutation on the other side. In general, amino acids involved in protein–protein or protein–nucleic acid interfaces should be constrained in sequence divergence.

For proteins in general, sequence divergence is constrained primarily by the need to fold the protein, and this varies considerably for different structural folds. Coiled coils all have a common structure, so they should all have the same constraint for folding. This constraint should be primarily at the *a* and *d* positions of the heptad, which need to form hydrophobic contacts. As discussed below, additional constraints will be imposed by binding interactions of surface residues.

3.2. Coiled coils acting as spacer rods show maximal divergence

Table 1 presents the sequence divergence of several coiled-coil proteins that had the highest divergences found in our analysis. We propose the protein giantin as the archetype of a coiled-coil that most likely serves as a spacer rod. It is the longest coiled-coil known, spanning 3150 residues when the short non-coil gaps are included. Giantin is proposed to tether COP2 vesicles to the Golgi apparatus (Barr and Short, 2003; Gillingham and Munro, 2003).

Table 1
Coiled coils with high divergences

Protein	Segments	Divergence (%)
<i>Giantin</i>		
Human/rat	Whole protein	26.7
	Coils (2712)	21.8
Human/chicken	Whole protein	64.0
	Coils	59.9
<i>NuMA</i>		
Human/mouse	Whole protein	18.4
	Coils (1462)	17.9
<i>Ndc80p</i>		
Human/mouse	Whole protein	16.2
	N-coil (173)	16.7
	C-coil (187)	21.2
Human/rat	Whole protein	14.8
	N-coil	15.9
	C-coil	16.3
<i>Nuf2p</i>		
Human/mouse	Whole protein	27.9
	Coil (313)	27.4
Human/rat	Whole protein	20.8
	Coil	21.5
<i>Kinectin</i>		
Human/mouse	Whole protein	18.7
	Coils (994)	17.7

The numbers in parentheses are the total number of amino acids in the human protein that was used as the reference sequence for comparison purposes. Details on how these numbers were determined are provided in the Section 2.

Giantin has a short non-coil sequence at the N-terminus that binds proteins of the Golgi, and a short transmembrane segment at the C-terminus that inserts into COP2 vesicles. This long coiled-coil provides a spacer bar of approximately 0.5 μ m between the Golgi and the vesicle, and it has no other known binding function.

Between human and rodents, giantin's sequence diverges 27% over the entire protein while the coiled-coil segment diverges 22%. The 22% sequence divergence in the coils is significantly higher than the average 12% sequence divergence for human/rat protein comparisons, as determined by Makalowski and Boguski (1998). With one or two exceptions the divergence appears to be spread randomly over the long coil. (A close inspection of the sequence alignment shows some stretches of 20–40 amino acids with no substitutions, but we have not attempted to determine the statistical significance of variation at this fine level.) Because this 22% sequence divergence is similar to that of several other coiled coils (discussed below), we suggest that it is characteristic of the class of coiled coils serving as an inert rod.

Table 1 also includes a comparison of giantin coils between human and chicken, since similar comparisons are made for the more conserved coiled coils to be discussed. The 60% amino acid sequence divergence of the coils segment is among the largest we observed for coiled coils between mammals and frogs or birds.

The protein NuMA localizes to the minus ends of microtubules and focuses them at the mitotic spindle

(Fant et al., 2004). NuMA has a coiled-coil of 1500 amino acids that separates proline rich globular domains of 24 and 44 kDa at the N- and C-termini, respectively. NuMA's coiled-coil diverges 18% between the human and rodent sequences suggesting that like giantin, it is serving primarily as a spacer rod. Although NuMA has been suggested as a potential nuclear cytoskeletal protein, its sequence is clearly not constrained by the packing constraints that are found in intermediate filaments and the lamins (see below).

