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Mimicry by a viral RHIM

Miguel Mompeán[1,2,†], Gunes Bozkurt[3,4,†] & Hao Wu[3,4,*]

Functional amyloids have recently attracted much attention due to their involvement in signalling pathways, with hybrid amyloid formation showcasing a key role in necroptosis. In this issue of *EMBO Reports*, Sunde and colleagues [1] unveil that hybrid amyloids are central to necroptosis more broadly, uncovering the amyloidal nature of viral-induced necrosome assemblies. They also prove that the mechanism by which murine cytomegalovirus unleashes necroptosis also relies on hybrid amyloid assembly by viral proteins, akin to that used by host cells. This study presents a way to selectively inhibit necroptosis in which amyloid assembly can be exploited further as a potential therapeutic target.

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Necroptosis is a mechanism of programmed cell death that operates in the absence of apoptosis. Execution of necroptosis, e.g. during viral infection, is mediated by the mixed lineage kinase domain-like (MLKL) protein following its activation by receptor-interacting protein kinase 3 (RIPK3). This process requires the formation of intermediate assemblies or *necrosomes* that activate MLKL for efficient host defence and elimination of infected cells (Fig 1A). Receptor-interacting protein kinase 3 (RIPK3) is central to the assembly of distinct necrosomes, all of which rely on protein–protein interactions through specific, conserved regions named RIP homotypic interaction motifs (RHIMs) with core tetrad sequences I(V)QI(V/L/C)G (Fig 1B). There are three RHIM-containing proteins that engage RIPK3 in three different necrosomes to signal MLKL for necroptosis execution in mammals, namely RIPK1, TRIF and ZBP1 (also known as DAI) [2]. The RIPK1-RIPK3 complex is considered the canonical necrosome, whereas TRIF-RIPK3 and ZBP1-RIPK3 are non-canonical necrosomes.

RHIMs are present not only in human necroptosis proteins, but also in proteins involved in immunity from fungi to flies. Herpesviruses also express RHIM-containing proteins, which are used as a mechanism to sustain viral infection of the host cells [2]. The M45 protein from the herpesvirus murine cytomegalovirus (MCMV) is an exemplary case of necroptosis inhibition. During viral infection, MCMV uses M45 to compete for RHIM-mediated interactions between RIPK1 and RIPK3 or between ZBP1 and RIPK3 to prevent the assembly of both canonical and non-canonical necrosomes, circumventing host cell death that eliminates the infected virus (Fig 1A) [2].

The RIPK1-RIPK3 canonical necrosome core is a functional hybrid amyloid [3] in which RIPK1 and RIPK3 oligomerize into heterotypic β-sheets by alternating their RHIMs (RIPK1-RIPK3-RIPK1-RIPK3) in the β-scaffold (Fig 1C). Intriguingly, homo-oligomeric amyloids formed by the RIPK3 RHIM core tetrad displayed an analogous fold to RIPK1-RIPK3, suggesting a common self- and cross-template mechanism in the assembly of RHIM-RHIM amyloids [4]. Recently, Gentle *et al* [5] showed that TRIF assembles into amyloids through its RHIM as well. These observations raise the important question of whether RHIM-mediated amyloid formation governs signalling pathways and viral regulation. Would non-canonical necrosomes such as ZBP1-RIPK3 be hybrid amyloids akin to the canonical RIPK1-RIPK3 necrosome? Could viral RHIM proteins such as M45 assemble into amyloids for the inhibition of host cell necroptosis?

In this issue of *EMBO Reports*, Sunde and coworkers [1] have convincingly answered these relevant questions, providing direct evidence at the single-molecule level of hybrid amyloid formation in necroptosis-associated, RHIM-containing proteins. The authors showed that ZBP1 and RIPK3 formed fibrillar assemblies with amyloid-like properties, such as fibrillar morphology, Congo red and thioflavin T binding, as previously observed in RIPK1 and RIPK3 [3], and corroborated here using single-molecule fluorescence. Strikingly, they also observed that mixtures of the different proteins tagged with distinct fluorophores co-localized within proteinaceous fibrils. These results indicate that ZBP1 and RIPK3 assemble into hybrid amyloids, confirming that both the canonical RIPK1-RIPK3 and the virus-induced, non-canonical ZBP1-RIPK3 necrosomes share a hetero-oligomeric, amyloidal nature.

