RIP2 filament formation is required for NOD2 dependent NF-κB signalling

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Activation of the innate immune pattern recognition receptor NOD2 by the bacterial muramyl-dipeptide peptidoglycan fragment triggers recruitment of the downstream adaptor kinase RIP2, eventually leading to NF-κB activation and proinflammatory cytokine production. Here we show that full-length RIP2 can form long filaments mediated by its caspase recruitment domain (CARD), in common with other innate immune adaptor proteins. We further show that the NOD2 tandem CARDs bind to one end of the RIP2 CARD filament, suggesting a mechanism for polar filament nucleation by activated NOD2. We combine X-ray crystallography, solid-state NMR and high-resolution cryo-electron microscopy to determine the atomic structure of the helical RIP2 CARD filament, which reveals the intermolecular interactions that stabilize the assembly. Using structure-guided mutagenesis, we demonstrate the importance of RIP2 polymerization for the activation of NF-κB signalling by NOD2. Our results could be of use to develop new pharmacological strategies to treat inflammatory diseases characterised by aberrant NOD2 signalling.
NOD2 belongs to the Nod-like receptor (NLR) family, which are characterised by three functional domains: a C-terminal ligand-binding domain comprising leucine-rich repeats (LRs), a central ATP-binding and oligomerization domain (nucleotide oligomerization domain, NOD) and an N-terminal effector death-domain (DD), which in the case of NOD2 is a double CARD (caspase recruitment domain)6,7. The downstream adaptor RIP2 belongs to the RIP kinase family and comprises an N-terminal kinase domain, a C-terminal CARD domain and a bridging intermediate domain8. Upon cognate ligand binding, NOD2 oligomerizes and recruits RIP2 via CARD-CARD interaction5,7,9-14. After RIP2 auto-phosphorylation and ubiquitination, RIP2 becomes a platform for downstream protein effectors including several ubiquitin E3-ligases 15.

Excessive or absent NOD2–RIP2 signalling is associated with several genetic and non-genetic inflammatory diseases, which lack specific and effective therapies. Loss-of-function single nucleotide polymorphisms (SNPs) in NOD2, which result in impaired epithelial mucosal barrier function, are one of the major nucleotide polymorphisms (SNPs) in NOD2, which result in several genetic and non-genetic inflammatory conditions. There is still an incomplete understanding of its underlying factors.

Recent studies of other intracellular innate immune signalling pathways have shown that ligand-induced oligomerization of NOD2 induces RIP2 activation is an important goal since it could lead to the development of new therapies for these clinical conditions.

Here we present biophysical and structural data, showing that full-length RIP2 can form filaments that are mediated by RIP2–CARD oligomerization. We report the atomic structure of RIP2CARD filaments, solved by high-resolution cryo-electron microscopy (cryo-EM), which reveals the molecular interactions underlying its assembly. We show that NOD2CARDs can bind to one end of the RIP2CARD filaments, suggesting that NOD2 activation could nucleate RIP2 filament formation. Consistent with this, we use structure-guided mutants, designed to specifically disrupt RIP2 filament formation, to demonstrate, in vitro and in cell based assays, the relevance of RIP2 polymerization for the activation of NF-κB signalling following NOD2 stimulation.

**Results**

**RIP2 forms filaments in vitro via its CARD domain.** Using the baculovirus system in sf21 cells, we expressed and purified recombinant full-length human RIP2 with a cleavable maltose-binding protein (MBP) tag at the N-terminus16 (MBP-RIP2fl) (Fig. 1a–c). Negative-stain EM of MBP-RIP2fl eluting from the amylose resin shows that the sample is a mixture of aggregates and oligomeric protein (Supplementary Fig. 1a). Addition of ATP and magnesium promotes elongation of the aggregates into a filamentous structure (Supplementary Fig. 1b, Supplementary Fig. 2). After MBP tag cleavage, the protein was further purified by size exclusion chromatography yielding a void volume (VV) fraction and a soluble, non-aggregated fraction denoted RIP2fl (Fig. 1b–c, Supplementary Fig. 2). Analysis of the non-aggregated RIP2fl by electrospray ionization (ESI) mass spectrometry confirmed that the protein is highly phosphorylated (Supplementary Fig. 1c) and an in vitro phosphorylation assay showed that it is capable of further self-phosphorylation and is thus functionally active (Fig. 1d).

Interestingly, the polymerized RIP2fl fraction contains a central filamentous core (Fig. 1e, Supplementary Fig. 1d–e), whereas imaging the RIP2fl fraction confirms that it is non-aggregated and presumably dimeric (Fig. 1c, f and Supplementary Fig. 2). When ATP and magnesium were added to the non-aggregated RIP2fl fraction, the protein oligomerized into long filaments of diameter 30–40 nm and variable length (0.1–1 μm) and which have a tendency to side-by-side aggregation (Fig. 1g). Extended filaments could also be obtained from the VV fraction by adding non-aggregated RIP2fl, ATP and magnesium, with the VV aggregates acting as seeds (Supplementary Fig. 1f).

We then investigated the importance of different nucleotides in promoting RIP2fl polymerization. For this, we used the uncleaved MBP-RIP2fl fusion protein (Fig. 2a) rather than tag-free RIP2fl. This is because when ATP and magnesium are added, MBP-RIP2fl polymerizes into short filaments (0.1–0.2 μm) that aggregate less compared to those made with tag-free protein, making them easier to visualise by negative-stain EM (Fig. 2b).

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expressed and purified from \textit{E. coli} recombinant RIP2CARD (residues 435–540) with a cleavable MBP tag at the N-terminus (MBP-RIP2CARD, Fig. 3a–c). Upon MBP tag cleavage by the Tobacco Etch Virus (TEV) protease, RIP2CARD mainly migrated in the void volume (VV) of the size-exclusion chromatography column (Fig. 3b). Negative-stain micrographs revealed that RIP2CARD from the VV forms long filaments (Fig. 3d), which have similar length to the RIP2 filaments, but a smaller diameter of less than 10 nm.

The structure of monomeric RIP2CARD. To aid structural analysis of the RIP2CARD filaments, we determined the X-ray crystal structure of RIP2CARD (residues 435–540), using a construct with crystallisable MBP\(^3\) fused at the N-terminus (crystMBP-RIP2CARD, Fig. 3a, e). This construct crystallised in space group \(P_2_1\) with four molecules per asymmetric unit (Supplementary Fig. 3). The structure was solved by molecular replacement using NLRP1 CARD domain with a crystallisable MBP at the N-terminus (PDB accession code 4IFP\(^{3,9}\)) as search model and refined at 3.3 Å resolution (Supplementary Table 1). RIP2CARD has the typical CARD fold comprising a Greek key helical bundle with the N- and C-termini oriented in the same direction and with helix H1 broken into two shorter helices: H1a and H1b (Fig. 3e, Supplementary Fig. 3–4). The RIP2CARD crystal structure is very similar to the previously reported solution NMR structure (Fig. 3f, PDB code: 2N7Z\(^{4,0}\)), with a root-mean-square deviation (RMSD) of all Ca positions of 0.95 Å. Interestingly H6 is absent in both the crystal and NMR structures and replaced by a long C-terminal loop, visible only in the NMR structure, which contains putative phosphorylation sites\(^4,1–4,3\).
The structure of RIP2CARD within filaments. We used solid-state NMR to study the structure of RIP2CARD within the filament. In order to obtain backbone resonance assignments, we recorded $^1$H-detected (H)CANH, (HCO)CA(CO)NH, (HCA)CB(CACO)NH, (H)CONH and (H)CO(CA)NH spectra on $^2$H, $^{13}$C, $^{15}$N-labeled and 100% back-exchanged RIP2CARD samples at 60 kHz magic angle spinning (MAS)\(^44\). This data was evaluated together with $^{13}$C-detected $^{13}$C-$^{13}$C DARR correlations on protonated samples, that were either uniformly $^{13}$C-labelled or selectivity-labelled using [2-$^{13}$C]- or [1,3-$^{13}$C]-glycerol as carbon source during protein expression\(^45,46\). The analysis of the $^1$H-detected data yielded the sequence specific assignment for residues Q441 to Q507 (Supplementary Table 2), except for the loop residues 448–451 and 497. The chemical shifts of the assigned residues of filamentous RIP2CARD closely match many chemical shifts of monomeric RIP2CARD in solution showing that the overall conformation is maintained upon filament formation (Fig. 3g). We were not able to assign any cross-peaks to the C-terminal 29 residues that were reported to be flexible by solution NMR investigations\(^40\). To check whether these signals are absent in our MAS NMR spectra, we inspected $^{13}$C-$^{13}$C correlation spectra of the samples with a 2- or 1,3-glycerol labelling pattern. At short mixing times, the amino acids Leu, Pro, Thr and Val lead to characteristic cross-peak pattern that allow for a counting of signals. We observed signals corresponding to 9 of 14 leucine residues, 6 of 6 Thr, 5 of 7 Val and only 1 of 4 proline residues (Supplementary Fig. 5). Relying on the distribution of the respective amino acid types in the sequence, this strongly suggests that the missing signals concern residues in the C-terminal segment from 512 to 540. Especially the absence of three proline signal sets, only one being detected, indicates strong structural heterogeneity or mobility in that region where they cluster. Furthermore, the number of missing Leu and Val signal sets corresponds to the number present in the C-terminus and thus corroborates the lack of an ordered structure there, indicating that H6 is also absent in filamentous RIP2CARD.