Ndc80p and Nuf2p are essential components of the kinetochore complex and form a heterodimeric coiled-coil with globular domains at their N-terminus. The C-terminus interacts with two other coiled-coil proteins, perhaps forming a short stretch of three-stranded coiled-coil (Wei et al., 2005). Nuf2p diverges 22–27% from human to rodent, which is the highest divergence in our data set for mammalian coiled coils. This is consistent with our hypothesis that it serves as a spacer rod. It is curious that its predicted partner, Ndc80p, is somewhat more conserved, but the 16–20% amino acid divergence between humans and rodents is still among the highest we observed in this study. We propose that Ndc80p's sequence divergence is representative of the lower end of amino acid sequence divergence for coiled coils functioning as spacer rods.

Kinectin is a kinesin-binding transmembrane protein of the endoplasmic reticulum that possesses a more highly conserved segment in the middle of the coils, while the ends are more divergent (Santama et al., 2004; Toyoshima et al., 1992). This suggests that the conserved segment may be binding another protein. The overall divergence of 18% is in the range that we consider typical for coiled coils serving as rods.

3.3. Coiled coils with extensive packing contacts have much lower sequence divergence

Conventional myosin II includes striated, smooth, and non-muscle isoforms and as such, provides a spectrum of coiled-coil proteins with different structural constraints. The coiled coils of striated muscle myosins (skeletal and cardiac) are highly conserved, with a divergence of only 2–3% between human and rodents (Table 2; head/neck/coiled-coil domains are identified as per Korn (2000)). As discussed in the Introduction, the coiled coils of the myosin tails use protein–protein interactions along their entire length and surface to assemble the thick filament. We suggest that this packing imposes the observed substantial constraint on sequence divergence.

The coiled coils of smooth muscle and non-muscle myosin II also self-associate to create filaments. Electron micrographs show that these filaments are not as organized or as regular as the thick filaments of striated muscle (Scholey et al., 1980; Suzuki et al., 1978). Smooth muscle myosin II assembles a side-polar filament as opposed to the bipolar thick filament found in striated muscle (Xu et al., 1996). Its sequence divergence of 4.5%, vs 2–3% for skeletal muscle

Table 2
Myosin II coil divergences

Protein	Segment	Divergence (%)
<i>Fast Emb skeletal</i>		
Human/rat	Head (767)	0.9
	Neck (71)	4.2
	Coil (1102)	2.1
<i>α-Cardiac</i>		
Human/mouse	Head (767)	3.7
	Neck (71)	2.8
	Coil (1094)	3.0
Human/rat	Head	3.1
	Neck	2.8
	Coil	3.0
<i>β-Cardiac</i>		
Human/rat	Head (766)	4.3
	Neck (71)	4.2
	Coil (1097)	1.7
<i>Smooth</i>		
Human/mouse	Head (771)	3.2
	Neck (71)	1.4
	Coil (1093)	4.5
Human/rabbit	Head	3.6
	Neck	0.0
	Coil	3.4
<i>Nonmuscle IIA</i>		
Human/rat	Head (764)	1.2
	Neck (71)	2.8
	Coil (1023)	5.4
<i>Myosin Va</i>		
Human/mouse	Coil (203)	7.0

myosin coiled coils is consistent with the less extensive packing observed in these side-polar filaments.

We also examined the shorter coiled-coil domain of the unconventional myosin Va which does not form filaments (Tyska and Mooseker, 2003). However, myosin V can form a “heteromotor” complex with kinesin, involving the 2nd and 3rd (medial and distal) coiled coils of myosin V (Langford, 2002). The 7% divergence of the coiled coils of myosin V is substantially higher than that of striated muscle myosin, but it is still well below the range we associate with coiled coils that act solely as rods. The formation of the myosin V/kinesin heteromotor complex may constrain myosin V's divergence.

Intermediate filaments are similar to the skeletal muscle myosin II tail in that the coiled coils of adjacent molecules pack tightly and extensively to form the 10 nm diameter filament. Desmin is the intermediate filament protein found in muscle cells (Bar et al., 2004; Paulin and Li, 2004). The coiled-coil segments of desmin diverge only 1–2% (Table 3), which is even more highly constrained than the myosin II tails. This no doubt reflects the fact that nearly the entire surface of the coiled coil is involved in highly specific interfaces with neighboring coiled coils.

