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1 **Structural and molecular basis of cross-seeding barriers in amyloids**

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57 **Abstract**

58 Neurodegenerative disorders are frequently associated with β -sheet-rich amyloid deposits.
59 Amyloid-forming proteins can aggregate under different structural conformations known as
60 strains, which can exhibit a prion-like behaviour and distinct patho-phenotypes. Precise
61 molecular determinants defining strain specificity and cross-strain interactions (cross-seeding)
62 are currently unknown. The HET-s prion protein from the fungus *Podospora anserina*
63 represents a model system to study the fundamental properties of prion amyloids. Here, we
64 report the amyloid prion structure of HELLF, a distant homolog of the model prion HET-s. We
65 find that these two amyloids, sharing only 17% sequence identity, have nearly identical β -
66 solenoid folds but lack cross-seeding ability *in vivo*, indicating that prion specificity can differ in
67 extremely similar amyloid folds. We engineer the HELLF sequence to explore the limits of the
68 sequence-to-fold conservation and to pinpoint determinants of cross-seeding and prion
69 specificity. We find that amyloid fold conservation occurs even at an exceedingly low level of
70 identity to HET-s (5%). Next, we derive a HELLF-based sequence, termed HEC, able to breach
71 the cross-seeding barrier *in vivo* between HELLF and HET-s, unveiling determinants controlling
72 cross-seeding at residue level. These findings show that virtually identical amyloid backbone
73 structures might not be sufficient for cross-seeding and that critical side-chain positions could
74 determine the seeding specificity of an amyloid fold. Our work redefines the conceptual
75 boundaries of prion strain and sheds light on key molecular features concerning an important
76 class of pathogenic agents.

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83 **Keywords:** Amyloid; prion; sequence-to-fold; cross-seeding; nuclear magnetic resonance

84 **Significance**

85 Amyloid folds, while performing functional roles in most domains of life, remain a key factor in
86 the emergence and development of multiple neurodegenerative disorders in humans. The
87 significance of our study is twofold: first, by structurally characterizing highly divergent natural
88 prion amyloids, we uncovered that functional amyloids can evolve in a regime of fold
89 conservation, withstanding extreme sequence diversification. Second, we found that virtually
90 identical amyloid backbone structures might not be sufficient for cross-seeding and that key
91 side-chain positions could determine the seeding specificity of an amyloid fold. This work thus
92 sheds light on the fundamental properties of a major category of pathogenic agents.

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111 **Introduction**

112 Amyloid-forming proteins undergo a phase transition to form insoluble, polymeric assemblies,
113 which can self-propagate *in vivo* as prions (1–3). Amyloid aggregates associated with
114 neurodegenerative diseases (i.e. Alzheimer’s, Parkinson’s etc.) have a prion-like behaviour (4, 5)
115 and can propagate under different structural conformations also known as prion ‘strains’
116 (conformational variants of identical protein sequence), which may be associated with distinct
117 phenotypes of the pathology (6–9). The cross-talk between an infectious amyloid conformation
118 (prion strain) and a naive homologous or heterologous amyloidogenic sequence, referred to as
119 ‘cross-seeding’ (10, 11), is a critical event in prion biology, representing the key aspect of
120 infectivity. Amyloid cross-seeding could play a role in the aetiology (12) and pathogenesis of
121 various proteinopathies (13, 14). However, our understanding of the precise molecular and
122 structural determinants allowing or limiting cross-seeding remains poor.

123 The fungal HET-s protein constitutes a highly favourable system to study the fundamental
124 properties of prion amyloids, as a high-resolution structure of the propagative prion state is
125 available (15, 16). The prion-forming domain (PFD) of HET-s contains two 21 amino acid
126 pseudo-repeats (R1 and R2), which are alternately stacked, each repeat forming four β -strands,
127 adopting a left-handed β -solenoid fold (15, 16). Noteworthy, several reports have stressed
128 structural similarities between the HET-s amyloid fold and pathological amyloids formed by the
129 human prion protein PrP (17) and tau (18).

130 Despite the structural similarities with some pathological amyloids, HET-s represents a
131 functional amyloid, which is integral to an immunity-related signal transduction pathway in
132 fungi (19). The β -solenoid fold ensures signal transduction from an activated NOD-like receptor
133 (NLR) to a downstream execution protein (HET-S, a pore-forming variant of the HET-s prion
134 protein). The fold represents a cell death trigger and functions on the basis of the prion
135 principle (19, 20). The structural templating of the HET-S PFD into the amyloid fold triggers the
136 cytotoxicity of the α -helical HeLo domain, which induces cell death targeting the plasma
137 membrane (21–23). Signal-transducing amyloids are widespread in fungi (24) and at least five

138 subfamilies of HET-s-related amyloid motifs (HRAMs) have been identified (25). The HRAMs
139 exhibit the two pseudo-repeats organization of HET-s and a specific pattern of hydrophobic and
140 polar amino acids, while each subfamily is being defined by the conservation of a set of HRAM-
141 specific residues (25). It has been speculated that the HRAMs may share a common β -solenoid
142 fold and that natural diversification is driven to preserve specificity of the signalling pathways
143 (between NLRs and cognate effectors) by limiting amyloid cross-seeding between distinct
144 HRAMs (19, 25). Here, we use the HRAMs as an experimental framework to investigate 1) the
145 sequence-to-fold relation in prion amyloids and 2) the molecular determinants defining prion
146 specificity, which allow or prevent cross-seeding.