NOD2CARDs bind to one end of the RIP2CARD filament. We have shown that both RIP2fl and RIP2CARD samples form filaments in vitro. However, in the cellular context, we expect that such polymerization is initiated by NOD2 oligomerization in response to cognate ligand binding. To recapitulate the core elements of this process, we set out to reconstitute in vitro a filamentous sample comprising the CARDS of both proteins that would be suitable for high-resolution structure determination by cryo-EM.

We first investigated whether NOD2 could be detected by immuno-gold labelling in RIP2CARD filaments formed in the presence of NOD2, following what was previously done for both...
the AIM2-ASC or NLRP3-ASC complexes. Using the baculovirus insect cell system we expressed and purified a truncated form of NOD2 with a TEV cleavable MBP tag, comprising the CARDs and NOD, but lacking the LRR domain (MBP-NOD2ΔLRR, residues 1–619) (Fig. 4a). This construct is presumed to be derepressed with the CARDs available for interaction. Indeed, a similar NLRP3 construct proved to be a more powerful ASC polymerization promoter compared with the full-length receptor.

Purified and tag-free NOD2ΔLRR eluted mainly in the void volume of a size-exclusion chromatography column (Fig. 4b–c) and consistent with this, negative-stain images showed that NOD2ΔLRR forms soluble aggregates (Fig. 4d). We mixed MBP-RIP2CARD with a less aggregated fraction of NOD2ΔLRR (Fig. 4b, d) and induced filament polymerization by addition of TEV. We then applied immuno-gold labelling against NOD2. As a control, we applied the same immuno-gold labelling to the NOD2ΔLRR sample in the absence of RIP2CARD (Fig. 4d). The control demonstrates that with the protocol used, NOD2ΔLRR can be specifically labelled, although with a heterogeneous number of gold-particles bound per aggregate. Micrographs of the NOD2ΔLRR sample mixed with RIP2CARD showed...
gold-particles on individual NOD2ΔLRR aggregates or NOD2ΔLRR aggregates bound to RIP2CARD filaments, mostly at one filament-end (Fig. 4e–f).

We then investigated by co-purification and immuno-gold labelling whether the NOD2CARDS are sufficient to interact with the RIP2CARD filament. For this, we used NOD2CARDS (residues 1–218) expressed with a cleavable N-terminal HIS-SUMO tag and a C-terminal SNAP tag (HIS-SUMO-NOD2CARDS) together with cleavable MBP-RIP2CARD (Fig. 5a). Due to the different requirements for optimal expression of these two constructs, we expressed them separately in E. coli and then mixed the pellets and co-puriﬁed the proteins. After clarification of the crude extract by centrifugation, the supernatant was applied to amylose resin and the eluate was analysed by SDS-PAGE and western blot (WB), using a speciﬁc antibody against the SNAP tag (Fig. 5b–c). The results showed that HIS-SUMO-NOD2CARDS co-elute with MBP-RIP2CARD (Fig. 5b–c). The diameter of filaments observed by negative-stain EM after HIS-MBP tag cleavage was the same as the homo RIP2CARD filaments, while their length ranged from 50 to 500 nm (Fig. 5d).

We then applied immuno-gold labelling against the SNAP tag. The results showed single gold-particles mostly sitting on filament-ends (Fig. 5d–e, Supplementary Fig. 6). In order to evaluate the binding position of NOD2CARD on RIP2CARD filament, two more controls were performed with the same immuno-gold labelling protocol: immuno-gold labelling on RIP2CARD filament without NOD2CARD (Control 1, C1) and immuno-gold labelling with only the secondary antibody on NOD2CARD-RIP2CARD filament (Control 2, C2) (Supplementary Fig. 6c, d). Control 1 was used to evaluate the speciﬁcity of the primary antibody, whilst Control 2 was used to judge the speciﬁcity of secondary antibody. We collected 20 random images on two different grids for each condition at a magniﬁcation of ×16,000 and evaluated the number and position of gold-particles.
in each micrograph (Supplementary Fig. 6f). Final statistics revealed that 70.9% of gold-particles are found on filaments, of which 91.7% are at one end and we never observe gold-particles at both ends. This shows that NOD2CARDs are preferentially bound at one end of the RIP2CARD filament. These data are consistent with the hypothesis that under physiological conditions activated NOD2 nucleates RIP2 filament formation yielding a polar assembly.

Cryo-EM of RIP2CARD filament. To elucidate the architecture of the RIP2CARD filament by cryo-EM, we optimized the protocols for production of both the RIP2CARD and NOD2CARDs-RIP2CARD filaments (Supplementary Fig. 7). For this we used a different RIP2CARD construct encoding for RIP2CARD (residues 431–540) with a P3C (human rhinovirus 3C protease) cleavable HIS-MBP tag at the N-terminus (HIS-MBP-RIP2CARD) (Supplementary Fig. 7a). This new RIP2CARD construct dramatically increased the tag cleavage efficiency (compare Fig. 3c with Supplementary Fig. 7c, f). We optimised the purification protocol for NOD2CARDs-RIP2CARD filaments, by introducing a size exclusion chromatography step before tag cleavage (Supplementary Fig. 7b). This allows aggregates to be discarded and the tagged NOD2CARDs-RIP2CARD complex to be separated from monomeric HIS-MBP-RIP2CARD. NOD2CARDs-RIP2CARD complexes and RIP2CARD were then recombined as described in the Methods. After cleavage,
HIS-MBP, HIS-SUMO tags and HIS-tagged proteases, were removed by affinity chromatography followed by dialysis with a high molecular weight cut off (Supplementary Fig. 7c). RIP2CARD filaments were prepared following the same protocol (Supplementary Fig. 7f). Negative-stain and cryo-EM micrographs of samples prepared under the same conditions show that RIP2CARD filaments with bound NOD2CARDs are shorter, straighter and have a lower tendency to aggregate than RIP2CARD filaments without NOD2 (Supplementary Figs. 7d, e, g, h). Therefore, the hetero-CARD filaments were used for cryo-EM data analysis and collection (Supplementary Table 3). Visual inspection of the individual cryo-EM images, 2D class-averages and corresponding power spectra indicate that the RIP2CARD filament has a helical symmetry (Fig. 6a–c). Indexing of the power spectra and symmetry refinement revealed a left-handed helix of 3.56 subunits/turn with an axial rise of 4.848 Å/subunit (Supplementary Fig. 8a, b). The final cryo-EM map at 3.94 Å resolution (Supplementary Fig. 8c) shows that the filament has an approximate outer diameter of ~75 Å with a central solvent channel of ~25 Å diameter (Fig. 6d). The crystal structure of RIP2CARD can be unambiguously fitted into the cryo-EM density, with both N- and C-terminal ends orientated towards the surface. In the case of the RIP2CARD, which lacks H6, we note independently conserved interactions (Fig. 7e and Supplementary Fig. 9c), these side-chains being specific to RIP2CARD (Supplementary Fig. 4). This interface is further reinforced by a polar interaction between N512 and Q458 (Fig. 7e). Moreover, Q497 (type IIa) can interact with the side chains of Q450, T452 and Q514 (type IIb) (Fig. 7e).

The type III interaction normally occurs between H3 (type IIIa) and a groove formed by H1-H2 and the H3-H4 loop (type IIIb). In the RIP2CARD filament, type IIb comprises the H3-H4 loop and N-terminal of H4, whilst the H1-H2 loop is only contributing to the type IIa surface as described above (Fig. 7f). P481, T482 (type IIIa) and A476 (type IIb) contribute hydrophobic interactions, whilst E472 and E475 from H3 (type IIIa) potentially form salt bridges with R488 and R483 (type IIb). Interestingly, both side chain of T482, T484 could swap from the original position in the crystal structure to interact with type IIIa instead of contributing to the intra-ahelical interactions (Fig. 7f).

