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Heterotypic amyloid interactions: clues to polymorphic bias and selective cellular vulnerability?

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Abstract

Recent years have witnessed an accelerating amount of atomic resolution structures of disease-associated amyloids. These studies have confirmed the polymorphic nature of amyloids but also the association of specific polymorphs to particular proteinopathies. These observations are strengthening the view that amyloid polymorphism is a marker for specific pathological subtypes (e.g. in tauopathies or synucleinopathies). The nature of this association and how it relates to the selective cellular vulnerability of amyloid nucleation, propagation and toxicity is still unclear. Here we provide an overview of the mechanistic insights provided by recent patient-derived amyloid structures. We discuss the framework organisation of amyloid polymorphism and how heterotypic amyloid interactions with the physiological environment could modify the solubility and assembly of amyloidogenic proteins. We conclude by hypothesizing how such interactions could contribute to selective cellular vulnerability.

Introduction

Protein aggregation is associated to debilitating diseases that are steadily affecting more and more people worldwide. From major neurodegenerative disorders, such as Alzheimer's and Parkinson's, to type 2 diabetes mellitus and systemic forms, this group of pathological conditions is characterized by the aberrant deposition of proteinaceous ordered fibrillar aggregates, known as amyloids [1-3]. Amyloid fibrils are typically formed by homotypic polymerization of single proteins into intermolecular β-rich assemblies. However, despite being considered as the major hallmark of these diseases, the formation of amyloid fibril aggregates alone does not suffice to explain the apparent complexity behind their toxicity, progression and selectivity [4] nor their ability to form conformationally variable amyloid structures, known as amyloid polymorphs or strains, upon given conditions [5,6]. Combined to lessons learned from spatial transcriptomic and proteomic approaches [7,8], heterocomplex assemblies from functional and pathological amyloids [9*] and accumulated experimental data on the role of cofactors [10], it is becoming evident that the inter-communication of amyloids with other molecules has a pivotal effect in their overall structure, function and derived pathology.

Here, we review current advances in our understanding of amyloid structure and polymorphism. We review how specific interaction interfaces of amyloids can be associated to cross-interplay with other biomolecules and discuss the structural effects of the latter in amyloid polymorphism and conformational diseases. Finally, we describe recently proposed mechanisms suggested to promote amyloid-protein cross-interactions and explore their potential impact in explaining complex disease phenotypes such as selective vulnerability and amyloid disease progression.

Framework amyloid polymorphism: a role for structural frustration and heterotypic interactions in polymorphism?

The structure of amyloid cores contains at least one and often multiple short sequence segments of about 5 to 10 residues that are essential for amyloid assembly [11-16]. These aggregation-prone regions (APRs) are kinetic hot spots that drive amyloid assembly by virtue

of their high cross-β propensity, which results from their sequence composition favouring a combination of high β-sheet propensity, hydrophobicity and side chain H-bond potential [17,18], whereas recent efforts have uncoupled sequence aggregation propensity from solubility [19]. Confirming the essential role of APRs for amyloid assembly, single point mutations neutralizing their cross-β propensity completely abolishes protein amyloidogenicity [20-22]. APRs can also form amyloid fibrils as isolated peptides [23,24]. Before the recent advent of full-length amyloid core structures and their polymorphs, X-ray structures of APR peptides provided the first insights both into the atomic structure of amyloid fibrils as well as the potential of APRs to contribute to structural polymorphism [25]. As isolated peptides, APRs form cross-β sheets that pack against each other to form homotypic steric zippers. The observation of alternative homotypic steric zipper arrangements for the same sequence immediately suggested the possibility of "packing polymorphisms" whereas the presence of several APRs in one amyloidogenic protein suggested additional opportunities for "segmental polymorphism" whereby alternative amyloid fibrils can be formed by incorporating different APRs [26,27] (**Fig. 1**). Combining these structures with NMR constraints suggested that APRs can also form heterotypic steric zippers between different APRs suggesting a third mechanism of "combinatorial polymorphism" (**Fig. 1**) [28]. The ability of short peptides to adopt amyloid-like structural conformations does not necessarily imply that they participate in a similar cross-beta architecture within the context of a full-length protein sequence. However, similar evidence supporting a model of segmental polymorphism was recently derived also from analysis of full-length proteins. For example, it has been demonstrated that Pmel17, which participates in the formation of functional amyloid, can form fibril polymorphs in which different segments shape the fibril core [29], whereas the low-complexity domain of FUS has been shown to form fibrils with different core-forming segments, depending on which construct is used [30].