147 **Results**

148 **Molecular characterization of HELLF**

149 We chose to work with a newly identified HELLF protein, encoded in the genome of *Podospora*
150 *anserina* (*SI Appendix*, Fig. S1), the natural host of the [Het-s] prion. HELLF consists of an N-
151 terminal HELL (HeLo-like) domain and a putative C-terminal PFD. The HELLF PFD carries
152 strongly divergent HRAM pseudo-repeats (R1 and R2) showing only 17% of sequence identity
153 with the PFD repeats of HET-S/s (Fig. 1A; *SI Appendix*, Fig. S1). HELLF pseudo-repeats belong to
154 the HRAM5 family (Fig. 1B). Strains expressing HELLF(209-277) showed phenotypic bistability
155 exhibiting either a $[\Phi^*]$ phenotype, where the protein remains soluble or a $[\Phi]$ phenotype with
156 formation of dot-like aggregates (Fig. 1C; *SI Appendix*, Fig. S1). The $[\Phi]$ strains triggered cell
157 death upon anastomosis (cellular fusion) with strains expressing full-length HELLF, while $[\Phi^*]$
158 strains formed viable heterokaryons (Fig. 1D). During the cell death reaction, as described for
159 HET-S (22), HELLF relocates to the cell membrane (*SI Appendix*, Fig. S1). The $[\Phi^*]$ strains
160 (aggregates-free) switched spontaneously (\sim 24 hours) to $[\Phi]$ strains (with aggregates), which
161 in turn could be cured (or reversed) back to $[\Phi^*]$ state through a sexual cross (*SI Appendix*,
162 Table S1, Table S2). These results establish HELLF as a distant HET-S homolog and demonstrate
163 that the HRAM5 pseudo-repeats bearing PFD of HELLF behaves as a prion in *P. anserina*.

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165 **Solid-state NMR (ssNMR) structure of HELLF prion domain**

166 We engaged in the structural characterization of HELLF(209-277). The protein self-assembled
167 into unbranched fibrils *in vitro* (Fig. 1E), exhibiting a typical cross- β signature by X-ray
168 diffraction (*SI Appendix*, Fig. S2). We took advantage of recent developments for fast magic-
169 angle spinning (MAS) NMR probes (26–28) to establish, and implement for the first time on a
170 fibril sample, a three-dimensional (3D) structure determination approach, based entirely on ^1H -
171 ^1H proximities. The approach, reminiscent of nuclear Overhauser effect (nOe)-based solution
172 NMR methods to solve soluble globular protein structures, allows a tremendous gain in
173 sensitivity and enables the use of simplified labelling schemes and minimal sample quantities.
174 Approximately 300 μg of fully protonated HELLF(209-277) were packed into a 0.7 mm ssNMR
175 rotor (Fig. 2A). Ultra-fast MAS performed at rates of ~ 110 kHz allowed the acquisition of high-
176 resolution ^1H -detected multidimensional spectra even in fully protonated samples (Fig. 2B; *SI*
177 *Appendix*, Fig. S3) with ^1H line widths of ~ 150 -200Hz enabling assignment of backbone and
178 side-chain protons (29). A unique set of resonances revealed the presence of a single
179 conformational polymorph in the fibrillar assembly, and conformation-dependent chemical
180 shifts (30) revealed a β -rich rigid core extending from residue Q221 to S272, including 8 β -
181 strands (Fig. S4). A flexible linker segment (G240-D247), comprising a β -breaker GxxxPG motif,
182 subdivides the rigid core in 2 regions with 4 β -strands each (Fig. S4). We employed a
183 combination of 3D H(H)CH and H(H)NH experiments (27) on a fully protonated, uniformly
184 $^{13}\text{C},^{15}\text{N}$ -labeled sample to derive 178 internuclear ^1H - ^1H distances (Fig. 2C). 3D amyloid
185 architectures render the distinction between intra- and intermolecular contacts in ssNMR
186 experiments difficult and usually require complex labelling schemes (16, 31). We designed a
187 new and simple labelling strategy based on an equimolar mixture of fully protonated proteins at
188 natural abundance, randomly co-aggregated with deuterated, extensively amide-reprotonated
189 and uniformly ^{15}N -labeled proteins (scheme denoted as (1/1) [(U- $^1\text{H},^{14}\text{N}$)/(U- $^1\text{H}^{\text{N}},^2\text{H},^{15}\text{N}$))] (Fig.
190 2E). Using this scheme, we observed 33 intermolecular ^1H - ^1H inter-strand aliphatic to amide
191 distances relying on a single 3D H(H)NH spectrum (Fig. 2D) with an asymmetric polarization

192 transfer (Fig. 2F). We identified 211 distance restraints in total, of which 176 are long-range
193 restraints ($|i-j| > 4$) (Fig. 2G) to derive a 3D structure of the HELLF fibrillar assembly at atomic
194 resolution with 20-conformer bundle r.m.s.d of 0.73Å for backbone atoms and 1.18Å for heavy
195 atoms (Fig. 3; *SI Appendix*, Table S3). Our 3D structure determination approach based on ^1H - ^1H
196 proximities compares favourably to benchmark studies of the HET-s prion domain amyloid
197 structure by ssNMR (16); we detected approximately 4.5 structurally meaningful restraints per
198 residue in the rigid amyloid core, a number approaching those used in high-resolution structure
199 determination protocols in solution NMR.