Figure 7g shows that the most significant chemical-shift differences between RIP2CARD in solution and within the filament, as determined by NMR, map to the subunit interfaces described above. These chemical-shift differences report on local conformational changes due to packing effects in the filament and therefore independently confirm the overall architecture of the intermolecular interfaces observed by cryo-EM. Notably, residues K443, R444, D495, L457, L456, K471 and Y474, located close at the type I interface, undergo strong chemical shift changes in the filament. Residue Y474 shows the largest effect. At its Cα resonance, multiple contacts in 13C-13C correlations employing long mixing times are observed, which indicates tight packing of Y474 in the filament. Similarly, conformational changes in the type IIa (M470) and type IIb (R483, T484) residues lead to significant chemical shift changes of the respective residues, indicating a change in environment of these residues.

Structure of the RIP2CARD filament. The RIP2CARD filament has a similar helical configuration to other CARD filaments already described, such as MAVS CARD, Caspase-1 CARD and the recently described BCL10 CARD filament30,31,34,35 (Supplementary Table 4). Following the established convention, the RIP2CARD assembly can be described through interactions at three major asymmetric interfaces, named type I, type II and type III47. Type I and II are inter-strand interactions, whilst type III is intra-strand along the helical strand trajectory (Fig. 7a–c).

The type I interface is defined as the interaction between helices H1 and H4 of one molecule (type Ia surface) with H2 and H3 of the adjacent one (type Ib surface). In the RIP2CARD filament, the type I interaction is electrostatic in nature involving several charged residues that form polar interactions (Fig. 7d). D461 and N457 from H3 (type Ib) interact with R444 and R448 from H1 and H4 (type Ia) respectively. The interaction between H1 (type Ia) and H2 (type Ib) is further reinforced by backbone contacts between the break in H1 and the C-terminus of H2 (Fig. 7d, Supplementary Fig. 9a-b). Additional charged or polar residues such as D492, D495, Q441, E445 and Q489 (type Ia) and D467, K471 (type IIb) contribute to the type I interface (Fig. 7d).

The type II interface is normally defined by the interaction between the C-terminal end of H4 and the H4-H5 loop (type IIa surface) and a groove defined by the H1 and H2 corner from one side and H6 helix and its preceding loop on the other (type IIb surface). In the case of the RIP2CARD, which lacks H6, we identified a somewhat different type II interface. The type IIa surface includes the C-terminal end of H4, the H4-H5 and H2-H3 loops, whilst the type IIb surface comprises the H1-H2 loop, the N-terminal of H2 and the visible part of the RIP2CARD C-terminal (Fig. 7e). At the type II interface, the side-chains of M470 (type IIa) and C455 (type IIb) make van der Waals interactions (Fig. 7e and Supplementary Fig. 9c), these side-chains being specific to RIP2CARD (Supplementary Fig. 4). This interface is further reinforced by a polar interaction between N512 and Q458 (Fig. 7e). Moreover, Q497 (type IIa) can interact with the side chains of Q450, T452 and Q514 (type IIb) (Fig. 7e).

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Mutational analysis of the RIP2CARD type II interface. Our immuno-gold labelling results show that NOD2CARDs bind at one end of the RIP2CARD filament, suggesting that an initial hetero-CARD complex might act as a nucleation point to promote unidirectional RIP2CARD filament growth. Available structures or models of hetero DD complexes, such as RIG-MAVS, the Myddosome, the PIDDosome and NLRP3-ASC, show that the DD belonging to the receptor protein continues the helical arrangement of the effector DDs, by forming a combination of the same type I–II–III interfaces. With a view to testing the effect on signalling of site-directed mutants that would uniquely disrupt the RIP2CARD filament structure and not the RIP2NODE2 hetero-CARD interaction, we modelled the hypothetical hetero-CARD type I, II, III interfaces and evaluated their importance based on the available interaction and mutagenesis studies12,14 (Supplementary Fig. 10). As the structure of NOD2CARDs is not yet available and the only reported direct interactions are to the N-terminal node2CARDs (residues 26–122), we computed a three-dimensional NOD2CARDa model using Swiss Model48 using as template the X-ray structure of the CARD of Nucleolar Protein 3 (PDB code:4UZ0)49, which shares 35% identity with NOD2CARDa (Supplementary Fig. 10a). The NOD2CARDa model structure obtained is similar to RIP2CARD (RMSD of 1.70 Å for all Ca positions of residues 433–518) and the presumed flexible C-terminus is not observed in the EM map.
Therefore, mutagenesis of RIP2 residues involved in these interfaces would affect both the interaction with NOD2 and filament formation. Conversely, none of the residues belonging to the observed RIP2 homo-type II interface have been explicitly implicated in the NOD2CARD-RIP2CARD interaction. We deduced that type II interactions within the RIP2CARD filament, notably involving RIP2CARD specific hydrophobic residues C455 and M470, could specifically stabilise homo-interactions within the filament. We therefore mutated the residues belonging to RIP2CARD type IIa and IIb surfaces (type IIa: M470 and Q497; type IIb: Q450, T452, E453, C455, Q458 and N512) by alanine and lysine substitution (serine for C455). We then assayed the ability of each mutated RIP2CARD construct to bind NOD2CARDSS, using the co-purification protocol described above (Fig. 8a, b). Mutant RIP2CARD domains were expressed at similar level to wild-type RIP2CARD and all the mutants displayed unimpaired binding to NOD2CARDSS, except for RIP2CARD T452K, which clearly...

Fig. 6 Cryo-EM structure of the RIP2CARD filament. a Cryo-EM image of NOD2CARDSS-RIP2CARD filaments used for structure determination. b, c 2D-class average (b) and corresponding power spectra (c) used for initial symmetry parameter estimation. d Final cryo-EM map of the three-dimensional RIP2CARD filament at 3.94 Å resolution (FSC in Supplementary Fig. 8c). Outer, inner and top view without (left) and with (right) RIP2CARD models fitted into one helical turn. e View of the RIP2CARD monomer fitted into the EM map showing only the main chain for clarity. f Zoomed-in views of the fitting of individual α-helices into the sharpened cryo-EM density. Helices are defined as in the sequence alignment (Supplementary Fig. 4) except for H1, which starts from I435 in the RIP2CARD monomer within the filament. Sidechains are shown as stick models. A few residues with clear EM density for sidechains are labelled.
Fig. 7 Structural analysis of the RIP2CARD filament assembly. a Ribbon diagram of RIP2CARD filament comprising 10 subunits. b, c Schematic diagram of the helical filament (b) and relative orientations of type I, type II and type III interfaces (c). Each subunit is represented as a hexagon with the same colour code as in the filament structure in (a). Each turn comprises 3.56 subunits. The fourth subunit is represented as a half-empty hexagon to highlight that it is shared with the next turn. Type I, II, III interfaces are represented as a single line, single-dashed line or double line respectively. d-f Ribbon diagram of RIP2CARD dimers interacting through (d) type I, (e) type II and (f) type III interfaces. Protein regions involved in the interface are highlighted using the colours as in (c). The insets show the interactions at relative type surface. H-bonds are represented by black dashed line. Backbone contacts are highlighted by blue dash line, respectively. Sulphurous groups involved in Van der Waals interactions in the type II surface are represented as spheres. g Sum of $^{13}$C$_\alpha$ and $^{13}$C$_\beta$ chemical shift differences between RIP2CARD in the solution and the solid state for each assigned amino acid. Residues that show a strong variation from the mean (1.2 p.p.m.; dotted red line) are labelled in the same colour code as used in (d-f). The secondary structure elements and positions of the interface surfaces are also shown.
showed lower binding in comparison to wild-type and all the other mutants. We then tested the ability of each construct to polymerize after tag cleavage by imaging the sample with negative-stain EM. The micrographs revealed that many constructs can still polymerise (Supplementary Fig. 11), but with a dramatic change in the filament quality notably for mutants T452K, E453K, C455S, M470A and M470K (Fig. 8c–h).

RIP2CARD-T452K forms long filaments but with multiple interruptions compared to wild-type and furthermore they have impaired binding to NOD2CARDS (Fig. 8b–d). This indicates that the mutation negatively affects both binding to NOD2CARDS and filament quality. Micrographs of RIP2CARD-E453K and RIP2CARD-M470A show particles that might correspond to protein aggregates with rare filaments (Fig. 8e, g). RIP2CARD-C455S forms irregular, more flexible filaments with a high tendency to aggregate (Fig. 8f). M470K micrographs show protein aggregation and absence of filaments (Fig. 8h).