The structures of full-length amyloid cores confirmed the packing promiscuity of APRs [31,32-33*,34-36,37*]. It also showed that the majority of steric interfaces involving APRs are constituted by heterotypic zippers between different APRs but also between APRs and non-APR segments (**Fig. 1**). Importantly, amyloid core structures also revealed that APRs constitute only about one third of the protein sequence incorporated into the amyloid core. Given that only APRs have a high propensity to form cross-β assemblies, this raises the

question as to how the other two-thirds of the residues within the amyloid core contribute to amyloid fibril stability and whether and how this feeds into amyloid polymorphism and polymorphic bias in disease [38]. A thermodynamic analysis of amyloid polymorphs, using FoldX [39], revealed that despite their sometimes very different tertiary packing, distinct polymorphs share common sequence segments of high stability, namely those constituting the APRs [40] (**Fig. 1**). Thus, APRs are (1) essential for amyloid assembly, (2) contribute most to the stability and (3) constitute the primary stable regions independent of polymorphism. This is confirmed by structural inspection. First, the local RMSD is the lowest between APR segments of different polymorphs. Second, main-chain cross-β H-bonds between amyloid rungs adopt ideal geometries within APR segments while this is not the case elsewhere. This is reflected by the generally more unfavourable energetic profile assigned by force field calculations to residues outside APRs suggesting that these regions are often structurally frustrated in the amyloid conformation and require additional modes of stabilisation to be incorporated in the amyloid core [40]. For instance, αS fibril formation was recently proposed to progress through gradual segmental folding driven by APRs, but was shown to require stabilisation by phospholipids, a cross talk that was proposed to transfer from monomer to fibrils [41*].

The observation that the same segments of high cross-β structural propensity dominate the stability of different polymorphs suggests a framework model [42] for polymorphism (**Fig. 1**) in which intrinsically favourable cross-β segments drive amyloid assembly and whereby less favourable sequence segments around and between these drivers need to be accommodated to the cross-β conformation by additional stabilizing interactions. This suggests that stabilizing modifications or additional interactions within these regions of lower cross-β propensity (including elements of the fuzzy coat around the amyloid core) potentially play an important role in steering polymorphism [43]. This is in agreement with the observation that *in vitro* polymorphic propagation in simple aqueous buffers is not robust and likely not representative of pathology. Supporting this notion, Strohäker et al. recently showed that de novo formation of in vitro-prepared aS fibrils results in morphologically differentiated fibrils compared to in vitro fibrils seeded with brain extracts derived from PD and MSA patients [33*], with similar results shown for AA fibril preparations [44*], while tau fibrils grown *in vitro* using additives such as heparin result in polymorphic conformations that

are not related to those found in patients [32]. In addition, *in vitro* propagated amyloid fibrils from patient seeds do not necessarily conserve the original polymorphic conformation, e.g. for α-syn from MSA patient [45*]. It is noteworthy, however, that the latter fibril species were produced by somewhat inefficient seeding reactions, as indicated by long lag phases during the kinetics experiments, which potentially puts into question the extent to which the resulting fibrils represent initial morphologies found in the patient-derived material or those produced by spontaneous in vitro aggregation. Understanding polymorphic bias in disease therefore requires understanding the context-dependent covalent modifications and interactions associated to each polymorph. Both post-translational modifications [46*] and familial disease mutations [47] associated to specific neurodegenerative pathologies have also been found to favour specific polymorphs. In addition a plethora of undetermined density islands in cryo-EM structures clearly indicate amyloids interact with a varied host of molecules, including protein fragments and non-proteinaceous ligands and co-factors ([48] and references therein).

Sequence specificity of lateral versus axial amyloid interactions

Amyloids fibrils are anisomorphic structures having a cross-section of about 70-120Å and lengths up to several μm. While amyloid elongation occurs at the extremities of amyloid fibrils [49], lateral interactions have been shown to provide for surface-assisted catalysis of amyloid nucleation [50], demonstrating that self-interaction and assembly can be mediated both by axial as well as lateral amyloid interactions. In the same manner both axial and lateral amyloid interfaces can engage in heterotypic interactions, however the nature (and therefore sequence-specificity) as well as the dimensions (and therefore the availability) of both types of interaction surfaces is expected to be very different. The axial interaction interface at growing extremities presents a fibril cross-section consisting of the entire amyloid core sequence (**Fig. 2**). Templated amyloid elongation favours incorporation of identical sequences likely because registered side-chain stacking maximizes cross-β H-bond saturation between amyloid rungs. The design of structure-based inhibitors exploits these structural properties [51] and has allowed the development of inhibiting peptides and peptidomimetics against tau [52], αS [53], Aβ [54], IAPP [55] and TTR aggregates [56]. Such inhibitors generally consist of

modified scaffolds embedding a homologous sequence containing point mutations that still allow to dock against the fibril tip but inhibit further growth [57-59]. These examples also highlight a certain degree of tolerance for mutations as single inhibitors targeting Aβ APRs were also found to interfere with tau and IAPP aggregation simultaneously [60,61*]. By the same token, amyloid cross-seeding and co-assembly is also found to be favoured by sequence homology [9]. A series of recent biophysical studies have recapitulated the importance of axial interactions for several amyloid-forming proteins [62-64]. Determined three-dimensional structures of amyloid heteromeric fibrils, such as in the case of the RIP1/RIP3 and $A\beta_{40}/A\beta_{42}$ heterocomplexes [65,66**], as well as structural analyses of synthetic co-assembled nanofibers [67] further validate axial heterotypic assembly. A systematic thermodynamic exploration of amyloid assembly confirmed the sequence-specificity of amyloid tip interactions but also revealed context-dependent structural rules for mutational tolerance showing how these interactions can affect amyloid protein solubility, the kinetics of amyloid aggregation and amyloid morphology [68,69]. This suggests that amyloid propensity and conformation can be shaped by amyloid tip-mediated heterotypic interactions with its surrounding proteome, a notion that was experimentally verified in terms of tau and Aβ aggregation [68,69].