200 **HELLF and HET-s prions share an identical backbone β -solenoid core**

201 The HELLF fibrillar architecture shows a β -solenoid fold, made by the intermolecular packing of
202 HELLF monomers along the fibril axis (Fig. 3). The intramolecular HELLF fold is composed of 8
203 β -strand structural elements separated by a short unstructured region (Gly240-Asp247) (Fig.
204 3). Intramolecular ssNMR restraints reveal a regular and rigid core, constituted by alternate
205 stacking of R1 (R1 β_1 to R1 β_4) and R2 (R2 β_1 to R2 β_4) pseudo-repeat regions (Fig. 4B, Fig. 3).
206 Each pseudo-repeat adopts a triangular shape, stabilized by hydrophobic side-chains interlaced
207 (*SI Appendix*, S5A) inside the amyloid core and protected from the solvent. Water molecules are
208 excluded from the amyloid core (*SI Appendix*, S5B-C). Several β -breaker glycine residues allow
209 for bending two consecutive β -arcs into the triangular arrangement of the amyloid core (Fig.
210 4B). R1 and R2 repeats are stacked through a hydrogen bond-rich pairing between β -sheets
211 (R1 β_i with R2 β_i , T255/S232 and T228/S259). To corroborate the HELLF intramolecular R1/R2
212 stacking, we performed scanning transmission electron microscopy (STEM) mass-per-length
213 (MPL) measurements and determined a MPL of 0.99 ± 0.10 kDa/Å corresponding to 1.1 ± 0.1
214 molecules per 0.94 nm (*SI Appendix*, Fig. S6). Considering a β -strand repetition of 0.47 nm in the
215 cross- β architecture as measured by X-ray diffraction (*SI Appendix*, Fig. S2), it leads to a fibril
216 layer (i.e. per 0.47 nm) composed of half of a HELLF molecule, in agreement with the fold
217 determined by ssNMR (Fig. 2G, Fig. 3). The inter-subunit packing consists of parallel, pseudo in-
218 register stacking and the overall intermolecular arrangement is consistent with cross- β stacked

219 solenoid architecture. In spite of the low sequence identity between HET-s and HELLF PFDs,
220 both prion domains adopt virtually identical backbone conformations (Fig. 4, A and C).

221 We took advantage of the high-resolution structures of HELLF and HET-s to perform molecular
222 modelling on the remaining HRAM families identified in fungal genomes (Fig. 4E). We found
223 that the observed sequence diversity in the HRAM superfamily is indeed compatible with a
224 unique structural solution underlying this amyloid fold (Fig. 4E). The findings underscore the
225 importance of the previously identified pattern of hydrophobic and polar amino acids as a
226 minimal requirement to adopt the HET-s-like β -solenoid fold (25).

227 In addition, we designed a protein sequence termed HED (HET-s distant) carrying two identical
228 repeats that share less than 5% identity with HET-s (one residue out of 21) (*SI Appendix*; Fig.
229 S7A, Table S5). HED was able to form a prion *in vivo* (*SI Appendix*, Fig. S7B-E). *In vitro*, HED
230 adopts a HET-s-like β -solenoid structure as seen by SSNMR (*SI Appendix*, Fig. S7F-G), implying a
231 β -solenoid fold conservation in spite of the extremely low sequence identity (5%) and a very
232 low sequence similarity (19%) to HET-s.

233 **HELLF and HET-s carry distinct prion specificities *in vivo***

234 Considering the structural similarity between HELLF and HET-s prion folds (Fig. 4A) and that
235 these proteins occur in the same species, we analysed the cross-seeding of [Φ] and [Het-s] prion
236 states *in vivo* (*SI Appendix*, Table S2). We found that [Φ] strains do not convert non-prion [Het-
237 s*] strains to the [Het-s] prion state nor do [Het-s] strains induce [Φ] prion formation (*SI*
238 *Appendix*, Table S2). HELLF PFD fibrils show no [Het-s] infectivity and HET-s PFD fibrils show
239 no [Φ] infectivity in transfection assays (*SI Appendix*, Table S4). HELLF and HET-s PFDs form
240 independent aggregates *in vivo* indicating that the two prions do not co-aggregate (Fig. 4D; *SI*
241 *Appendix*, Fig. S8). Strains co-expressing HELLF and HET-s can display four alternate epigenetic
242 states ([Het-s*] [Φ *], [Het-s] [Φ] but also [Het-s*] [Φ] and [Het-s] [Φ *]) confirming that [Het-s]
243 and [Φ] are independent prions (*SI Appendix*, Fig. S8, Table S2). Since the backbone structures
244 in the amyloid fold of HET-s and HELLF PFDs are nearly identical (Fig. 4A), it appears that

245 strong structural similarity and interactions of the cross- β backbones are insufficient for
246 amyloid templating and/or co-aggregation.

247 At the level of side-chain packing, HELLF has more hydrophilic (Q²²¹, Q²³⁶, Q²⁴⁸ and Q²⁶³) side-
248 chains pointing inside the amyloid core compared to HET-s (N²²⁶ and N²⁶²). A striking difference
249 is observed at the end of the 3rd β -strand, composed of Q²⁴⁰L (R1) and L²⁷⁶I (R2) in HET-s and
250 M²³⁵Q (R1) and F²⁶²Q (R2) in HELLF. Therefore, the lack of cross-seeding between HET-s and
251 HELLF could result from unfavourable hydrophobic-hydrophilic packing between solvent
252 exposed HET-s Q²⁴⁰ and HELLF F²⁶² as well as HET-s L²⁴¹ and HELLF Q²⁶³.

253 **HRAM-specific residues control cross-seeding between HELLF and HET-s**

254 The results suggested that side-chain residues, decorating the amyloid backbone, could play a
255 key role in defining prion specificity and cross-seeding. Thus, we decided to test whether by
256 varying HRAM-specific residues between HET-s and HELLF PFDs, the cross-seeding barrier
257 between the two prions could be breached. We replaced five residues on each of the two 21
258 amino acids HRAM5 pseudo-repeats of HELLF with residues from the corresponding positions
259 found in the HRAM1 pseudo-repeats of HET-s. The engineered sequence was termed HEC (HET-
260 s closer). We targeted for replacement residues that are highly conserved inside each HRAM
261 (Fig. 1.B) and essential for distinguishing HRAM5 (HELLF) from HRAM1 (HET-s) (25) (Fig. 5A).
262 The five amino acid substitutions for each pseudo-repeat of HELLF were introduced in two
263 strongly conserved HRAM5-specific sub-motifs of the protein – the QxFG (position 1-4, where x
264 is any possible amino acid residue) and QG(Q/I) (position 16-18) sub-motifs (Fig. 5A, Fig. 1B).
265 The substitution of Q in the QG(Q/I) sub-motif would also remove one of the unfavourable
266 hydrophobic-hydrophilic interactions in the potential cross-seeded interface.