We next investigated the effect of these mutations on the activation by NOD2 of transcription factor NF-κB, by using a luciferase reporter assay (Fig. 9 and Supplementary Fig. 12). We transiently transfected HEK293T cells with HA tagged RIP2CARD mutants (Fig. 9a) together with a plasmid encoding firefly luciferase under the control of NF-κB promoter. NF-κB activation was induced using the specific NOD2 activator MDP, and cells were lysed 20 h later to record luciferase activity and assay protein expression. In agreement with the in vitro data, mutants Q450, Q458, Q497, N512 show unimpaired NF-κB signalling; with the particularity that Q450K, Q497A and Q497K...
show high levels of auto-activation compared with the wild-type RIP2 (Fig. 9b, d). In contrast, T452K, E453K, C455S and M470K failed to transmit the signal from NOD2 to NF-κB, as the luciferase value is equal or lower than the control empty vector (Fig. 9b, d). The less drastic alanine mutants T452A, E453A, C455A and M470A also showed lower or zero activity (Fig. 9b, d). All the mutants show similar protein expression to wt RIP2 (Fig. 9c, e and Supplementary Figure 12).
Discussion

Recent studies on several innate immune systems have shown that recognition of cognate ligands by PRRs that contain a DD (e.g. CARD and PYD) triggers their oligomerization and interaction with the downstream adaptor resulting in the formation of a higher-order filamentous assembly called a signalosome\(^9,^{31}\). Here we focus on the NOD2-RIP2 signalling pathway, a receptor-adaptor protein combination that shares close structural similarities with PRRs involved in signalosome formation. Specifically, we investigated whether the recruitment of RIP2 by NOD2 via CARD-CARD interactions could lead to the formation of such a signalosome (‘nodosome’). Our biophysical, structural and functional data show that RIP2, via its CARD, can form helical filaments, plausibly nucleated from one end by activated NOD2. Furthermore, we show that RIP2 polymerization is essential for NF-κB activation by NOD2, presumably by favouring recruitment of downstream effectors such as the RIP2 ubiquitin ligase XIAP\(^17\).

The starting point was our finding that phosphorylated and active RIP2fl forms filaments in vitro in the presence of ATP and magnesium. The subsequent observation that RIP2CARD also spontaneously forms more slender filaments, suggests that the CARD domain forms the core of the RIP2fl filaments, while the kinase domain (RIP2K) is on the exterior. Interestingly we observed that not only ATP, but also non-hydrolysable adenosine nucleotides together with magnesium promote polymerization of RIP2fl. This suggests that enhanced RIP2 polymerization by nucleotide-binding results from RIP2K structure stabilization rather than any increase in RIP2K auto-phosphorylation activity. Our previously published biophysical data\(^20\), show that stable activation of RIP2K involves the coupling of kinase dimerization with auto-phosphorylation of the activation loop. We therefore speculate that CARD polymerization promotes kinase dimerization, by increasing the local RIP2 concentration. Dimerization favours the kinase domain being in the active conformation and therefore able to bind any adenosine-derived nucleotide, independently from the phosphorylation state of the activation loop. In a physiological context, RIP2 could either be already phosphorylated, as it was reported that auto-phosphorylation contributes to protein stabilization\(^36,^{52}\) or further phosphorylated upon dimerization to stabilize the active conformation. These observations are in line with a recent study showing that an active conformation of the RIP2 kinase, rather than necessarily a catalytically active kinase, is essential for NOD2 signalling, since it permits interaction with the E3 ligase XIAP\(^17\).

In order to investigate the assembly mechanism of the RIP2 filament core, we co-purified RIP2CARD and NOD2CARDs and used immuno-gold labelling to show that NOD2CARDs bind preferentially at one of the two filament-ends, forming a polar assembly. We then successfully imaged this sample using cryo-EM, obtaining an EM density map at 3.94 Å resolution, where we observed the X-ray structure of RIP2CARD either to bind NOD2CARDs or to polymerize in vitro. We speculate that alanine mutations have a milder effect, compared to changing the charge, that cannot be detected by negative-stain EM, except for the case of M470A, whose severe disruptive effect highlights the importance of this RIP2 specific residue in forming the type II interface in the filament. In conclusion, we provide evidence for the existence and biological importance of a NOD2-RIP2 polar filamentous assembly, which is likely the core of the nodosome complex. Based on the results described here, other published data and analogy to other signalosome systems, we propose that nodosome assembly occurs as follows (Fig. 10): (1) binding of MDP to NOD2 LRR domain activates the receptor causing derepression of the CARDs; (2) NOD2 oligomerises via its NOD and CARD domains; (3) oligomerized NOD2 recruits RIP2 via its CARD domain forming the hetero-CARD complex (4) cumulative binding of RIP2 to the hetero-CARD complex promotes filament elongation to form the helical assembly here described; (5) polymerization of RIP2CARD...
in the presence of ATP stabilises the active antiparallel dimeric form of RIP2K; (6) E3 ligases, such as XIAP bind the active from of RIP2K; and (7) RIP2 becomes K63-ubiquitinated enabling it to recruit downstream effector proteins.

Finally, by combining our biophysical, structural and functional analysis with existing data, we provide essential information that could potentially be used to explore new therapeutic options for inflammatory diseases characterised by aberrant NOD2-RIP2 signalling.

Methods

Constructs, protein expression and purification. Constructs described in this paper were generated from pcDNA3 plasmids encoding human RIP2 and human NOD2. The N-terminally HIS-tagged TEV (Tobacco Etch Virus) and P3C (human rhinovirus 3C) proteases used in this paper were produced at the Protein Expression and Puriﬁcation Core Facility at EMBL, Heidelberg, Germany.

Recombinant human RIP2 and human NOD2ΔLRR(1–619) were produced using the baculovirus system in sf21 insect cells. The DNA sequence of RIP2 and NOD2ΔLRR were cloned from pcDNA3 into the vector pFastBacHTB using NcoI and HindIII cloning sites. Using the In-Fusion cloning technology (Takara Clontech), the original TEV cleavable HIS tag was replaced with a TEV cleavable maltose-binding protein (MBP) tag, which improved both expression and stability of recombinant proteins during insect cells expression (MBP-RIP2f and MBP-NOD2ΔLRR). MBP-RIP2f and MBP-NOD2ΔLRR were expressed and purified following the same protocol. Virus generation and amplification, insect cell infection and protein expression were performed at the EMBL Eukaryotic Expression Facility. Briefly, Sf21 cells at 0.6 x 10^9 cells ml^{-1} were infected with a virus shot able to stop cells growing in 24 h. On the fourth day post-infection, cells were harvested and re-suspended in 1 x 10^{-3} (v/v) ratio of lysis buffer 20 mM Tris pH 7.5, 50 mM NaCl, 2 mM β-mercapto-ethanol (βMe), 0.01% NP40 supplied with protease cocktail inhibitor (Complete, Roche). Using a douncer, cells were homogenized and afterwards centrifuged at 18,000 x g for 30 min. The resulting supernatant solution was incubated for at least 2 h with amylace-affinity chromatography resin (New England Biolabs), whilst gently shaking at 4 °C. The fusion protein was then eluted using the same lysis buffer supplemented with 40 mM maltose. Upon overnight TEV cleavage, either RIP2f or NOD2ΔLRR were applied to size exclusion chromatography, using a similar buffer composition of lysis buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM TCEP).

For crystallization purposes, RIP2CARD (435–540), was cloned into pETXM1 plasmid using the NcoI and XhoI cloning sites, resulting in a protein construct with an N-terminal crystallisable MBP (crystMBP-RIP2CARD) spaced by a three alanine linker. The construct was expressed in E. coli Rosetta 2 (Novagen) by growing the bacterial culture at 37 °C until an OD_{600 nm} of 0.6 and inducing with 0.3 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) overnight at 16 °C. The cells where harvested, re-suspended in 1 10^{-1} (v/v) ratio of lysis buffer 20 mM Tris pH 8, 50 mM NaCl, 2 mM (β-Me) containing protease cocktail inhibitor (Complete, Roche) and lysed by sonication. The crude extract was centrifuged for 30 min at 18,000 x g and the soluble fraction was applied to amylase-affinity chromatography resin (New England Biolabs) and purified as described above. After elution, the fusion protein was applied to a prepacked anion exchange chromatography column (GE Healthcare) with a 0 to 1 M NaCl gradient. The protein was further purified on...
A Superdex 200 size exclusion chromatography column (GE Healthcare), in buffer containing 20 mM Tris pH 8 and 50 mM NaCl. The protein corresponding to the monomeric peak was used immediately for crystallization purposes.