While they provide sequence-specific modes of interaction, the ratio of axial versus lateral interaction surface decreases rapidly with fibril growth. Therefore, axial interactions are probably more important at early stages of amyloid nucleation and assembly, while lateral interactions probably dominate once larger fibrils are formed. Lateral surfaces do not provide the sequence information displayed at amyloid tips. They are formed by the external surface of the fibril, forming homogeneous longitudinal grooves and ridges along the fibril axis the periodicity of which is determined by the helical pitch of the fibril (**Fig. 2**). This periodic layout of shape, charge and hydrophobicity mediates protofibrillar assembly and provides the substrate for interaction with various dyes reporting amyloid structure [70]. Lateral surfaces also allow stabilizing the amyloid fibril by interaction with polyanionic molecules such as heparin or polyphosphates [71]. In addition, it has been proposed that small structural imperfections along the length of the fibril might provide additional interaction points [72]. Finally, CryoEM structures often display unresolved densities at their surfaces, further illustrating their propensity to interact with their environment [48]. Although the relative

importance of axial versus lateral interactions is far from resolved, evidence therefore suggest that lateral interactions are probably more promiscuous. Diverse structural polymorphs, formed by different protein precursors, were shown to commonly cross-interact with several proteins found in biological fluids, in contrast to less ordered or amorphous aggregates [73**]. While Aβ fibril surfaces mediate secondary self-nucleation, it also facilitates templating of S100A9 amyloid formation [74], whereas a similar sensing mechanism has been proposed to modulate YAP activity upon conversion of PMEL functional amyloids to melanoma disease [75]. Blocking such surface interactions has also been used as a strategy to interfere with secondary nucleation of aggregating proteins. An engineered β-wrapin construct was shown to bind and inhibit the secondary nucleation of α-synuclein [53]. TTR [76] and BRICHOS [77] cross-interactions, as well as surface lateral arrangements between variant protofibrils [78] inhibit secondary nucleation of Aβ. However, TTR has also been linked to primary nucleation inhibition [76], indicating that despite recent progress, the overlapping importance of these different amyloid binding modes remains unclear.

Amyloid interactions and co-aggregation in the cell

Single-cell transcriptomics and mass spectrometry studies are starting to provide insights towards the molecular context in which amyloid aggregation occurs in cells and tissues and with what proteins they interact and/or co-aggregate [79*,80**]. These findings suggest several non-exclusive mechanisms that could contribute to selective cellular vulnerability. First, context could be provided by an association-by-function mechanism for co-aggregation (**Fig. 3A**), whereby proteins that directly interact with the amyloidogenic protein have a higher probability to be entrapped in fibrillar inclusions. Aβ-cross-reactive amyloidogenic proteins linked to positive regulation of AD progression have been extensively documented as major components of amyloid plaque deposits [81]. Such a mechanism was also proposed for food amyloids in the case of hen egg-white proteins [82]. A particular case of co-aggregation by proximity consists in the association of elements of the proteostatic machinery with protein inclusions, including chaperones but also ribosomal and proteasomal components [83], although the heterotypic activity of the latter with amyloids has also been related to the generation of toxic oligomeric species [84]. The association of chaperones to

amyloids can be a protective mechanism [85] but has also been associated to inducing proteostatic collapse of metastable proteins (**Fig. 3B**) [86]. The proteomes of neurons and other brain cell populations seem to be enriched in proteins that are meta-stable, meaning that their physiological expression levels exceed their intrinsic solubility [87*]. Their native state is therefore less stable than the amyloid state and needs to be kinetically controlled. Chaperones are crucial for this process and their depletion e.g. by aggregation of an amyoidogenic protein could therefore result in the collapse of metastable sub-proteomes, thereby contributing to selective vulnerability in ALS and AD [88,89].

Heterotypic interactions are also considered a major force of the "stickers and spacers" and "scaffold-client" models proposed to drive liquid-liquid phase separation, (LLPS) as such intermolecular interactions can mediate the structural and dynamical properties of biomolecular condensates [90]. Droplet conversion was also shown to be promoted by heterotypic interactions induced by amyloidogenic peptide additives [91]. In a turn of events, however, phase separation has also been proposed to facilitate heterotypic sequestration of amyloidogenic proteins, although this mechanism could also potentially explain the molecular entrapment of co-interactors in amyloid deposits (**Fig. 3C**) [92,93*]. An in-depth analysis using network-free stochastic modeling indicated that the balance of heterotypic multicomponent LLPS condensates and their transition to growing large aggregates can be described in large by thresholds set by a solubility product constant [94].