267 We expressed HEC in a $\Delta het-s \Delta hellf$ strain and tested the ability of the protein to propagate as a
268 prion and to carry [Het-s] and/or [Φ] prion specificity *in vivo*. Strains expressing GFP-HEC
269 showed two distinct phenotypes; [HEC*] strains presented diffuse GFP-HEC fluorescence, did
270 not induce [Het-s] or [Φ] prions, and were unable to trigger cell death by incompatibility with
271 HET-S or HELLF expressing strains (*SI Appendix*, Fig. S9, Table S6, Table S7). We observed that

272 some [HEC*] strains did, spontaneously and at low rate (~10%), transition into a [HEC] state
273 characterized with the appearance of fluorescent dot-like aggregates (*SI Appendix*, Fig. S9, Table
274 S6). None of the spontaneous [HEC] strains were able to induce [Het-s] or produce a barrage
275 with a HET-S strain. Yet, [HEC] strains induced cell death with HELLF-expressing strains,
276 indicating that the engineered HEC sequence shows [Φ] prion specificity and we termed the
277 phenotype [HEC Φ] (Fig. 4B; *SI Appendix*, Fig. S9). We were equally able to induce the [HEC Φ]
278 phenotype by exposing [HEC*] strains to [Φ] strains (*SI Appendix*, Table S7). Spontaneously
279 formed (or [Φ]-induced) [HEC Φ] strains converted [HEC*] strains into [HEC Φ] prion state after
280 contact, demonstrating the ability of aggregated HEC to self-propagate (behave as prion) (*SI*
281 *Appendix*, Fig. S9).

282 Next, we found that after a contact with a [Het-s] strain, the prion-free [HEC*] strains could
283 equally be converted to a [HEC] state, characterized by the appearance of fluorescent GFP-HEC
284 dot-like aggregates and the ability to induce cell death with HELLF, indicating that the prion
285 state of HEC can be induced both by HELLF and HET-s (*SI Appendix*, Table S7). Importantly, we
286 found that [Het-s]-induced [HEC] strains, unlike the [HEC Φ] strains, were capable of converting
287 [Het-s*] strains into [Het-s] strains and to induce cell death with HET-S (Fig. 5, B and C; *SI*
288 *Appendix*, Fig. S9). Hence, we termed this phenotype [HEC ζ]. Importantly, [HEC Φ] and [HEC ζ]
289 phenotypes remained stable in time and faithfully self-propagated over several passages (*SI*
290 *Appendix*, Table S7, Table S8). In addition, we observed that GFP-HEC aggregates from [HEC ζ]
291 strains partially co-localized with [Het-s] and not with [Φ], while fluorescent HEC aggregates
292 from [HEC Φ] strain produced the opposite results and co-localized predominantly with [Φ] (*SI*
293 *Appendix*, Fig. S9). We concluded that the engineered HEC sequence could propagate as two
294 distinct prion strains [HEC Φ] and [HEC ζ] and was capable of breaching the cross-seeding barrier
295 between [Het-s] and [Φ] prions. A likely explanation could be found in the reduced hydrophilic
296 – hydrophobic clashes between HEC and the sequences of the two fungal prions (HET-s and
297 HELLF), following the replacement of two strongly conserved hydrophilic HRAM5-specific
298 residues (Q236 and Q263 in HELLF) with HRAM1-specific hydrophobic residues (L241 and

299 I277 in HET-s). Because HEC was designed by the targeted replacement of HRAM-specific
300 residues, we concluded that such residues play a key role in the control of prion propagation,
301 allowing or preventing cross-seeding between HRAM families.

302 **Cross-seeding induced structural plasticity of HEC near HRAM-specific residues**

303 To pinpoint determinants of the cross-seeding at structural level, we investigated the
304 engineered protein sequence *in vitro* using ssNMR. Based on the resonance assignment of
305 recombinant ¹³C,¹⁵N-labelled sample, HEC adopts a cross-β fold, highly similar to HET-s and
306 HELLF (*SI Appendix*, Fig. S10, Fig. S11B). We then assembled HEC fibrils *in vitro* in presence of
307 5% of unlabelled HET-s or HELLF, respectively termed HEC^s and HEC^ϕ. The resulting fibrillar
308 states were analysed using solid-state NMR and the detected spectral fingerprints were similar
309 between the three HEC preparations (HEC, HEC^s and HEC^ϕ), while exhibiting slight structural
310 differences at some amino acid positions (*SI Appendix*, Fig. S11). Both co-aggregation
311 experiments (HEC + 5% HET-s and HEC + 5% HELLF) produced relatively similar chemical shift
312 perturbations in HEC, suggesting similar interaction surfaces between the peptides (*SI*
313 *Appendix*, Fig. S11). To gain further insight into the structural differences between HEC co-
314 aggregated with HET-s or HELLF, we compared both chemical shift sets and plotted the
315 differences as a function of the primary sequence (Fig. 5D). We detected small CA chemical shift
316 variations on residues constituting the amyloid core of HEC^s and HEC^ϕ, suggesting slight
317 conformational changes within the assemblies (Fig. 5, D and E). Remarkably, most of the
318 residues showing highest backbone chemical shift difference between HEC^s and HEC^ϕ were part
319 of or adjacent to the HRAM-defining positions that were modified in HELLF to engineer HEC
320 (highlighted in red in Fig. 5D). Chemical shift differences are not only observed at the level of
321 backbone conformation but also on side-chain NMR signals (e.g. M235 and F262). Additional
322 NMR signals were observed (peak doubling, i.e. A223) between HEC^s / HEC^ϕ and HEC (Fig. 5E)
323 indicating a second local structural conformation for some residues (Fig. 5F). Considering
324 chemical shift differences and presence of peak doubling, we observed that the QxFG and
325 QG(Q/I) sub-motifs, which distinguish HELLF (HARM5) from HET-s (HRAM1) and have been