RHIM-mediated M45 interactions with RIPK1, RIPK3 and ZBP1 have been shown to underlie necroptosis inhibition [6,7]. The work by Sunde and colleagues [1] reveals that M45 constructs protecting against necroptosis in human cells formed both homo-oligomeric and hybrid amyloids with RIPK1, ZBP1 and RIPK3, via the viral RHIM. Not only these findings consolidate a prominent role for hybrid amyloids as an emerging principle in signal transduction, but also showcase how viral proteins exert necroptosis inhibition by imitating hetero-oligomeric amyloidal assembly.

What structural factors may underlie inhibition of canonical RIPK1-RIPK3 and

1 Instituto Regional de Investigación Científica Aplicada (IRICA), University of Castile-La Mancha, Ciudad Real, Spain
2 Department of Chemistry, Columbia University, New York, NY, USA
3 Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA. E-mail: wu@crystal.harvard.edu
4 Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA
†These authors contributed equally to this work
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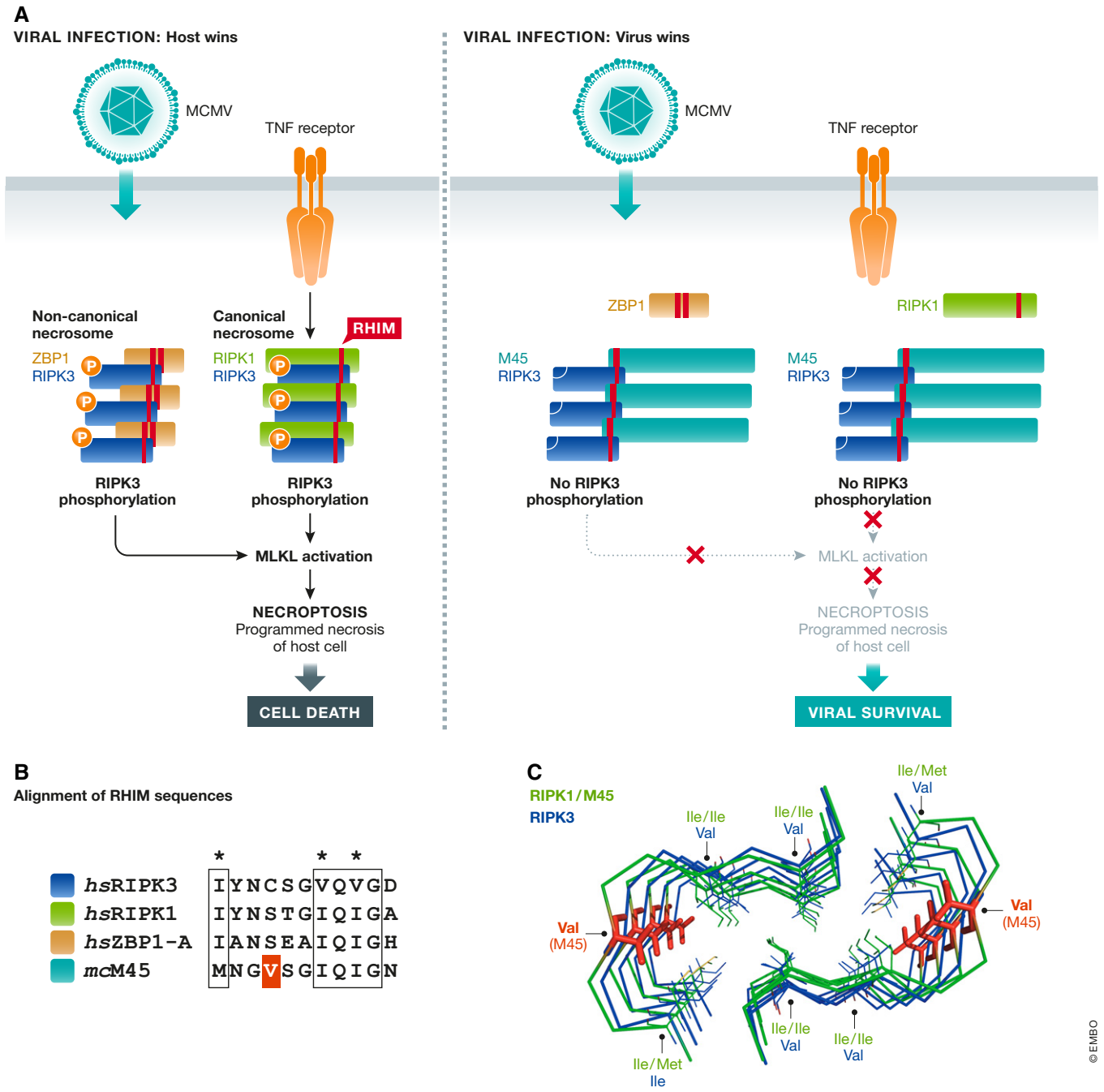