A major driver of the initiation of age-related amyloid diseases is believed to be the waning of proteostatic control especially the ability of cells to degrade (misfolded) proteins with age [95]. Once susceptible proteins start to aggregate the above-mentioned mechanism likely all contribute to the selective cellular propagation and toxicity of amyloids. But can interacting and co-aggregating molecules also contribute to the selective susceptibility of amyloid initiation? And can sequence-specific amyloid interactions affect the intrinsic propensity of amyloidogenic proteins to assemble into amyloids? Recently it was shown that Sup35 prion variants utilize heterotypic interactions as a conformational mechanism to regulate species-specific transmissibility barriers [96**]. Similarly, IAPP is now considered a co-trigger factor in AD due to its homology to Aβ [97], whereas the ability of the otherwise functional CsgA subunit to accelerate aS amyloid formation implies a role for the gut microbiome in Parkinson's disease progression [98]. Perhaps what is the most representative

example of targeted heterotypic interactions, however, is the case of the evolutionary shaped co-aggregating signaling necrosome complex (**Fig. 3D**) [65]. This amyloid-complex driven pathway has even been re-purposed by sequence-dependent viral counterparts in an effort to promote viral infectivity (**Fig. 3D**) [99**]. Utilizing a similar sequence-driven mechanism, we showed that the sequence promiscuity of APRs from major amyloidogenic proteins, such as Aβ and tau, increase cellular vulnerability to aggregation spreading by supporting heterotypic interactions with homologous sequence hotspots found in proteins with pivotal roles in AD progression (**Fig. 3E**) [68,69]. Together, the above indicate that heterotypic interactions contribute both to cause and effect of amyloid aggregation. Sequence-specific amyloid interactions can both influence the solubility and the kinetics of amyloid assembly as well as being co-precipitated by the aggregation process itself. Finally, heterotypic amyloid interaction can affect the structure of mature amyloid fibrils [68,69].

Conclusions

The increasing amount of disease-associated amyloid structures has provided molecular detail to the structural principles determining amyloid polymorphism and has confirmed polymorphic bias in different tau and a-synucleinopathies, but also in other amyloid diseases. Although much still needs to be learned on the role of polymorphism and to what extent it is a cause or an effect in disease, it nevertheless remains that polymorphs are the result of their interaction with their environment. Structural inspection of amyloid structures reveals that amyloid cores are not uniformly stable but that they consist of segments with high cross-β propensity interspersed with segments that need to be accommodated to the cross-β backbone by additional stabilising interactions. This can be achieved by a variety of factors such as post-translational modifications, familial disease mutations, binding to other proteins or cofactors that can all contribute to polymorphic selection. Heterotypic interactions can occur both at the amyloid extremities as well as on their lateral surfaces and can be both amyloid sequence-specific or sterically determined. These interactions have been shown to affect the solubility of amyloid proteins, the rate and mechanism of amyloid assembly as well as amyloid structure. The fact that those contextual elements are absent *in vitro* probably explains the inability of *in vitro* seeding to accurately perpetuate disease polymorphs but also the less efficient seeding potential of seeds generated in simple aqueous buffers. Conversely it could be hypothesized that cell- and

context-specific interactions of amyloids *in vivo* could not only direct polymorphic bias but also that once formed propagated seeds would be entropically primed to interact more efficiently with specific cellular factors in successive vulnerable cells. It also implies that, in order to maintain polymorphic bias, these interactions need to be present in successive cells (**Fig. 4**). Together this could explain the disease-specific tropism of different polymorphs, as well as cellular vulnerability patterns [31-37].