326 partially replaced in HELLF to engineer HEC, are the most impacted regions during the cross-
327 seeding experiments of HEC (Fig. 5, Fig. 1B). Thus, the results indicate that the engineered HEC
328 sequence adopts similar amyloid fold to HET-s and HELLF, while exhibiting limited structural
329 plasticity near several different HRAM-specific residues during cross-seeding. Although these
330 conformational changes are subtle, they are in agreement with the observed HEC prion strains
331 *in vivo* and highlight the role of HRAM-specific residues as potential hotspots defining prion
332 infectivity and controlling cross-seeding.

333 **Discussion**

334 Our study exploits the natural diversity occurring in a superfamily of fungal prion domains to
335 document the limits of sequence-to-fold conservation in amyloids and the molecular and
336 structural determinants of cross-seeding. First, by structurally characterizing highly divergent
337 natural prion amyloids of the HRAM family, we uncovered that functional amyloids can evolve
338 in a regime of fold conservation, withstanding extreme sequence diversification. Our work thus
339 indicates that the sequence-to-fold evolutionary interplay for functional amyloids is similar to
340 what has been described for globular (35) and membrane proteins (36) with fold conservation
341 occurring even at very low levels of sequence identity and similarity. These findings are in
342 agreement with previous studies documenting the high resistance of the β -solenoid fold to an
343 extensive array of amino acids substitutions (32, 33). The apparent rigidity of β -solenoids has
344 been proposed as a key feature for this class of proteins to perform as scaffolding devices (34).
345 Here, the robustness of the β -solenoid fold would equally allow for the diversification of the
346 HRAM signalling specificities (25).

347 The experimental HELLF SSNMR structure as well as the study of synthetic HRAM-derived
348 proteins (HEC and HED) confirms structural and functional importance of the pattern of
349 hydrophobic/polar side chain arrangement in HRAM families. This combination of
350 bioinformatics and experimental approaches now opens the possibility to predictably engineer
351 the generic HRAM β -solenoid fold.

352 Second, this study sheds light in an unprecedented way on the determinants of amyloid cross-
353 seeding. We found that virtually identical amyloid backbone structures might not be sufficient
354 for cross-seeding and that critical side-chain positions could determine the seeding specificity of
355 an amyloid fold. Extrapolating our conclusions to amyloids causing neurodegeneration,
356 especially considering the structural similarities between the HRAM β -solenoid fold and β -
357 solenoid folds of pathological amyloids in humans (17, 18, 37, 38), provides a conceptual update
358 on key features of the molecular behaviour for this category of pathogenic agents.

359

360 **Materials and Methods**

361 ***Prion propagation and incompatibility assays.*** Incompatibility phenotypes were determined
362 by confronting strains of solid corn meal agar medium and a 'barrage' reaction was assessed 2-3
363 days post-contact. Prion propagation was assayed as the ability to transmit the $[\varphi]$ prion
364 phenotype from a $[\varphi]$ -donor strain to a $[\varphi^*]$ prion-free tester strain after confrontation on solid
365 medium. Transformants were confronted to wild type strains either directly or after contact
366 with a $[\varphi]$ -donor strain 6, 11 and 17 days after transfection to evaluate $[\varphi^*]$ and $[\varphi]$ phenotypes
367 frequencies and spontaneous $[\varphi]$ prion propagation. Protein transfection experiments with
368 amyloid fibrils of recombinant HELLF(209-277) or HET-s(218-289) were carried following the
369 general protocol described by Benkemoun *et al.* (39) with minor modifications. In brief, an agar
370 piece (~ 5 mm³) covered with fresh (24 h of growth) prion-free mycelium is placed in a 2 ml
371 screw cap tube containing 500 μ l of STC buffer (0.8 M sorbitol, 50 mM CaCl₂, 100 mM Tris-HCl
372 pH 7,5) in addition to 50 μ l of amyloids (2-3 mg.ml⁻¹). The mycelium is fragmented using a
373 mechanical cell disruptor (FastPrepTM FP120). Two consecutive runs of 30 s each at speed of 6
374 m/s were realized and fractions of the suspension (20-25 μ l) were directly spotted on corn meal
375 medium to be assessed for prion conversion after 4-5 days of regeneration at 26°C.

376 ***Protein purification.*** Cells were sonicated on ice in a lysis buffer (Tris 50 mM, 150 mM NaCl, pH
377 8) and centrifuged to remove *E. coli* contaminants. Proteins were expressed in inclusion bodies
378 due to their insoluble properties and purified under denaturing conditions. The supernatant

379 was discarded and the pellet incubated with lysis buffer supplemented with 2 % Triton X-100.
380 The membrane pellet containing inclusion bodies was extensively washed with lysis buffer to
381 remove Triton X-100 traces and incubated at 60 °C overnight with 8 M guanidine hydrochloride
382 until complete solubilization. After a centrifugation step at 250 000 g, lysate was recovered and
383 incubated for 2 h with pre-equilibrated Ni-NTA beads (Ni Sepharose 6 Fast Flow, GE Healthcare
384 Life Sciences) in a binding buffer (50 mM Tris, 0.5 M NaCl, 20 mM imidazole, 7 M urea, pH 8).
385 Proteins were eluted from the beads with 10 mL of elution buffer (50 mM Tris, 0.5 M NaCl, 500
386 mM imidazole, 7 M urea, pH 8). After the affinity chromatography step, proteins were loaded on
387 a HiPrep 26/10 desalting column (GE Healthcare) to exchange buffer for 1 % acetic acid and
388 remove low molecular weight compounds. All the purification steps were realized in 100 %
389 H₂O. It allowed amide proton back exchange under denaturing conditions for the mixed (1/1)
390 [(U-¹H/¹⁴N)/(U-¹H_N/²H/¹⁵N)] sample.