The nuclear lamins are closely related to intermediate filament proteins, and in *X. laevis* they form 10 nm filaments on the nuclear envelope (Stuurman et al., 1998);

Table 3
Cytoskeletal protein coil divergences

Protein	Segment	Divergence (%)
<i>Desmin</i>		
Human/mouse	Whole protein	2.4
	N-coil (164)	1.8
	C-coil (119)	0.8
<i>Lamin A</i>		
Human/mouse	Whole protein	3.6
	Coil (200)	2.0
Human/rat	Whole protein	3.3
	Coil	1.5
<i>Lamin B</i>		
Human/mouse	Whole protein	4.1
	Coil (198)	2.0
Human/rat	Whole protein	4.6
	Coil	2.0
<i>Kinesin (kif5b)</i>		
Human/mouse	Whole protein	2.8
	Coils (239)	2.0
Human/rat	Whole protein	2.6
	Coils	1.0
<i>Kinesin (kar3)</i>		
Human/mouse	Whole protein	20.6
	Coil (162)	14.2
<i>Kinesin (kif4)</i>		
Human/mouse	Whole protein	13.3
	Coils (518)	8.7
Human/rat	Whole protein	13.8
	Coils	10.4

however, their structure is less well understood. More than 180 mutations, scattered equally throughout the coiled-coil and globular domains, are known to cause genetic defects known as laminopathies (Ben Yaou et al., 2005), demonstrating clearly the important nuclear function of lamins. Their sequence divergence of 1.5–2% (Table 3) is similar to that of desmin, consistent with the idea that they pack into a structure like an intermediate filament.

We extended our analysis to include kinesins, which are motor proteins with coiled coils that are not involved in packing interactions for filament formation. We expected these coiled coils to diverge as the inert rods, but we found that some members of the kinesin family were highly conserved. For example, conventional kinesin (kif5b/Table 3) has only 1–2% sequence divergence, which matches that of desmin and suggests that the surface of the coiled-coil is involved in substantial binding interactions. The myosin Va/kinesin heteromotor complex formation discussed above is one likely constraint. Another is that the coils may be involved in the intramolecular regulatory mechanism. The coiled-coil of this conventional kinesin has a hinge near its middle, and in the absence of cargo, the molecule folds back on itself so that the N-terminal domain binds the C-terminal motor and inactivates it (Coy et al., 1999; Friedman and Vale, 1999; Stock et al., 1999). The major interaction stabilizing the compact conformation has been thought to be that of the N- and C-terminal domains. How-

ever, interaction of the N- and C-terminal halves of the coiled coil may also be involved in stabilizing the folded conformation. This interaction could constrain the sequence divergence. Consistent with this, no such folding mechanism has been identified for the minus-end directed kinesin kar3. This coiled coil has a 14% divergence (Table 3), suggesting that it is serving primarily as a rod, with no extensive binding interactions. Kinesin 4 (kif4) has coils with an intermediate level of sequence divergence, 8–10%. It is interesting to note that for all three kinesins the coils are more conserved than the head domains. This is also true for several myosin IIs (Table 2).

3.4. SMC proteins with less constrained coiled coils

SMC5/6 is involved in DNA repair (Jessberger, 2002). The coiled coils of SMC5 diverge 13–16% between humans and rodents (Table 4), which is somewhat less than the ~20% observed for the more divergent coils reported in Table 1. Its partner, SMC6, is somewhat more constrained with 10–12% divergence (Table 4). The SMC proteins differ from those in Table 1 in that their coiled coils are anti-parallel. The rules determining the anti-parallel structure are not clearly defined, and they may impose an additional constraint on sequence divergence even if they are functioning primarily as spacer rods.

The coiled coils of the condensins (SMC2/4) diverge from 9 to 16% (Table 4), the same range as the SMC5/6 pairs. The SMC2/4 dimer tends to fold into a linear rod, with the two coiled coils contacting along their entire length (Anderson et al., 2002). This contact between the two coiled coils may impose some constraint on sequence divergence.

Although our major focus is on divergence between mammals, we extended the analysis to compare human and *D. melanogaster*, *D. melanogaster*, and *D. pseudoobscura*, and *D. melanogaster* and *A. gambiae* (mosquito) for SMC2/4. As shown in Table 4, these proteins show very high divergences. The human/fly divergence of 72–79% in the coils is the highest we observed in this study.