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Figure Legends

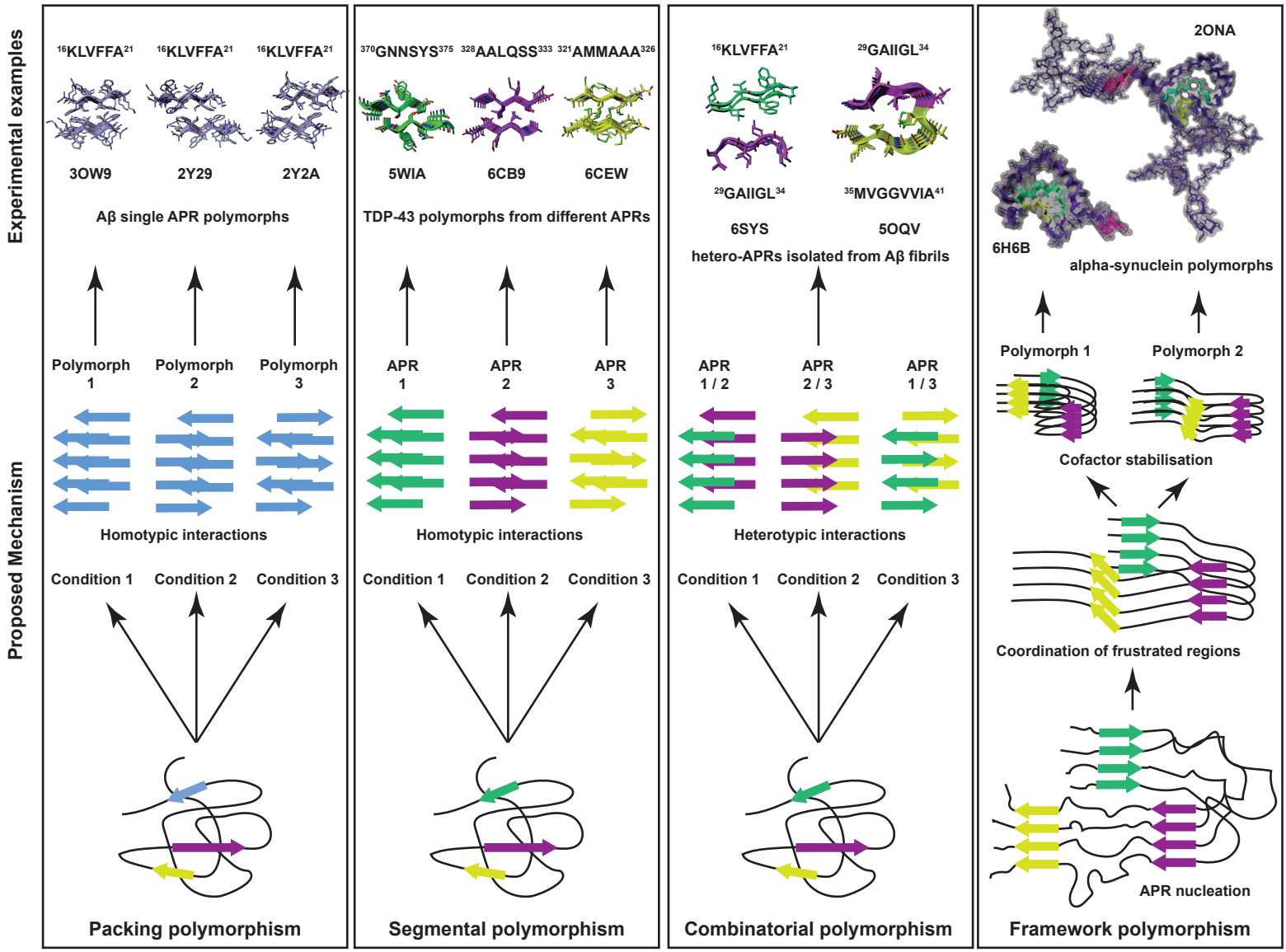
Figure 1. Proposed models for amyloid polymorphism and the role of heterotypic interactions in each process. Packing and segmental polymorphism were based on the early findings on structural polymorphism produced by a single (e.g. the polymorphic KLVFFA APR from Aβ) or multiple APRs derived from a single amyloid protein (e.g. multiple TDP43-derived APRs form distinct polymorphs). Different conditions (pH, temperature etc) or stabilising