For immuno-gold labelling experiments and solid state NMR, RIP2CARD (435–540) was cloned in pETM40, which results in RIP2CARD with N-terminal MBP tag (MBP-RIP2CARD). Protein was expressed in E. coli Rosetta 2 5, growing the bacterial culture at 37 °C until OD600 of 0.6 and inducing with 0.3 mM IPTG for 4 h at 18 °C. Protein was either purified as described for crystMBP-RIP2CARD, or combined with NOD2CARDs as described in the immuno-gold labelling section.

For production of the NOD2CARDs-RIP2CARD filament sample, RIP2CARD (431–540) was cloned into pETM15 using the restriction sites NcoI and Xhol. By applying the technology in-Fusion, a TEV cleavable HIS-SUMO tag was added to the C-terminus (HIS-SUMO-NOD2CARDs). The construct was transformed and expressed in E.coli Rosetta 2 by growing the bacterial culture supplied with 0.04% (w/v) glucose at 37 °C until an OD600 nm of 0.6 and inducing with 0.3 mM IPTG overnight at 16 °C.

For production of RIP2CARD filaments bound to NOD2CARDs, 1.1 of RIP2CARD culture (or ~5 g of bacterial pellet) was combined with 100 ml of NOD2CARDs (or ~0.9 g of bacterial pellet) and resuspended in 100 ml of lysis buffer (20 mM Tris pH 8, 50 mM NaCl, 1 mM TCEP and Complete protease inhibitors (Roche)). The sample was then lysed by sonication and crude extract was let on gel-casting for 30 min on 4 °C. After centrifugation at 18,000 × g for 30 min, the soluble fraction was incubated for 2 h with amylase-affinity chromatography resin. The sample was then eluted using lysis buffer supplemented with 40 mM maltose and complex formation was checked on SDS-PAGE. The amylose eluate was successfully passed through size exclusion chromatography (Superdex 200, GE Healthcare) equilibrated in 20 mM Tris pH 8, 50 mM NaCl, 1 mM TCEP to separate aggregates, NOD2CARDs-RIP2CARD complex and RIP2CARD monomer. NOD2CARDs-RIP2CARD complex at 0.25-0.35 mg ml⁻¹ and monomeric RIP2CARD at 0.45-0.55 mg ml⁻¹ were then combined at the desired ratio (usually 1 x 10⁻³/v/v) and filament formation was checked by adding protease overnight at 4 °C. HIS-MBP and HIS-SUMO tags, proteases and uncleaved protein were removed by applying the sample to a Ni-NTA resin (Takara). Further purification of filaments was achieved by dialysis overnight using a membrane with a 300 kDa cut off. SDS-PAGE and negative-stain EM were used along the purification procedure to check sample homogeneity and filament formation. Filaments containing only RIP2CARD were obtained and purified following a similar protocol. To avoid CARD protein aggregation all the purification steps were carried out at 4 °C.

Gel filtration calibration curve. The calibration curve of the Superdex 200 column (GE Healthcare) was used to purify RIP2fl, RIP2CARD and NOD2CARD (Supplementary Fig. 2) was produced by applying the instructions kit of NATURE COMMUNICATIONS| DOI: 10.1038/s41467-018-06451-3 | www.nature.com/naturecommunications

In vitro radioactive phosphorylation assay. We used an in vitro radioactive assay to analyse the autophosphorylation activity of RIP2CARD. 1.2 μg of freshly purified RIP2CARD was mixed with 10 μM ATP (10:1 ATP-gamma-32P) and 10 mM MgCl₂. The reaction was incubated at 30 °C and blocked at 1, 3, 5, 10, 15 min by adding SDS loading buffer. Resulting samples were used at 12% SDS-PAGE gel and results were revealed using a Typhoon scanner (GE Health).

Liquid chromatography/electrospray ionization mass spectrometry. The phosphorylation profiles of freshly purified and non-aggregated RIP2fl by Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC/ESI-MS) was analysed using a 6200 TOF mass spectrometer coupled to a HPLC system (1100 series, Agilent Technologies). The mass spectrometer was calibrated with tuning mix (ESI-L, Agilent Technologies) and the following settings were used: gas temperature (nitrogen) 300 °C, drying gas (nitrogen) 7 L min⁻¹, nebulizer gas (nitrogen) 10 psig, Vcap 4kV, fragmentor 250 V, skimmer 60 V, Vpp (octopole RF) 250 V, and extractor 0 V. A number of peptides were prepared using mobile phase A composition was: H₂O 95%, ACN 5%, TFA 0.03%. When mobile phase B was ACN 95%, H₂O 5%, TFA 0.03%. Two samples were analysed: freshly purified and non-aggregated RIP2fl (phosphorylated RIP2fl) and the same RIP2fl sample supplemented with 0.3 U of lambda protein phosphatase (New England Biolabs) per μg of protein (de-phosphorylated RIP2fl). The second sample was measured after 1.5 h of incubation at room temperature. Using a C8 reverse phase micro-column (Zorbax 300SB-C8, 5μm, 5 x 0.3 mm, Agilent Technologies) the protein samples were desalted on-line for 3 min at a flow rate of 0.1 ml min⁻¹ with 50 mM NaCl; the samples were then eluted at 50 μl min⁻¹ with 70% of mobile phase B, spectra were acquired in the positive ion mode in the 300-3000 m/z range. Data were processed with MassHunter software (v. B.02.00, Agilent Technologies) and the number of RIP2fl phosphorylation sites was calculated by comparing the two spectra obtained.

Negative-stain EM. Two different protocols were used to prepare negative-stain EM. For RIP2CARD sample, 4 μl of sample were applied to the clean side of the carbon膜 on a carbon-mica interface, letting the sample absorb for 20 s. The carbon film was then floated on a drop of 2% (w/v) uranyl acetate, picked up with a 400-mesh copper grid (Electron Microscopy Science) and dried on filter paper (Whatman).

For all the other samples, 6 μl of protein solution were applied to glow-discharged carbon coated copper grid (300 mesh, Electron Microscopy Science) and let adsorb for 30 s. Grids were then washed twice in 25 μl drop of protein buffer and stained twice for 30 s with 6 μl of 2% (w/v) uranyl acetate. Between each step excess of protein/buffer solution/staining was blotted off using a filter paper. Grids were dried on adsorbing paper for at least 5 min before storage.

Negative-stain preparations were imaged either with a JOEL 1200 EX II microscope at 100 kV on photographic film or with a Tecnai 12 (FEI) TEM at 120 kV on a Ceta 16 M camera, at a nominal magnification of 16,000× to 48,000×. Negative-stain preparations were imaged to investigate the effect of nucleotides on RIP2 polymerization (see below), were imaged using a Tecnai 12 (FEI) at a nominal magnification of 30,000×.

Polymerization of MBP-RIP2fl and RIP2fl. Polymerization of RIP2fl was induced by mixing 20 μl of purified tag-free RIP2fl at 0.3 mg ml⁻¹ with 5 mM ATP (Sigma) and 10 mM MgCl₂. After one hour of incubation at room temperature, sample was visualised by negative-stain EM.

In order to assay the relevance of ATP for RIP2 polymerization, MBP-RIP2fl was purified as RIP2fl, omitting the tag cleavage step. Protein was concentrated until 1 mg ml⁻¹ in final buffer 20 mM Tris pH 7.5, 50 mM NaCl and 2 mM MgCl₂. Nucleotides (ATP, AMPPCP ADP, AMP, from Sigma) were prepared as follows: nucleotide stocks were dissolved in Milli-Q water at 100 mM and stored at ~80 °C. For each experiment a new aliquot was quickly thawed and diluted twice in 1 M Tris pH 7.5, 100 mM MgCl₂ and successively diluted 10 times in protein sample. MBP-RIP2fl was then incubated with 5 mM ATP, ADP, AMP or AMPPCP (Beta, Gamma-methylene-adenosine 5’-triphosphate) overnight at room temperature. As controls we also prepared MBP-RIP2fl and MBP-RIP2fl supplemented with 10 mM MgCl₂. For each sample one negative-stain grid preparation was made and micrographs collected. The entire experiment was repeated twice.