3.5. Factors that might constrain sequence divergence of coiled coils

We have already suggested that extensive packing interactions are probably the basis for the constrained sequence divergence myosin and desmin. An intermediate level of sequence divergence might be thought to reflect binding interactions of other proteins to the coiled coil. However, it seems unlikely that the binding of one or even a few globular proteins would significantly constrain the coiled coil. A typical globular protein is only ~5 nm in diameter, so it would only be able to contact a fraction of a 50 nm long coiled-coil. For example, Nse2 binds to the coiled coil of SMC5 (Sergeant et al., 2005), but it is only 267 amino acids. Even if it were a coiled coil (which it is not) it would cover only half the length of SMC5, and more importantly it could only contact residues along one side. As a globular

Table 4
SMC protein and coil divergences

	SMC2 whole protein	SMC2 N-coil	SMC2 C-coil	SMC4 whole protein	SMC4 N-coil	SMC4 C-coil
Total AAs	1197	Hsa 178–503	Hsa 658–936	1288	Hsa 272–588	Hsa 767–1020
% Divergence						
Human/mouse	7.9%	11.3%	9.7%	10.5%	10.4%	15.1%
Human/rat	6.7%	8.8%	9.4%	10.5%	9.9%	16.2%
Human/frog	18.6%	22.6%	24.2%	23.0%	27.8%	32.1%
Human/fly	58.9%	72.2%	75.3%	60.2%	77.1%	79.1%
<i>DmelDps</i>	N/A			25.5%	24.4%	35.1%
<i>DmelAga</i>	46.2%	56.1%	55.4%	48.6%	58.7%	67.4%
	SMC5 whole protein	SMC5 N-coil	SMC5 C-coil	SMC6 whole protein	SMC6 N-coil	SMC6 C-coil
Total AAs	1101	Hsa 207–458	Hsa 649–930	1091	Hsa 215–456	Hsa 660–914
% Divergence						
Human/mouse	11.7%	14.3%	14.7%	9.7%	11.2%	10.4%
Human/rat	11.2%	12.7%	16.2%	12.4%	12.4%	11.4%
Human/frog	36.6%	45.8%	47.7%	36.1%	53.1%	49.8%
Human/chicken	37.3%	42.0%	48.2%	40.4%	48.7%	51.8%
Chicken/frog	45.6%	45.3%	50.4%	41.6%	49.7%	50.0%
	SMC1 whole protein	SMC1 N-coil	SMC1 C-coil	SMC3 whole protein	SMC3 N-coil	SMC3 C-coil
Total AAs	1233	Hsa 173–507	Hsa 672–1031	1217	Hsa 173–504	Hsa 668–1022
% Divergence						
Human/mouse	0.6%	0.3%	0.7%	0.1%	0.0%	0.0%
Human/rat	0.4%	0.6%	0.0%	0.6%	0.0%	1.1%
Human/cow	0.2%	0.0%	0.0%	1.5%	0.0%	0.0%
Human/chicken	5.4%	6.8%	1.4%	0.7%	0.6%	0.0%
Human/frog	5.9%	7.1%	5.4%	3.3%	4.2%	4.8%
Human/fly	50.3%	58.5%	57.9%	48.1%	40.7%	59.7%
<i>DmelDps</i>	6.6%	5.9%	9.8%	3.3%	3.4%	4.4%
<i>DmelAga</i>	30.9%	38.6%	36.5%	21.2%	29.4%	28.1%

AA numbers and totals are as in Fig. 1. N/A means that the *D. pseudoobscura* sequence for SMC2 is not available.

protein it would contact only a small fraction of the surface of the SMC5 surface. Consistent with this, SMC5 is even somewhat more divergent than its partner, SMC6.

We can, however, suggest two other mechanisms that might constrain sequence divergence of a coiled coil. One is that constraints may be imposed by mechanical properties. If the function of a molecule required a rod of greater or lesser flexibility, this might be achieved by a particular sequence, such as at the *a* and *d* positions of the heptad repeat. This is presently just a speculation, as there is little information on the mechanical properties of different coiled coils. The other possibility is that some coiled coils may need a surface designed to avoid weak interactions with other proteins or DNA. We will return to this idea for the mechanism of cohesin.