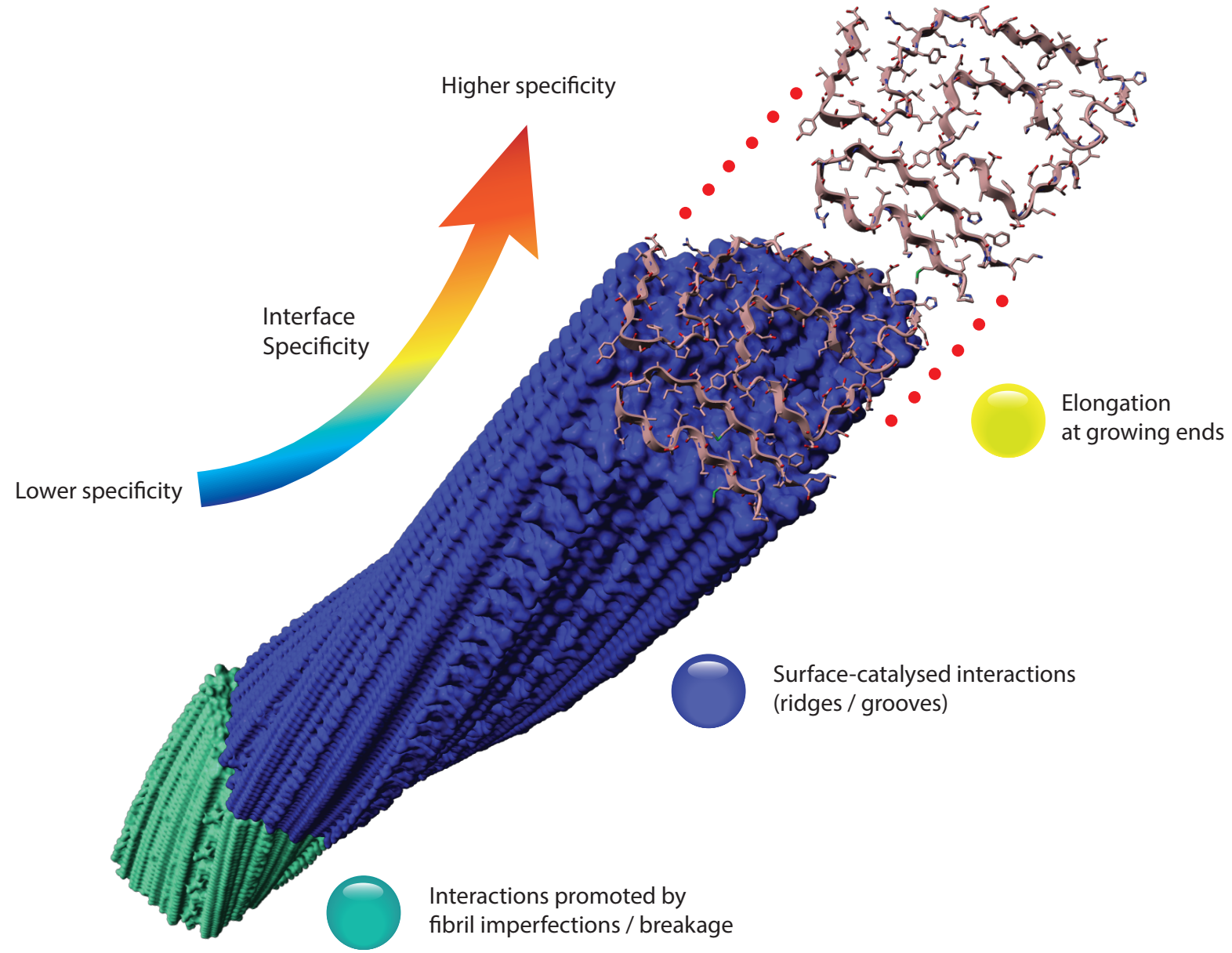
cofactor heterocomplexes (for instance metals, structural waters, poly-ions etc) could contribute to the derived polymorphic layouts. In combinatorial polymorphism, despite the role of cofactors, it was suggested that different APRs may also form heterotypic steric zipper interfaces that promote polymorphism, a notion that can be seen in isolated structural segments of full-length amyloid fibrils. During framework polymorphism, APRs act as stabilising nuclei that promote the amyloid fold, whereas surrounding frustrated regions steer polymorph formation based on heterotypic stability with cofactors. Identified cavities or unidentified EM densities in the periphery of cryoEM amyloid structures support the structural and stabilising importance of amyloid-cofactor heterocomplexes.

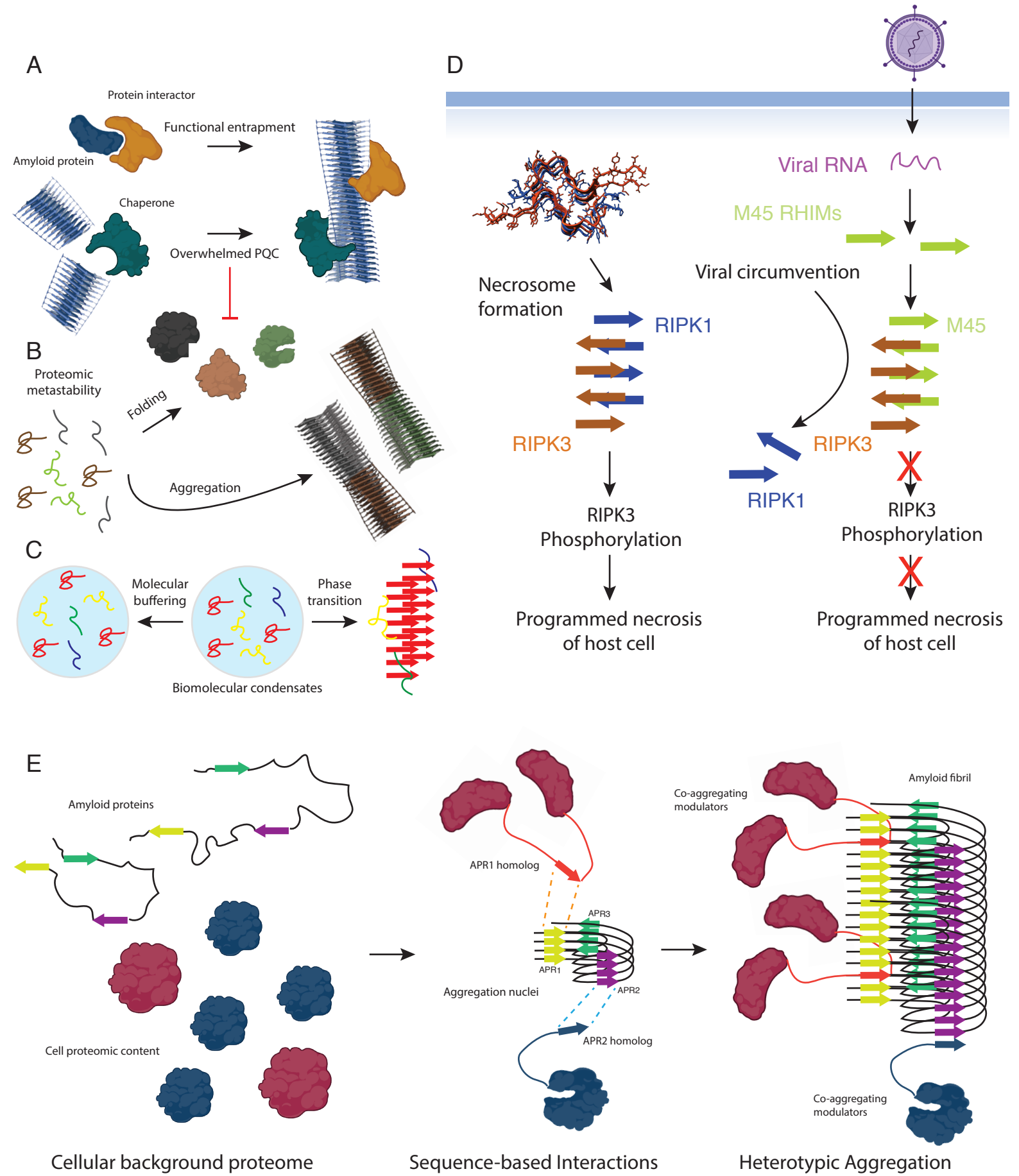
Figure 2. Levels of specificity of potential amyloid interfaces. Lateral interaction sites formed along the surface of amyloid fibrils as fragmentation sites, or as grooves and ridges shaped by the pitch of the filament, can promote promiscuous coupling to other biomolecules. On the contrary, interactions that take place at the tip of amyloids are considered to be limited by the higher specificity required and imposed by stacking and packing interactions with the entire amyloid core sequence.