Structure determination of crystMBP-RIP2CARD. Freshly purified crystalMBP-RIP2CARD was concentrated to 6 mg ml⁻¹ and used immediately for crystalization trials. Initial crystalization conditions were established by testing several commercial screens at the EMBL High Throughput Crystalization Laboratory (Grenoble, France) using a Cartesian robot. The best crystals were obtained at 4 °C with the sitting drop method from solutions containing 6 mg ml⁻¹ of crystalMBP-RIP2CARD and 37.5% w/v NaOAc and 27% w/v 16 M camera, at a nominal magnification of 16,000× to 48,000×. For controls we also prepared MBP-RIP2fl and MBP-RIP2fl supplemented with 10 mM MgCl₂. For each sample one negative-stain grid preparation was made and micrographs collected. The entire experiment was repeated twice.
described was used. Deuteriation was achieved by using D₂O instead of H₂O for all medium components.

Purification was done in the presence of 2 M urea to prevent aggregation or too early filament formation as reported. Cells from 1.2 L culture resuspended in 20 mM Tris pH 8, 20 mM NaCl, 2 M urea, 2 mM β-Me, 1 mM Pefabloc (Sigma), 5 mM MgCl₂, and 15 μM Benzonase (Merck) were disintegrated by high pressure (Microfluidizer LM10, Microfluidics). After centrifugation at 22,000 x g, for 1 h at 4 ºC, the supernatant was filtered (0.45 μm) and incubated with about 12 ml 50 % amyllose resin (New England Biolabs) at 15 ºC on a rotator for 3 h. MBP-RIP2CARD was eluted with 10 mM maltose in 20 mM Tris pH 8, 20 mM NaCl, 2 M urea, 2 mM β-Me after a washing step. The fusion protein, at about 1 mg ml⁻¹, was cleared by TEV protease overnight at 22 ºC. The next night was used for dialysis against 20 mM Tris pH 8, 20 mM NaCl, 2 mM β-Me at 8 ºC to remove urea and to begin filament formation lasting a further 48 h at 20 ºC. Filaments were collected by ultracentrifugation at 35000 x g for 1 h to produce a pellet for solid state NMR and quality checking by negative-stain EM.

Solid state NMR. The RIP2CARD filaments were packed into the respective rotor by ultracentrifugation at 100000 x g for 1 h using a custom-made filling device. All proton-detected experiments were recorded on a wide-bore 800 MHz spectrometer equipped with a 1.3 mm triple-resonance MAS probe (Bruker, Karlsruhe, Germany). Typical n/2-pulse lengths were 2.5 μs for 1H, 5 μs for 13C and 7 μs for 15N. The MAS frequency was set to 60 kHz and the sample temperature was kept at approximately 295 K. For the backbone assignment, a standard set of experiments, (H)CANH, (HCO)(CA)(CNH) and (HCA)(CB)(CNH), was recorded on 2H, 13C, 15N-labeled RIP2CARD samples. Water suppression was achieved using the MISSISSIPPI sequence and WALTZ-16 was used for 13C and 15N decoupling during proton detection. The spectra were processed with NMRPipe employing shifted-sinebell and Lorentzian-to-Gaussian apodization functions. The 1H-13C 15N DARR correlation spectra were recorded on wide bore 600 and 700 MHz spectrometers equipped with 3.2 mm triple-resonance MAS probes (Bruker, Karlsruhe, Germany). Typical n/2-pulse lengths were 3.1 μs for 1H, and 5 μs for 13C. All 2D spectra were recorded at either 13 333 (on the 600 MHz) or 15 555 Hz (700 MHz) MAS frequency and a sample temperature of approximately 285 K. Various mixing times, with durations of 10, 50, 150, 300 and 500 ms were applied after MBP and HIS-SUMO tags cleavage without further purification. The two straight 2D class-averages were subsequently used as templates for automatic picking of helical segments in RELION, using a maximum curvature parameter of 0.14, and a minimum segment length of 805 Å. The coordinates of filament extremities were converted into EMAN2 format, while shortening them by 180 Å at each extremity, to avoid including the hetero-CARD complex. This resulted in 4443 filament sections for a total length of 260 μm. All subsequent processing steps were performed in the helical reconstruction software SPINDLE SPRING. The complete model was obtained by 3D auto-picking from the manually picked data set and the symmetry parameters as estimated from the 2D class average power spectrum indexing, with low-pass filtering to 20 Å. The auto-picked filament coordinates were used to extract 37043 segments using a segment length of 400 Å and segment step size of 70 Å. The symmetry parameters were further refined with the module Segre13Dgrid, by defining a 11*11 grid spanning between 17.2-17.4 Å (step 0.02 Å) for the pitch and between 3.4-3.6 (step 0.02) for the number of units per turn. The maximum of the amplitude correlation between experimental and reprojection power spectra was found at a pitch of 17.26 Å, 3.56 units per turn (Supplementary Fig. 8b), corresponding to a left-handed helix with a 14.24 Å axial rise. The symmetry parameters, we performed a high-resolution structure refinement using the auto-picked segments with a strict segment selection during refinement, based on geometrical restraints, namely filament straightness (70% of straightest filaments kept) and forward-backward difference (limited to 5 Å). This resulted in a final reconstruction including 43566 segments (corresponding to 135,254 asymmetric units after symmetrization) at a resolution of 3.94 Å (FSC between half data set maps, cut off 0.143, Supplementary Fig. 8c). For visual display and the model building, the EM map was filtered to 3.9 Å and sharpened using a B-factor of 200 Å.

Visualization of the resulting map and initial rigid body fitting of the crystallographic structure was done using Chimera. Atomic model refinement was done using PHENIX real space refinement and manual adjustment with Coot. The crystallographic model was used to assign side chains for the residues without clear EM density map. Structure validation was done with Molprobity. The model was co-produced with Phenix (PDB: 6E6L) and Chimera. The model was installed and configured by SBDgrid. Data collection, image processing and refinement statistics are reported in Supplementary Table 3 and Supplementary Figure 5d.

Production of RIP2CARD mutants and Western blots. RIP2CARD mutants were produced by PCR mutagenesis of the HIS-MBP-RIP2CARD construct, using the oligos and their complements as reported in Supplementary Table 5. Expression was performed using E. coli BL21 and CO110s.

For co-production of RIP2CARD mutants with NOD2CARDs, 0.250 ml (~ 2.5 g of bacterial pellet) of HIS-MBP- RIP2CARD mutants culture was resuspended in 50 ml of lysis buffer (20 mM Tris pH 8, 50 mM NaCl, 1 mM TCEP and Complete protease inhibitor cocktail (Roche)), could be suing thus be treated as described for NOD2CARDs-RIP2CARD filament sample. After elution from the amyllose resin, complex formation was checked on SDS-PAGE, and presence

Cryo-EM movies where automatically collected on an FEI Polara electron microscope operated at 300 KV. Micrographs were recorded on a K2 direct electron detector operated in counting mode using the software Latitude S, at a nominal magnification of 41270 × (corresponding to 1.21 Å/pixel at the specimen level), with a defocus range of -1.5 to -3.5μm. 720 movies of 40 frames were collected with a total dose of 50 electrons Å⁻², were motion-corrected and dose-weighted using MotionCor2. The defocus estimation was performed with CTFIND4D/CTFFIND.

Initially, 4125 sections of filaments (total length 97 μm) were manually boxed using the e2helixbox module of EMAN2, while avoiding to include filament ends where the hetero-CARD complex could be situated. This manually picked data set was analysed with RELION, to obtain 2D class-averages used both as templates for automatic picking, and for initial symmetry estimation from their power spectrum. To this end, the filaments were segmented with an inter-box distance of 25 Å into 23742 segments of size 420×420 pixels, which were used for 2D classification, asking for thirty classes. Three significantly populated 2D class-averages were obtained, amongst which two correspond to straight segments, and show a clear repetitive pattern along the helical axis (Fig. 6b). The individual power spectra of these two 2D class-averages were nearly identical, and showed layer lines up to -1/4.9 Å⁻¹ (Fig. 6c), which enabled a first estimation of the helical period as follows (Supplementary Fig. 8a). The layer lines were regularly spaced at multiples of -1/155.8 Å⁻¹, giving an estimate of the repeat c Å meridional line l = 32 (Bessel order n = 0), with a height of 1/4.87 Å⁻¹ indicated the axial rise between subunits. A strong layer line l = 9 with a first intensity maximum near the meridian (n = 1), at 1/17.3 Å⁻¹, was attributed to the pitch P, as observed for other CARD domains filamentous assemblies (Supplementary Table 4). Therefore, the structure repeats after u = 32 subunits (32 × 4.87 Å = 155.8 Å), in t = 9 turns, resulting in a number of units per turn (u/t) of ~3.56.