391 ***Assembly of HELLF(209-273) fibrils in vitro.*** The pure protein recovered after HPLC
392 purification in 1% acetic acid was concentrated in Amicon Ultra-15 (cut-off 3 kDa) centrifugal
393 filter units (Merck Millipore) to reach the final protein concentration of 1 mM. For the mixed
394 (1/1) [(U-¹H/¹⁴N)/(U-¹H_N/²H/¹⁵N)] sample, monomers were solubilized in 1% acetic acid at 1
395 mM concentration at a molar ratio of 1:1. Fibrils were formed by adjusting the pH with 3 M Tris
396 to a pH value of 7.5. The protein solution was allowed to self-assemble for 2 weeks at room
397 temperature under slow shaking. Fibrils were then centrifuged at 20000 g and washed several
398 times with water supplemented with 0.02 % NaN₃ and transferred to the ssNMR rotor.

399 ***Solid-state NMR spectroscopy of HELLF(209-277).*** ssNMR spectra were recorded on a 23.5 T
400 (1 GHz ¹H frequency) spectrometer (Bruker Biospin, Germany) equipped with a 0.7 mm triple
401 resonance (¹H, ¹³C, ¹⁵N) MAS probe. Sample spinning frequency was 100 kHz. Spectra were
402 referenced according to 4,4-dimethyl-4-silapentane-1-sulphonic acid (DSS) signals.

403 ***Backbone and side-chain resonances assignment of HELLF.*** We used a set of eight 3D ¹H
404 detected experiments: (HCA)CB(CA)NH [4 scans, 11 ms (t₃) x 4 ms (t₂) x 20 ms (t₁)],
405 (HCO)CA(CO)NH [16 scans, 11 ms (t₃) x 8 ms (t₂) x 20 ms (t₁)], (H)CANH [4 scans, 11 ms (t₃) x

406 8 ms (t2) x 20 ms (t1)], (H)CONH [8 scans, 11 ms (t3) x 8 ms (t2) x 20 ms (t1)], (H)CO(CA)NH
407 [32 scans, 11 ms (t3) x 8 ms (t2) x 20 ms (t1)], (HCA)CBCAHA [4 scans, 8 ms (t3) x 4 ms (t2) x
408 20 ms (t1)], (H)N(CO)CAHA [8 scans, 14 ms (t3) x 6 ms (t2) x 20 ms (t1)] and (H)NCAHA [4
409 scans, 14 ms (t3) x 8 ms (t2) x 20 ms (t1)]. The combination of these experiments allowed the
410 connectivities between each ^1H - ^{15}N couple to intra-residual or sequential CA, CA, CO and HA
411 resonances, necessary to perform the entire backbone assignment. Side-chain proton
412 assignment was performed using a ^1H - ^{13}C CP-based sequence followed by a WALTZ mixing step
413 (H)CwaltzCH [2 scans, 6 ms (t3) x 6 ms (t2) x 20 ms (t1)]. We used as a starting point the
414 previously assigned CA and HA chemical shifts to correlate unambiguously all the carbons and
415 protons of the side-chains step by step (27). Spectra were analysed using the CCPNmr Analysis
416 Software (40).

417 ***Collection of intra- and inter-molecular restraints for HELLF from solid-state NMR.*** 211

418 distance restraints per monomer were collected from ^1H detected ssNMR spectra to determine
419 the 3D structure of the HELLF(209-273) amyloid fibril. We added to these restraints 34 dihedral
420 angles (phi/psi) estimated from chemical shifts using the TALOS+ software. 143 long-range
421 intra-molecular restraints were assigned on 3D (H)CHH [4 scans, 6 ms (t3) x 4 ms (t2) x 20 ms
422 (t1)], HhNH [8 scans, 8 ms (t3) x 4 ms (t2) x 20 ms (t1)] and H(H)CH [4 scans, 6 ms (t3) x 4 ms
423 (t2) x 20 ms (t1)] spectra using a radio frequency-driven recoupling (RFDR) ^1H - ^1H mixing. 33
424 inter-molecular restraints were assigned on a 3D H(H)NH spectrum [48 scans, 9 ms (t3) x 4 ms
425 (t2) x 20 ms (t1)] using a RFDR ^1H - ^1H mixing, using a mixed (1/1) [(U- $^1\text{H}/^{14}\text{N}$)/(U- $^1\text{H}_\text{N}/^2\text{H}/^{15}\text{N}$)]
426 sample for the unambiguous detection of intermolecular restraints. A RFDR mixing followed by
427 a ^{15}N -edited CP allowed a magnetization transfer from all the protons of the (U- $^1\text{H}/^{14}\text{N}$) HELLF
428 monomers to ^{15}N atoms of the (U- $^1\text{H}_\text{N}/^2\text{H}/^{15}\text{N}$), back protonated monomers adjacent in the fibril,
429 followed by the ^1H detection of the amide protons