3.6. The extraordinary conservation of the coiled coils of cohesin, SMC1/3

The cohesin subunits SMC1 and SMC3 have the most highly conserved coiled coils that we have found, more constrained even than skeletal muscle myosin and intermediate filaments (Table 4). For example, there is not a single amino acid change between human and bovine sequences in the 603 and 685 amino acids of the coiled coils of SMC1 and SMC3. Other mammals have only 1–4 amino acid changes over the same regions. For SMC3, this very high level of conservation is also observed between human and chicken,

and the human–frog comparison shows a only slightly larger (4%) divergence of the coils. The high sequence conservation in SMC1 and SMC3 from mammals to avians and amphibians is in striking contrast to the 42–50% divergence of SMC5/6 coiled coils between humans and chickens (Table 4), and the 30% divergence of SMC4 between humans and frogs (Table 4).

The availability of insect SMC1/3 sequences allowed us to analyze the sequence divergence between humans and flies. Consistent with the mammalian comparisons, the human/fly divergences for SMC1/3 are substantially lower than the SMC2/4 human/fly divergences. Also of interest is the divergence between insect species. Although the absolute rates of divergence are substantially higher than between vertebrates, SMC1/3 is much more constrained than SMC2/4 when comparing *D. melanogaster* to *D. pseudoobscura* or *A. gambiae* (Table 4).

Given the very high sequence constraint observed for cohesin at the amino acid level, the question arises whether there might be a similar constraint at the DNA level. The mRNA-derived cDNA sequence of rat and human SMC1 are 90.5% identical. Makalowski and Boguski (1998) have provided a similar analysis for a large group of proteins (see also their Appendix 1 at their new web site http://warta.bio.psu.edu/htt_doc/Makalowski/PNAS/). β -adap-tin, which is 100% identical at the protein level between human and rat, showed 91.5% identity at the DNA level. Therefore, the degree of genomic conservation observed

with SMC1 is consistent with the normal frequency of synonymous substitutions.

3.7. Sequence variation by heptad position

We were interested to know how sequence variation depended on the position within the heptad repeat. In particular, would the sequence be more constrained at the hydrophobic *a* and *d* positions than in the outward facing *bcefg* positions? We examined giantin and NuMA, embryonic skeletal muscle myosin, desmin, and all of the SMCs to ensure we covered a range coiled-coil proteins from the different classes (Table 5).

If the variation were randomly distributed among the 7 positions, we would expect 1/7 (14%) of the total variation to occur at each of the *a* and *d* positions, and 5/7 (71%) at the combined, outward-facing *bcefg* positions. For 7 of the 10 proteins we analyzed, the *a* and *d* positions were more constrained than would be expected for random variation. This is most convincing for the large proteins giantin and NuMA, with 568 and 205 total changes respectively. This agrees with our suggestion that their coiled coils are serving primarily as rods. The *a* and *d* positions are needed to form

the coiled coil, so we would expect them to be more conserved than the outward-facing residues, which are presumably not involved in specific interactions. In contrast, skeletal muscle myosin shows a much larger constraint at the *bcefg* positions (54% of all changes, vs 71% for a random distribution). This result also fits our interpretation that these outward-facing residues are constrained by the extensive packing contacts to assemble the myosin thick filament. Desmin similarly shows more constraint at the *bcefg* positions, but the number of changes (4) is too small to make a significant statement. It is important to note that both myosin and desmin are also much more constrained at the *a* and *d* positions than giantin or NuMA. This is probably because the side chains of the *a* and *d* positions are not completely buried, but project from the coiled coil and can participate in packing interactions.