The two straight 2D class-averages were subsequently used as templates for automatic picking of helical segments in RELION, using a maximum curvature parameter of 0.14, and a minimum segment length of 805 Å. The coordinates of filament extremities were converted into EMAN2 format, while shortening them by 180 Å at each extremity, to avoid including the hetero-CARD complex. This resulted in 4443 filament sections for a total length of 260 μm. All subsequent processing steps were performed in the helical reconstruction software SPINDLE SPRING. The complete model was obtained by 3D auto-picking from the manually picked data set and the symmetry parameters as estimated from the 2D class average power spectrum indexing, with low-pass filtering to 20 Å. The auto-picked filament coordinates were used to extract 37043 segments using a segment length of 400 Å and segment step size of 70 Å. The symmetry parameters were further refined with the module Segre13Dgrid, by defining a 11*11 grid spanning between 17.2-17.4 Å (step 0.02 Å) for the pitch and between 3.4-3.6 (step 0.02) for the number of units per turn. The maximum of the amplitude correlation between experimental and reprojection power spectra was found at a pitch of 17.26 Å, 3.56 units per turn (Supplementary Fig. 8b), corresponding to a left-handed helix with a 14.24 Å axial rise. The symmetry parameters, we performed a high-resolution structure refinement using the auto-picked segments with a strict segment selection during refinement, based on geometrical restraints, namely filament straightness (70% of straightest filaments kept) and forward-backward difference (limited to 5 Å). This resulted in a final reconstruction including 43566 segments (corresponding to 135,254 asymmetric units after symmetrization) at a resolution of 3.94 Å (FSC between half data set maps, cut off 0.143, Supplementary Fig. 8c). For visual display and the model building, the EM map was filtered to 3.9 Å and sharpened using a B-factor of 200 Å.

Visualization of the resulting map and initial rigid body fitting of the crystallographic structure was done using Chimera. Atomic model refinement was done using PHENIX real space refinement and manual adjustment with Coot. The crystallographic model was used to assign side chains for the residues without clear EM density map. Structure validation was done with Molprobity. The model was co-produced with Phenix (PDB: 6E6L) and Chimera. The model was installed and configured by SBDgrid. Data collection, image processing and refinement statistics are reported in Supplementary Table 3 and Supplementary Figure 5d.
HIS-SUMO-NOD2CARD5 confirmed by western blot (WB). For WB, 8 μg of total protein was loaded on either a 17% SDS-PAGE gel (Fig. 5b) or stain-free 4–12% gradient SDS-PAGE gel (Bio-Rad) (Fig. 8b). Rabbit anti-SNAP at 1:2000 dilution (P9310, New England Biolabs) was used for detection of NOD2CARD5. For WB revelation, a goat anti-rabbit secondary antibody linked to alkaline phosphatase was used at 1:3000 dilution (A3676, Sigma). Uncropped blots are shown in Supplementary Fig. 13.

Filament polymerization was stimulated by adding proteases for 4 h at room temperature, at a protein concentration of 2.5 mg ml−1. Negative-stain EM was used to check the polymerization state of each sample.

Experiments were repeated twice for constructs that showed impaired RIP2 CARD polymerization.

Mammalian cell culture and plasmids. A HEK293T cell line (from the laboratory of W. Filipowicz) was used. The cell line has not been authenticated but has been tested and shown to be free of mycoplasma. Cells were maintained in DMEM medium (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS) and non-essential amino acids ( Gibco), at 37°C and 5% CO2. Constructs for in cell based assays, were generated from pCDNA3 plasmids encoding for human RIP2(1-540). The original pcDNA3-RIP2(1-540) was modified into pcDNA-HA-RIP2(1-540), using the oligos reported in Supplementary Table 5, which resulted in a construct encoding for full-length RIP2 with an HA tag at the N-terminus and a GSAGSA linker between tag and protein. Single amino acids mutants were obtained by site-directed PCR mutagenesis of pcDNA3-HA-RIP2(1-540), using the oligos listed in Supplementary Table 5.

In cell reported luciferase assay and Western blots. HEK293T cells were seeded in 12-well plates 24 h prior transfection. Transfection was performed with Lipofectamine reaction (SigmaGen). Each well was transfected with 5 μg pCDNA3 plasmid encoding for pCDNA3-HA-RIP2 or corresponding mutant, 1 ng of pCMVpRL (500 ng of pCMVpRL-NF-κB), encoding a firefly luciferase reporter gene under NF-κB promoter and 50 μg pRenilla-TK plasmid. As negative control, transfection mix with pCDNA3-HA-RIP2 replaced with empty vector was used. pRenilla-TK was used to correct for the transfection efficiencies. Each transfection mixture was prepared in double and completed with either the NOD2 activator MDP or its corresponding control cMDP (Invivogen). Cells were lysed 24 h after transfection in 250 μl of lysis buffer, accordingly to the manufacturer protocol Dual Luciferase assay (Promega). Sample was then used for luciferases activities measurement and for western blots (WB). Firefly and Renilla luciferase activities were measured using a Clariostar microplate reader using the double injector system (BMG Labtech). Data were analyzed using the software CLARIOSTAR Plus v3.1 (BMG Labtech). The luminescence of each well was normalized to the corresponding Renilla luciferase readings. The Student t-test was performed and the p-values were calculated with GraphPad software (GraphPad Prism,

Statistical analysis. Statistical analysis on the luciferase reporter assay was performed using GraphPad (GraphPad Prism) with the aim of quantitatively finding evidence of significant differences between the two experiments, namely with NOD2 ligand MDP and the control cMDP. Their mean response was assessed using a parametric method; hence each of the samples was evaluated using a Shapiro-Wilk test for normality. The null hypothesis assumed normality, and all samples failed to reject this hypothesis test showing at 95% of significance that they were all normally distributed. Moreover, samples were assumed to have homogeneous variances to satisfy the requirements of the parametric tests. The Student t-test on two independent samples (two-sided) was conducted for each pair of conditions. Firstly, wild-type RIP2 and vector showed, without ambiguity according to p-value, that their respective means were different. Then, wild-type RIP2 was compared to the mean of each mutant. Except for wild-type RIP2 vs mutant Q548A, Q548K, N512A and N512K where no significant differences from their means was observed, all other mutants rejected more or less strongly the null hypothesis. In other words, their mean responses were significantly different from that of wild-type RIP2. Finally, the Student t-test comparison of two independent samples were conducted on “MDP” and “cMDP” version of each mutants, including both vector and wild-type RIP2. All conditions rejected the null hypothesis, leading to the conclusion that the means were different at 95% significance. Once we were definite evidence about differences from MDP and cMDP experiment was confirmed, a boxplot was generated to visualise the results (Fig. 9).

Data availability
Coordinates and structure factors for crystalMBP-RIP2CARD are deposited in the PDB with accession code 6GJT [https://www.rcsb.org/structure/6GJT], Coordinates for the RIP2CARD filament have accession code 6GGS [https://www.rcsb.org/structure/6GGS]. The cryoEM map has accession code EMD-4399. The solid-state NMR data is deposited in the BMRB database with accession number 27555 (this data can be retrieved using the repeat ID 2018-07-16.deposit.bmrb.wisc.edu.80.13422729 at http://deposit.bmrb.wisc.edu/bmrb-addt-access.html).