430 ***NMR structure calculation of HELLF.*** The structure of HELLF fibrils was determined in several
431 cycles of structure calculations and restraint analysis with ARIA 2.3 (41). Cross-peak
432 assignments for ^1H - ^1H correlations were converted into distance restraints with an upper-

433 bound of 8.5Å. Backbone dihedrals angles were predicted with TALOS+(30) from ^1H , ^{13}C and ^{15}N
434 chemical shifts. TALOS predictions for residues in secondary structure elements (R1 β_{1-4} , R2 β_{1-4}
435 and C-terminal α -helix) were converted in dihedral angle restraints with an error range
436 corresponding to ± 1.5 times the TALOS error with a minimum of $\pm 15^\circ$. HELLF fibrils structure
437 was calculated as a pentamer with five copies of HELLF(220-272) using simulated annealing
438 performed with CNS 1.2 (42). The ladder topology was maintained during the calculation
439 through distance restraints ensuring that the distance between equivalent C α atoms in
440 neighbouring monomers is constant throughout the pentamer, without fixing a particular
441 distance value, i.e. $d_{m/m+1} = d_{m+1/m+2} = d_{m+2/m+3} = d_{m+3/m+4} = d_{m+4/m+5}$ (43, 44). Additionally, an NCS
442 restraint was added to minimize the r.m.s difference between atomic coordinates of the
443 monomers(44). For every ARIA iteration 100 structures were calculated and the 10 lowest-
444 energy structures from the last iteration were refined in a shell of water molecules (45). On the
445 basis of the identified β -strands and the in/out distribution of side-chains from an initial ARIA
446 calculation using NMR restraints only, intra- and inter-monomer hydrogen bond restraints
447 between equivalent β -strands from the R1 and R2 pseudo-repeats were included in subsequent
448 rounds of ARIA calculation. Final restraints and structure statistics are given in SI Appendix,
449 Table 3.

450 ***Solid-state NMR spectroscopy of HEC, HEC, HEC^S and HEC^Φ D.*** Proton-detected ssNMR spectra
451 were recorded on a 23.5 T (1 GHz ^1H frequency) and 14.1 spectrometers (Bruker Biospin,
452 Germany) equipped with 0.7 mm, 1.3 mm and 3.2 mm triple resonance (^1H , ^{13}C , ^{15}N) MAS
453 probes. Spinning frequency was maintained at 100 kHz (0.7 mm) and 60 kHz (1.3 mm) for ^1H
454 detection and 11 kHz (3.2 mm) for ^{13}C detection, respectively.

455 2D (H)NH experiments of HEC and HED were recorded at 100 kHz MAS (1 GHz ^1H frequency
456 spectrometer). Resonance assignment of HED, recorded using a 1.3 mm probe (1 GHz ^1H
457 frequency spectrometer), was performed using the following set of experiments: (H)CONH,
458 (H)(CO)CA(CO)NH, (H)NCAH, (H)COCAH. 2D ^{13}C - ^{13}C experiments of HEC^S and HEC^Φ were
459 recorded at 11 kHz MAS (600 MHz ^1H frequency spectrometer).

460 Experimental data were processed using TopSpin and analyzed using Sparky (46) or CCPNMR.

461 **Modeling of HRAMs families structures.** 3D models of the prion forming domain (PFD) of
462 representative proteins of HRAM-2, HRAM-3 and HRAM-4 were constructed with the program
463 MODELLER (47) using the HET-s(218–289) (PDB 2KJ3) (43) and HELLF(220-272) structures.
464 For each HRAM family, the sequence of the R1 and R2 repeats of HET-s and HELLF were aligned
465 on the predicted repeats of the PFD to be modeled. HRAMs models were built as trimers using
466 the atomic coordinates of the R1 and R2 repeats of HELLF and HET-s structures as templates.

467 **Data Availability**

468 All data from this work are included in the main text and the SI Appendix of the paper.

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482

483 **Author contributions**

484 D.M., L.B.A., J.S., G.P., B.H., N. E.M and A.L. performed ssNMR experiments; D.M., N. E.M. G.P., B.H.
485 and A.L. analyzed ssNMR data; B.B. implemented and performed structure calculations; D.M.,
486 M.B., A.N. expressed, purified, and polymerized HELLF fibrils for NMR; A.D., V.C., S.J.S. carried

487 out microscopy, prion infectivity and in vivo experiments; B.K. performed x-ray diffraction
488 experiment; J.S.W. performed STEM experiments; A.D., D.M., B.H., A.L. and S.J.S. wrote the paper;
489 all authors discussed the results and commented on the manuscript.

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491 **Declaration of Interests**

492 The authors declare no competing financial interests.

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604

605 **Fig. 1 HELLF is a distant HET-s homolog encoded in the genome of *Podospora anserina*.**

606 **A.** Sequence similarity between HELLF and HET-s PFDs. The two pseudo-repeats are boxed.
607 Conserved residues are shown in Taylor colour scheme and intensity of colour reflects degree of
608 conservation. **B.** MEME cartoons showing the conservation of HRAM-specific residues. **C.** Dot-
609 like aggregates of the HELLF PFD (GFP-HELLF(209-277)) formed in *P. anserina*. Scale bar is 5
610 μ m. **D.** HELLF controls a programmed cell death reaction as determined by the 'barrage'
611 phenotype (white arrowhead) between strains expressing full-length HELLF and strains
612 expressing the HELLF PFD in the $[\varphi]$ prion state. **E.** Electron micrograph of fibrillar assemblies
613 of the HELLF PFD, scale: 100nm.