SMC2/4 and SMC5/6 generally show higher constraint at *a* and *d*, similar to giantin, and consistent with their functioning primarily as rods. SMC1/3 had too few substitutions from human to mouse to draw any meaningful conclusions. Extending the SMC1/3 analysis to chicken and frog suggests higher constraints at the *a* and *d* positions versus the *bcefg* positions. However, there are only 2–27 total

Table 5
Sequence divergence of the *a* and *d* residues of heptad repeats

Comparison	Total AA changes	Total <i>a</i> changes	Total <i>d</i> changes	Total <i>bcefg</i> changes
<i>Giantin</i>				
Human/rat	568	68 (12.0%)	52 (9.1%)	448 (78.9%)
<i>NuMA</i>				
Human/mouse	205	26 (10.3%)	22 (8.7%)	205 (81%)
<i>Emb Skel myosin tail</i>				
Human/rat	22	4 (18.2%)	6 (27.3%)	12 (54.5%)
<i>Desmin</i>				
Human/mouse	4	2 (50%)	0	2 (50%)
<i>SMC1</i>				
Human/mouse N + C-coils	3	0	1 (33.3%)	2 (66.7%)
<i>SMC1</i>				
Human/chicken N + C-coils	26	0	2 (7.7%)	24 (92.3%)
<i>SMC3</i>				
Human/mouse N + C-coils	0	0	0	0
<i>SMC3</i>				
Human/chicken N + C-coils	4	0	0	4 (100%)
<i>SMC3</i>				
Human/frog N + C-coils	31	3 (9.7%)	1 (3.2%)	27 (87.1%)
<i>SMC2</i>				
Human/mouse N + C-coils	72	8 (11.1%)	2 (2.8%)	62 (86.1%)
<i>SMC4</i>				
Human/mouse N + C-coils	88	5 (5.7%)	10 (11.4%)	73 (82.9%)
<i>SMC5</i>				
Human/mouse N + C-coils	73	5 (6.8%)	10 (13.7%)	58 (79.5%)
<i>SMC6</i>				
Human/mouse N + C-coils	50	4 (8.0%)	3 (6.0%)	43 (86.0%)

We tabulated the number of amino acid changes at the *a* and *d* positions, and the sum of all changes in *bcefg* positions. The numbers in parentheses give the percent of changes that occurred at the position. If the changes were randomly distributed, we would expect 1/7 = 14.3% at *a* and *d* and 5/7 = 71.4% at *bcefg*.

substitutions at the *bcefg* position, and 2–4 at *a* and *d*, so the numbers are of questionable significance.

Overall, the analysis in Table 5 shows that the *a* and *d* positions are more constrained than *bcefg* for most proteins, in particular for those functioning as structural rods.

3.8. Biological significance of cohesin sequence conservation

The much higher sequence conservation of SMC1/3 relative to other SMC proteins was noted previously by Cobbe and Heck (2004), in their comprehensive review of SMC proteins. However, these authors did not speculate on the possible functional significance. We now suggest that this extraordinary constraint on the sequence divergence indicates that the entire length and surface of the coiled coils are intimately involved in the cohesion mechanism.

The function of cohesin is to hold sister chromatids together until the beginning of anaphase. Earlier models for cohesin function postulated that the SMC1 head bound to one chromatid, and the SMC3 head bound to the other, with the coiled coils forming a long link between the sister chromatids (Hirano, 2002). A more recent and very intriguing model proposes that sister chromatids are held together by cyclization of the cohesin molecule (Haering et al., 2002), produced when the two heads of a single cohesin are linked by the *scc1* subunit and form a ring (Haering et al., 2002) (Fig. 2). Initially, the cohesin ring encircles one DNA strand. When this strand is replicated (the DNA polymerases can fit through the SMC ring), it leaves both strands entrapped in the same ring. Anaphase is initiated by a specific protease that cuts the *scc1* and opens the ring. There is much intriguing evidence for this model, but some questions and controversy remain (Gruber et al., 2003; Haering et al., 2002).

In both models, and in the general thinking about SMC proteins, the N- and C-terminal domains are thought to be the functional units, while the coiled coils provide a long rod or spacer arm. If this were the case we would expect the terminal domains to be highly conserved, but the coiled coils would fall in the class of inert rods, with an expectation of ~20% sequence divergence. This appears to be true for SMC2/4 and SMC5/6. However, as noted above and in Table 4, SMC1 and SMC3 are among the most highly conserved proteins in mammals, and the conservation is as high in the coiled-coil segments as in the heads. This extreme constraint on sequence variation means that these coiled coils are not simply structural rods. We will present three speculations on possible reasons for this extreme constraint.