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References
1. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. Cell 140, 805–820 (2010).
2. Ogura, Y. et al. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κappaB. J. Biol. Chem. 276, 4812–4818 (2001).
3. Girardin, S. E. et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. 278, 8869–8872 (2003).
4. Inohara, N. et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn’s disease. J. Biol. Chem. 278, 5509–5512 (2003).
5. Magalhaes, J. G. et al. Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands. Eur. J. Immunol. 41, 1435–1445 (2011).
6. Ting, J. P. et al. The NLR gene family: a standard nomenclature. Immunity 28, 285–287 (2008).
7. Mackawa, S., Ohno, U., Shibata, T., Miyake, K. & Shimizu, T. Crystal structure of NOD2 and its implications in human disease. Nat. Commun. 7, 11813 (2016).
8. Humphries, F., Yang, S., Wang, B. & Moynagh, P. N. RIP kinases: key decision makers in cell death and innate immunity. Cell Death Differ. 22, 225–236 (2015).
9. Boyle, J. P., Parkhouse, R. & Monie, T. P. Insights into the molecular basis of the NOD2 signalling pathway. Open biology 4, https://doi.org/10.1098/ ribi.140178 (2014).
10. Philipps, D. J., Sorbara, M. T., Robertson, S. J., Croitoru, K. & Girardin, S. E. NOD proteins: regulators of inflammation in health and disease. Nat. Rev. Immunol. 14, 9–23 (2014).
11. Rubino, S. J., Selvanantham, T., Girardin, S. E. & Philpott, D. J. Nod-like receptors in the control of intestinal inflammation. Curr. Opin. Immunol. 24, 398–404 (2012).
12. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallographica D Biological Crystallography. 66, 213–221 (2010).
13. Fridj, V. & Rittinger, K. The tandem CARDS of NOD2: intramolecular interactions and recognition of RIP2. PLoS ONE 7, e34375 (2012).
14. Wagner, R. N., Proeck, M., Kuefer, T. A. & Schwarzenbacher, R. Evaluation of nod-like receptor (NLR) effector domain interactions. PLoS ONE 4, e4931 (2009).
15. Damgaard, R. B. et al. The ubiquitin ligase XIAP recruits LUBAC for NOD2 signaling in inflammation and innate immunity. Mol. Cell 46, 746–758 (2012).
16. Bertrand, M. L. et al. Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. Immunity 30, 789–801 (2009).
17. Goncharov, T. et al. Disruption of XIAP-RIP2 association blocks NOD2-mediated inflammatory signaling. Mol. Cell 69, 551–565 (2018). e557.
18. Windheim, M., Lang, C., Peggie, M., Plater, L. A. & Cohen, P. Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochem. J. 404, 197–200 (2007).
19. Homer, C. R. et al. A dual role for receptor-interacting protein kinase 2 (RIP2) kinase activity in nucleotide-binding oligomerization domain 2 (NOD2) dependent autophagy. J. Biol. Chem. 287, 25565–25576 (2012).
20. Hugot, J. P. CARD15/NOD2 mutations in Crohn’s disease. Ann. N. Y. Acad. Sci. 1072, 9–18 (2006).
21. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. Nature reviews. Gastroenterol. & Hepatol. 12, 720–727 (2015).
22. Liu, J. Z. & Anderson, C. A. Genetic studies of Crohn’s disease: past, present and future. Best. Pract. & Res. Clin. Gastroenterol. 28, 373–386 (2014).
23. Wehkamp, J. & Stange, E. F. Paneth cell apoptosis: a new player in Crohn’s disease. J. Crohn’s & Colitis 4, 523–531 (2010).
24. Miceli-Richard, C. et al. CARD15 mutations in Blau syndrome. Nat. Genet. 29, 19–20 (2001).
25. Caso, F. et al. Autoinflammatory granulomatous diseases: from Blau syndrome and early-onset sarcoidosis to NOD2-mediated disease and Crohn’s disease. RMD Open 6, e000977 (2020).
26. Henckaerts, L. & Vermeire, S. NOD2/CARD15 disease associations other than Crohn’s disease. Inflamm. Bowel Dis. 13, 235–241 (2007).
27. Kanazawa, N. et al. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome? Blood 115, 1143–1147 (2010).
28. Xie, T. et al. Structural insights into RIP1-mediated necroptotic signaling. Cell Rep. 5, 70–78 (2013).
29. Wu, H. & Fuxreiter, M. The structure and dynamics of higher-order assemblies: amyloids, signalosomes, and granules. Cell 165, 1055–1066 (2016).
30. Hou, F. et al. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell 146, 448–461 (2011).
31. Wu, B. et al. Molecular imprinting as a signal-activation mechanism of the viral RNA sensor RIP1-IRG-1. Mol. Cell 55, 511–523 (2014).
32. Xu, H. et al. Structural basis for the prion-like MAVS filaments in antiviral innate immunity. eLife 3, e01489 (2014).
33. Lu, A. et al. Unified structural parameters for the assembly of ASC-dependent inﬂammosomes. Cell 156, 1193–1206 (2014).
34. Lu, A. et al. Molecular basis of caspase-1 polymerization and its inhibition by a new capping mechanism. Nat. Struct. Mol. Biol. 23, 416–425 (2016).
35. David, L. et al. Assembly mechanism of the CARMA1–BCL10–MALT1–TRAF6 signalosome. Proc. Natl Acad. Sci. USA https://doi.org/10.1073/pnas.1707611115 (2018).
36. Pellegrini, E., Signor, L., Singh, S., Boeri Erba, E. & Cusack, S. Structures of the inactive and active states of RIP2 kinase inform on the mechanism of activation. PLoS ONE 12, e0171617 (2017).
37. Jang, T. H. et al. Structural study of the RIPPotosome core reveals a helical assembly for kinase recruitment. Biochemistry 53, 5424–5431 (2014).
38. Wraight, D. S. Crystal structures of MBP fusion proteins. Protein Sci.: a Publ. Protein Soc. 25, 559–571 (2016).
39. Jin, T., Curry, J., Smith, P., Jiang, J. & Xiao, T. S. Structure of the NLRP1 new capping mechanism. J. Biol. Chem. 287, 2377–2387 (2012).
40. Lin, Z. et al. Structural basis of death domain signaling in the p75 neurotrophin receptor. J. Biol. Chem. 287, 16569–16578 (2012).
41. Daub, H. et al. Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. J. Struct. Biol. 185, 15–26 (2014).
42. Dinh, D. et al. Crystal structure of caspase recruiting domain (CARD) of apoptosis repressor with CARD (ARC) and its implication in apoptosis. Sci. Rep. 5, 9847 (2015).
43. Ohm, M., Li, Y., Cheng, Y. & Walz, T. Negative staining and image classification – powerful tools in modern electron microscopy. Biol. Proced. Online 8, 23–24 (2010).
44. Flot, D. et al. The ID23-2 structural biology microfocus beamline at the ESRF. Acta Crystallogr. D. Biol. Crystallogr. 66, 125–132 (2010).
45. Karplus, P. A. & Diederichs, K. Linking crystallographic model and data bias in small molecule X-ray crystallography. Science 336, 105–108 (2012).
46. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
47. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D. Biol. Crystallogr. 53, 240–255 (1997).
48. Emaley, P. J. & Cowtan, K. K. moist: model-building tools for molecular graphics. Acta crystallographica. Section D. Biol. Crystallogr. 60, 2126–2132 (2004).
49. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D. Biol. Crystallogr. 66, 12–21 (2010).
50. DeLano, W. L. PyMOL. Molecular Graphics System http://www.pymol.org (2002).
51. Kabsch, W. Xds. J. Appl. Crystallogr. 36, 758–763 (2003).
52. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).
53. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. J. Struct. Biol. 142, 334–347 (2003).
54. Yang, G. et al. EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol. 197, 38–46 (2007).
55. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 (2012).
56. Desfosses, A., Ciuffa, R., Gutsche, I. & Sachse, C. SPRING – an image processing package for single-particle based helical reconstruction from electron cryomicrographs. J. Struct. Biol. 185, 15–26 (2014).
57. Sachse, C. et al. High-resolution electron microscopy of helical specimens: a fresh look at tobacco mosaic virus. J. Mol. Biol. 371, 812–835 (2007).
58. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
59. Morin, A. et al. Collaboration gets the most out of software. eLife 2, e01456 (2013).
60. The R-core team. The R Project for Statistical Computing http://www.r-project.org/ (2017).
61. Wickham, H. ggplot2: Elegant Graphics for data analysis (Springer-Verlag, New York, 2009).
62. Wishart, D. S. & Sykes, B. D. The 3C chemical-shift index: a simple method for the identification of protein secondary structure using 13C chemical-shift data. J. Biomol. NMR 4, 171–180 (1994).
63. Wishart, D. S. & Sykes, B. D. Chemical shifts as a tool for structure determination. Methods Enzymol. 239, 363–392 (1994).

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Author contributions
E.P. and S.C. conceived and directed the project. E.P. performed biochemical and biophysical experiments alone or together with I.G. and H.M. (negative-stain EM on RIP2, Fig. 1 and Supplementary Fig. 1), G.K. (crystallisation of RIP2 CARD, Fig. 3 and Supplementary Fig. 3; Purification of NOD2 ΔLRR, Fig. 4), S.G. (Co-purification and immuno-gold labelling experiments, Figs 4, 5), M.H. (preparation and screening of cryo-EM grids, Fig. 6 and Supplementary Fig. 7) and W.M.S (in vitro analysis of RIP2 CARD mutants, Fig. 8 and Supplementary Fig. 11). K.R. purified protein for NMR experiments. A.W. and H.O. measured and analysed solid-state NMR data. E.P., H.M. and C.S. performed initial EM data processing. G.S. did the Polara cryo-EM data collection. E.P. and A.D. performed the helical reconstruction. E.P. and S.C. performed the crystallographic structure determination. P.M. performed statistical analysis. L.S. performed mass spectrometry experiments. E.P. and S.C. wrote the manuscript with input from all authors.

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