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618 **Fig. 2 Solid-state NMR (ssNMR) characterization of HELLF(209-277) fibrils.** **A.** ssNMR
619 sample preparation of HELLF(209-277), requiring minimal sample quantity (<300ug) using a
620 0.7 mm ssNMR rotor. **B.** Extracts of ssNMR spectra for ^1H , ^{13}C and ^{15}N sequential assignments. A
621 combination of (HCA)CB(CA)NH (red), (HCO)CA(CO)NH (black), (H)CANH (purple), (H)CONH
622 (green) and (H)CO(CA)NH (blue) was used to assign HELLF(209-277) fibrils. **C.** ^1H - ^{13}C ssNMR
623 spectra of fully protonated HELLF(209-277) amyloid fibrils. **D-F.** Collection of ssNMR distance
624 restraints. **D.** Intramolecular distances based on a H...(H)CH experiment on fully protonated
625 HELLF sample. **E.** ssNMR approach to detect intermolecular interactions, based on a (1/1) [(U-
626 $^1\text{H}/^{14}\text{N}$)/(U- $^1\text{H}_\text{N}/^2\text{H}/^{15}\text{N}$)]-labelled HELLF sample. **F.** Intermolecular distances based on a
627 H...(H)NH experiment on (1/1) [(U- $^1\text{H}/^{14}\text{N}$)/(U- $^1\text{H}_\text{N}/^2\text{H}/^{15}\text{N}$)]-labelled HELLF(209-277). **G.**
628 ssNMR restraints shown on the HELLF(209-277) rigid core.

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633 **Fig. 3 Solid-state NMR structure of HELLF(209-277) amyloid assembly.** **A.** Side view of a
634 ribbon representation of HELLF(209-277) structure, representing five monomers stacked in the
635 fibrillar arrangement. Individual molecules are represented in different colours. **B.** Top view of
636 the SSNMR HELLF(209-277) amyloid structure with main fibril axis and labelled β -strands. **C.**
637 Top view of the solenoid-forming pseudo-repeats, R1 (top) and R2 (bottom), of HELLF(209-
638 277). Hydrophobic residues are shown in white, acidic residues in red, basic residues in blue
639 and others green.

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644 **Fig. 4 HELLF(209-277) amyloid fold presents strong similarity with HET-s(218-289),**

645 **while the two prion domains lack *in vivo* cross-seeding.** **A.** Backbone structural alignment of
646 HELLF PFD (in blue) and HET-s PFD (in yellow) ssNMR structures. **B-C.** Shown are four
647 cartoons representing the hydrophobic triangular core of amyloid fibrils formed by the
648 successively stacked pseudo-repeats – R1 (left panels) and R2 (right panels) – of HELLF (**B**) and
649 of HET-s (**C**). Amino acid residues decorating the amyloid backbones are drawn as beads of
650 different colours. Hydrophobic residues are shown in white, acidic residues in red, basic
651 residues in blue and others green. **D.** Fluorescent microscopy images of strains co-expressing
652 HET-s-RFP ([Het-s*] or [Het-s] state) and the cytotoxic-dead HELLF(L52K)-GFP mutant ([Φ*] or
653 [Φ] state) exhibiting distinct epigenetic combinations. Full panels are given in SI Appendix, Fig.
654 S8. Scale bar: 2 μm. **E.** 3D models for the two pseudo-repeats of different HRAM families. Below
655 the molecular models are shown the protein sequences used to generate the models for each
656 HRAM family. The sequences GenBank/NCBI reference IDs are as follows: XP_009252118.1
657 (HRAM1), CDM29511.1 (HRAM2), XP_007744431.1 (HRAM3) and XP_007838484.1 (HRAM4).

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661 **Fig. 5 HELLF-derived chimeric protein HEC breaches the prion cross-seeding barrier**
662 **between HELLF and HET-s.** **A.** Sequence alignments of the two pseudo-repeats (R1 and R2),
663 constituting the PFDs of HELLF, HET-s and the engineered protein HEC. HELLF-specific residues
664 are shown in blue colour. HET-s-specific HRAM1-defining residues are shown in red. Residues
665 shared between all three proteins are shown in purple. **B.** Representation of barrage
666 phenotypes between strains expressing full-length HET-S or HELLF in confrontations with HEC-
667 expressing strains of [HEC^S] (carrying HEC prion strain induced by contact with [Het-s] prion)
668 or [HEC^Φ] (HEC prion strain induced by [Φ]) phenotypes. The barrage reaction is shown as a
669 line separating two incompatible strains (green circles). **C.** Induction of the [Het-s] prion by
670 HEC-expressing strains *in vivo*. [Het-s] induction is measured in percentage of prion-free [Het-
671 s*] strains converted (by the cited strains) to prion-infected [Het-s] strains. Negative control is

672 indicated with a minus sign. Experiments were performed at least in triplicate. P value ($a \neq b$) <
673 0.0001, one-way ANOVA with Tukey's multiple comparisons test. **D.** C α chemical shift
674 differences between HEC seeded with [Het-s] and [Φ]. Non-identical residues between HEC and
675 HELLF are coloured in red. Asterisks indicate unassigned residues because of spectral
676 ambiguities. **E.** Extracts of ^{13}C - ^{13}C ssNMR spectra of HEC fibrils seeded with [Het-s] (red) or [Φ]
677 (blue). **F.** Cartoon representation of the amyloid backbone of HEC pseudo-repeats. Residues
678 with highest conformational changes between HEC seed by HET-s or HELLF, as measured by
679 difference in chemical shift on C α , are shown in yellow. Green stars highlight residues in two
680 conformations.

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repeat 1 (R1) repeat 2 (R2)

HELLF ATRACRTGQKFGEMKIDDHSIAMQGIIVGVAQ----PG-----VDQSFGLTITTKSSRAFGQGM DAGSFSNLF SK
HET-S KIDAIIVGRNSAKDIRTEERARVQLGNVVTAAALHGGIRISDQTTNSVETVVVGKGESRVLIGNEYGGKGFWDN--