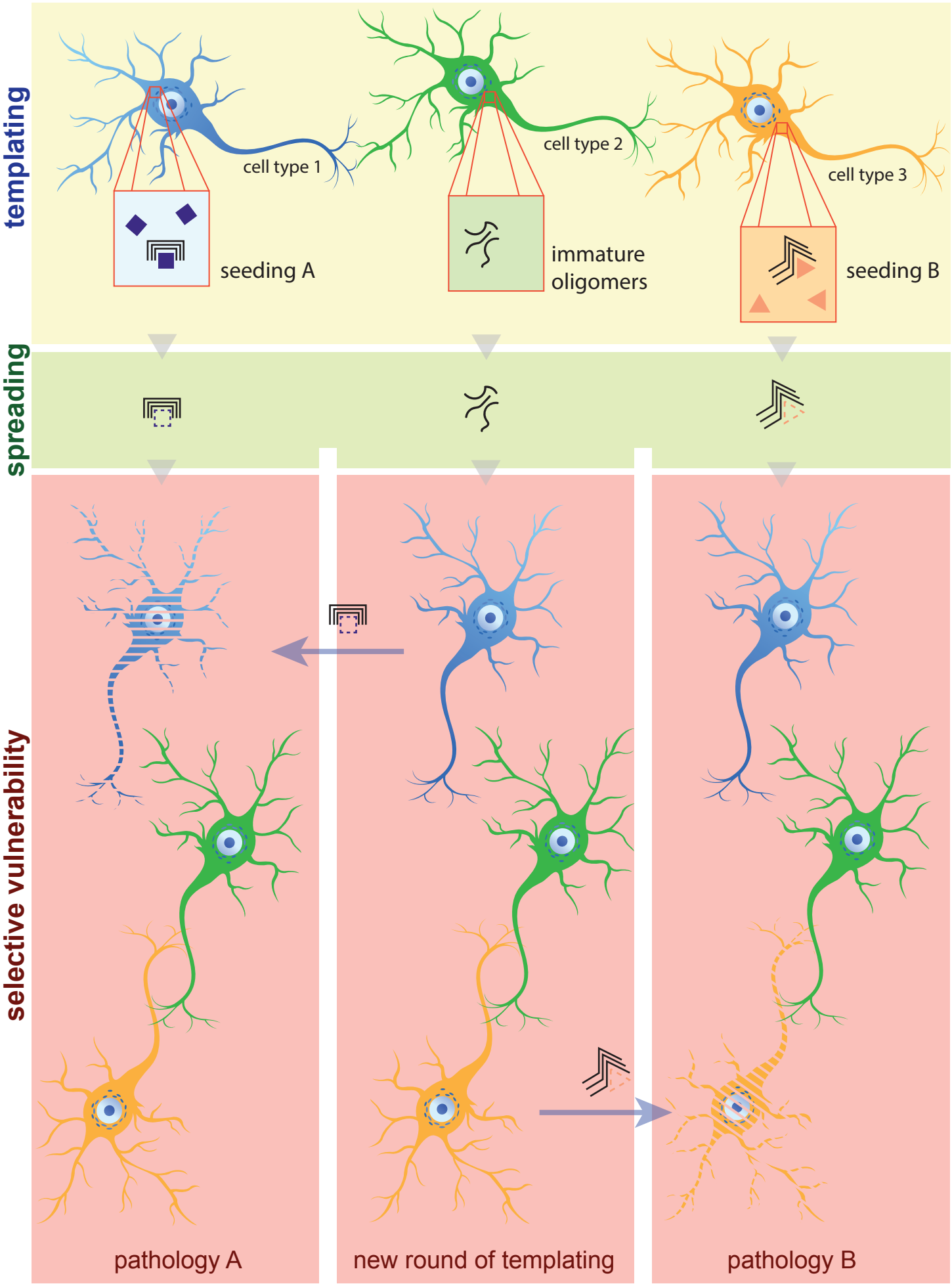
Figure 3. Proposed model mechanisms of heterotypic amyloid assembly and their variable specificity. (A) Proximity-induced co-assembly has been proposed as a loss-of-function mechanism of co-aggregation. This includes proteins that are physiologically related in function to amyloid proteins (e.g. co-aggregation of Aβ to Reelin [100]), as well as a collapse of the cellular proteostatic quality control machinery (PQC). (B) PQC deterioration has also been linked with a shift in the delicate equilibrium of supersaturated proteins that can induce massive aggregation by widespread collapse. (C) The formation of multicomponent condensates has been proposed as a precursor step to amyloid aggregation, but also as a mechanism of heterotypic buffering against aggregation prone cellular components. (D) Sequence-specific mechanism of programmed necrosis involves the formation of heterotypic RIPK1/RIPK3 amyloid complexes. Homologous viral counterparts have been evolutionarily shaped to override necrosome formation, thus ensuring host and viral survival. (E) Cell-specific protein components that harbour sequence stretches with high sequence homology to APRs of amyloid proteins can co-aggregate and promote cellular susceptibility to amyloid spreading.