Figure 1. Hybrid amyloid formation in necroptosis-associated proteins.
(A) Canonical (RIPK1-RIPK3) and non-canonical (ZBP1-RIPK3) necrosomes rely on hybrid amyloid assembly engaging RIPK3 to activate MLKL and execute cell death by necroptosis (left). Viral proteins containing RHIMs (e.g. M45) compete for RIPK3 recruitment into hybrid amyloids to sustain viral infection by inhibiting necroptosis of the host cell (right). (B) Alignment of the RHIM sequences in human RIPK1, RIPK3, ZBP1 (first RHIM, named here ZBP1-A) and murine cytomegalovirus (MCMV) M45. Asterisks denote non-polar residues forming the amyloid interface. A Val residue in M45 may underlie the basis for RIPK3 binding (see panel C). (C) M45-RIPK3 structural model built using the RIPK1-RIPK3 amyloid structure [4]. Since M45 and RIPK1 share the IQIG core tetrad, the remaining flanking residues in RIPK1 were mutated to the M45 RHIM sequence. RIPK1 and M45 (green) are assumed to have analogous folds. RIPK3 is shown in blue. An additional Val residue, exclusive to M45, would contribute a fourth non-polar side chain to the fold (red).

non-canonical ZBP1-RIPK3 necrosome assembly by M45, likely through a preferred M45-RIPK3 interaction? RIPK1 and ZBP1 share identical RHIM core tetrads, with sequence IQIG (Fig 1B), which are known to be optimal for RIPK3 (VQVG) binding [4]. Although ZBP1 contains a second RHIM, with core tetrad sequence VQLG, it is its first RHIM (IQIG) the most crucial for engaging RIPK3 [6,8]. Interestingly, the viral RHIM of M45 also contains the IQIG sequence. Therefore, the basis for RIPK3

recruitment by M45 to mediate necroptosis inhibition by competing with RIPK1 and ZBP1 might be found outside the boundaries of the core tetrads. Sequence alignment of the distinct RHIMs illustrates that flanking residues around the RHIM of ZBP1 permit the same key stabilizing interactions observed in the RIPK1-RIPK3 structure, including the Asn and Cys/Ser ladders, as well as the oblong hydrophobic core enclosing three non-polar side chains (Fig 1B and C). Altogether, these observations may illustrate alternative (RIPK1-RIPK3-triggered and ZBP1-RIPK3-triggered) yet common mechanisms (hybrid amyloids) to activate MLKL for necroptosis execution via RIPK3. Interestingly, RHIM flanking residues in M45 feature a Val residue that would replace an otherwise Ser-Cys ladder (in RIPK1-RIPK3 and ZBP1-RIPK3 amyloids) by a Val-Cys ladder (in M45-RIPK3 amyloids; Fig 1C). Should all hybrid amyloids display analogous structures as hypothesized here, this extra hydrophobic Val in M45 might assist its assembly into M45-RIPK3 hybrid amyloids. This larger hydrophobic interface would be consistent with a longer inter-β-sheet distance (~1 Å) in M45 [1], and most likely in M45-RIPK3 amyloids (both containing the Val residue), with respect to RIPK1-RIPK3 fibrils [3], and presumably ZBP1-RIPK3 fibrils.

Finally, another important result from this work confirms that the RIPK1-RIPK3 hybrid amyloid previously observed by co-expression of the two proteins [3,4] also forms when the two proteins are independently expressed and reassembled [1], which validates the specificity of RHIM-mediated hybrid assembly.

The results presented by Sunde and coworkers [1] will certainly stimulate further structural studies to afford a better understanding of viral modulation of hybrid amyloids in signalling. Incorrect activation of necroptosis has been recently linked to death of motor neurons in amyotrophic lateral sclerosis (ALS) [9] and suppression of the immune response against cancer [10]. Therefore, the binding of RIPK3 by M45 to prevent distinct necrosome assemblies poses amyloid interfaces as potential targets for selective inhibition of necroptosis in pathological settings.

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