The first possibility is that the sequence conveys an essential structural property to the rods, either rigidity or flexibility. This seems unlikely, since it is difficult to see how conservative substitutions in the outward-facing *bcefg* positions could have a major effect on the mechanical properties.

A more attractive possibility is that the coiled coils are involved in binding interactions over their entire surface. A prime candidate for the binding partner is chromatin fibers. This is a novel suggestion—that the binding of chromatin to the coiled-coil domains could be an important part of the mechanism of sister chromatid cohesion. This possibility is not presented as an alternative to the cyclization model, but as a potential additional mechanism.

A third possibility is that the sequence constraint is not to preserve binding interactions, but the opposite—to maintain a surface structure that minimizes adhesion to chromatin. This may be important for the demonstrated ability of cohesin to slide over the chromatin in response to transcription (Glynn et al., 2004; Lengronne et al., 2004).

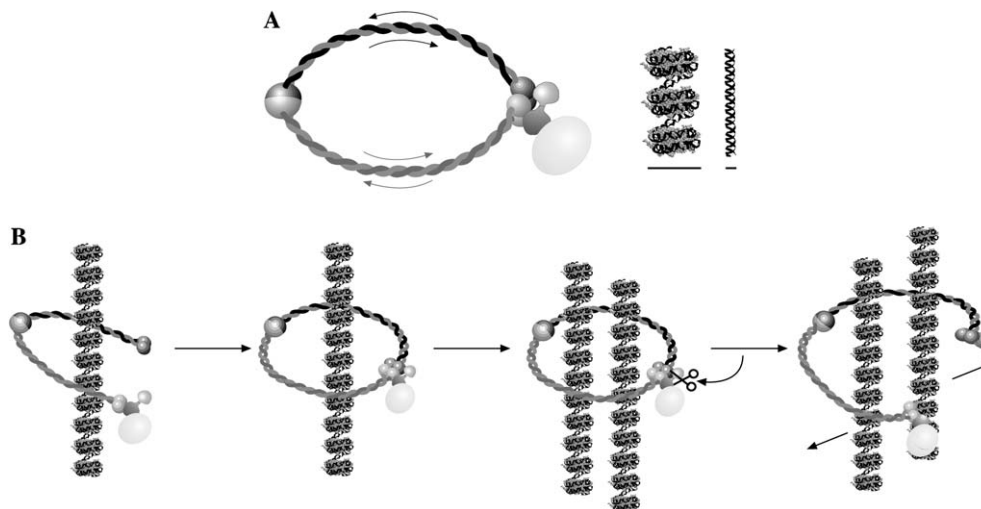


Fig. 2. The cyclization model of cohesin action. (A) The SMC1/3 dimer forms a circle when its heads are bridged by accessory proteins, in particular *Scc1* (see Fig. 2). To the right of the circle is shown to scale a 10 nm chromatin fiber of DNA wrapped around nucleosomes, and a double-stranded DNA molecule. (B) Before chromosome replication, the cohesin molecules wrap around the chromatin fiber and form closed circles, with the two heads linked by the *Scc1* subunit. Following replication the chromatin fibers of the sister chromatids are trapped in the SMC circle. They are released to start anaphase when the protease separase clips *Scc1* and opens the circle. The figure is reprinted from Haering et al, *Molec Cell* 9:773–88, 2002 with permission of the publisher.

Indeed both adhesion and anti-adhesion may be involved. The association of cohesin to the centromeric region involves specific DNA sequences (Gerton, 2005), which might bind the coiled coils, while association to the chromosome arms may be primarily via cyclization, with a need to provide smooth sliding in response to transcription.

The mechanisms involved in sister chromatid cohesion are still incompletely understood. The coiled coils have been largely ignored in past models. The extraordinary constraint in sequence divergence of the coiled coils suggests they are not just structural rods, but are an intimate part of the mechanism of sister chromatid cohesion.

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