Figure 4. Polymorphic bias as a mechanism promoting cellular susceptibility. The templating bias that structural polymorphs exhibit explains how certain cellular types sharing similar intrinsic polymorphic components are particularly vulnerable to amyloid spreading of entropically primed seeds (left and right column) and why particular polymorphs are tethered to certain forms of disease (seeding A to pathology A, seeding B to pathology B). This also precludes that the polymorphic content of cells may also potentially capture and shape unspecific early oligomeric forms to promote an additional layer of biased spreading that further enhances cellular vulnerability and distinct pathologies (middle column) at a second level.


Experimental examples
Proposed Mechanism
16KLVFFA21
16KLVFFA21
16KLVFFA21
3OW9
2Y29
2Y2A
Aβ single APR polymorphs
Polymorph 1
Polymorph 2
Polymorph 3
Homotypic interactions
Condition 1
Condition 2
Condition 3
Packing polymorphism
370GNNSYS375
328AALQSS333
321AMMAAA326
5WIA
6CB9
6CEW
TDP-43 polymorphs from different APRs
APR 1
APR 2
APR 3
Homotypic interactions
Condition 1
Condition 2
Condition 3
Segmental polymorphism
16KLVFFA21
29GAIIGL34
29GAIIGL34
35MVGGVVIA41
6SYS
5OQV
hetero-APRs isolated from Aβ fibrils
APR 1 / 2
APR 2 / 3
APR 1 / 3
Heterotypic interactions
Condition 1
Condition 2
Condition 3
Combinatorial polymorphism
2ONA
6H6B
alpha-synuclein polymorphs
Polymorph 1
Polymorph 2
Cofactor stabilisation
Coordination of frustrated regions
APR nucleation
Framework polymorphism


Higher specificity
Interface
Specificity
Lower specificity
Elongation
at growing ends
Surface-catalysed interactions
(ridges / grooves)
Interactions promoted by
fibril imperfections / breakage


A
Protein interactor
Functional entrapment
Amyloid protein
Chaperone
Overwhelmed PQC
B
Proteomic metastability
Folding
Aggregation
C
Molecular buffering
Phase transition
Biomolecular condensates
D
Viral RNA
M45 RHIMs
Viral circumvention
Necrosome formation
RIPK1
RIPK3
M45
RIPK3 Phosphorylation
Programmed necrosis of host cell
RIPK1
RIPK3
RIPK3 Phosphorylation
Programmed necrosis of host cell
E
Amyloid proteins
Cell proteomic content
APR1 homolog
APR3
APR1
APR2
Aggregation nuclei
APR2 homolog
Co-aggregating modulators
Amyloid fibril
Co-aggregating modulators
Cellular background proteome
Sequence-based Interactions
Heterotypic Aggregation


templating
cell type 1
cell type 2
cell type 3
seeding A
immature
oligomers
seeding B
spreading
selective vulnerability
pathology A
new round of templating
pathology B