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REVIEW

The role of the HORMA domain proteins ATG13 and ATG101 in initiating autophagosome biogenesis

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Autophagy is a process of regulated degradation. It eliminates damaged and unnecessary cellular components by engulfing them with a *de novo*-generated organelle: the double-membrane autophagosome. The past three decades have provided us with a detailed parts list of the autophagy initiation machinery, have developed important insights into how these processes function and have identified regulatory proteins. It is now clear that autophagosome biogenesis requires the timely assembly of a complex machinery. However, it is unclear how a putative stable machine is assembled and disassembled and how the different parts cooperate to perform its overall function. Although they have long been somewhat enigmatic in their precise role, HORMA domain proteins (first identified in Hop1p, Rev7p and MAD2 proteins) autophagy-related protein 13 (ATG13) and ATG101 of the ULK-kinase complex have emerged as important coordinators of the autophagy-initiating subcomplexes. Here, we will particularly focus on ATG13 and ATG101 and the role of their unusual metamorphosis in initiating autophagosome biogenesis. We will also explore how this metamorphosis could potentially be purposefully rate-limiting and speculate on how it could regulate the spontaneous self-assembly of the autophagy-initiating machinery.

Keywords: ATG101; ATG13; autophagy; membrane contact site; protein metamorphosis

A hallmark of macro-autophagy (autophagy hereafter) is the *de novo* formation of double-membrane autophagosomes. They capture and transport cellular components to lysosomes, where the captured biomolecules are degraded for recycling [1]. Autophagy has been proven to play a wide range of roles in cellular housekeeping, including the removal of damaged or unneeded organelles, intracellular pathogens and protein aggregates [2]. It is an essential biological pathway that promotes organismal health, longevity and helps combat cancer and neurodegenerative diseases [3]. However, *de novo* autophagosome biogenesis is complicated. It requires the generation of intricate proteinaceous membrane contact sites between a 'lipid source' and a cup-shaped membrane ('phagophore' or 'isolation membrane'). Dozens of simultaneous autophagosomes can be formed, requiring the transport of hundreds of millions of lipid molecules [4–6]. The phagophore will expand and eventually close to form the autophagosome. Conceptionally, these membrane contact sites perform distinct but highly integrated functions: they are responsive to signals to assemble on-demand, where they create a tether between the growing phagophore and the lipid source and allow lipids to flow into the growing autophagosome. The exact composition

Abbreviations
ATG, autophagy-related; HORMA, protein domain named after HOP1-MAD2-REV7; MEF, murine embryonic fibroblast; PAS, pre-autophagosome structure; PI3, phosphatidylinositol 3-phosphate; PP2C, phosphatases type 2C; RB1CC1, RB1 inducible coiled-coil 1; ULK, Unc-51-like kinase.

of the contact site, how it is assembled and disassembled and the molecular mechanism of its integrated activities are unclear.

The past three decades of autophagy research have shown the complexity of autophagosome biogenesis machine. The current challenge is to appoint biochemical and biophysical functions to individual proteins and functional complexes and understand how they collaborate. In this review, we will concentrate on ATG13 and ATG101, whose 'scaffolding' role in the autophagosome biogenesis machinery is coming into focus. The structures of ATG13 and ATG101 surprisingly showed that both adopt an HORMA domain fold (Fig. 1A) [7–11]. This domain was first identified in the *Saccharomyces cerevisiae* proteins Hop1, Rev7 and Mad2, hence its name [12]. More HORMA domains were later identified, with ATG13 and ATG101 most closely related to the meiotic HORMADs [13]. These proteins are a conserved family of metamorphic signalling proteins that scaffold key signalling complexes in a variety of biological pathways. The presence of ATG13 and ATG101 in the upstream ULK-kinase complexes means that they too are ideally placed to regulate the scaffolding of autophagy effector complexes.

ATG13, AG101 and the autophagosome biogenesis machinery

After the induction of autophagy, transient 'puncta' called the pre-autophagosome structure (PAS) are formed [14–16]. The formation of this site of autophagy initiation is an essential step preceding autophagic membrane nucleation [14–16]. Through mechanisms that are not yet fully resolved, the inhibition of mTORC1 activity triggers the recruitment of the first subcomplexes of autophagy-related (ATG) proteins to these 'puncta' in starvation-induced autophagy. The coalescence of the ULK1 complex, the class III phosphatidylinositol 3-phosphate (PI3)-kinase complex I complex and ATG9A vesicles are required for the formation of an 'isolation membrane' or 'phagophore', the first step in the maturation of the autophagosome [17]. Early work suggested a model where the initiation complexes are recruited in a strict hierarchical order. The ULK1 complex [consisting of the ULK1 or -2 kinase, RB1CC1 (RB1 inducible coiled-coil 1)/FIP200, and HORMA domain proteins ATG13 and ATG101] is the most upstream and central regulation node within the autophagy network [18–22]. The recruitment of the ULK-kinase complex is followed by the PI3-kinase complex I [composed of PIK3C3 (phosphatidylinositol 3-kinase catalytic subunit type 3)/VPS34, PIK3R4 (phosphoinositide-3-kinase regulatory subunit 4)/VPS15, BECN1 and ATG14] and the transmembrane protein ATG9A, a lipid scramblase that resides in small, highly dynamic vesicles [23–27].

Initiation of bulk autophagy is tightly connected to nutrient levels and the target of rapamycin complex 1 (TORC1) in yeast or its homologue mammalian/mechanistic target of rapamycin (mTOR). Active TORC1 inhibits bulk autophagy by negatively regulating Atg1 kinase (the yeast version of ULK-1 and ULK-2) activity *via* phosphorylation of Atg13 [16]. Mammalian ATG13 has been proposed to act as a signalling hub that integrates various upstream pathways, in conjunction with the mTOR-ULK1/2 axis [28]. TOR1 and PKA both phosphorylate Atg13 in budding yeast [29], while mammalian ATG13 is directly phosphorylated by mTOR and AMPK [30]. *In vitro* hyper-phosphorylation of Atg13 by TOR1 inhibits the ability of Atg13 to bridge Atg17–Atg29–Atg31, resulting in an impeded PAS formation [16,31,32]. Atg13 is dephosphorylated during starvation by the PP2C phosphatases, leading to its multivalent interactions with Atg17 [33]. Similarly, in mammalian cells the phosphorylation of ATG13 dramatically changes upon autophagy induction, but this does not appear to influence ATG13 binding affinity to ULK1 and ATG101 [34]. Upon recruitment, Atg1/ULK1 kinase subsequently phosphorylates itself and other components of the ULK1 complex, Atg9/ATG9A and all subunits of the PI3-kinase complex I [35–38]. The recruitment of Atg9/ATG9A and Atg14/ATG14, however, mainly relies on Atg13/ATG13 rather than Atg1/ULK1 [7,27,39–41]. The disruption of ATG13 binding to ULK1/2 does not completely abolish autophagy in response to amino acid deprivation [42]. Autophagy is however completely halted in *ATG13*-deficient KO murine embryonic fibroblasts (MEFs) and DT40 cells in both normal and nutrient-deprived conditions [28,40]. Both ATG13 and FIP200 are required for ULK1 to be appropriately localized to the phagophore [21], and the knockout of either *ATG13* or *FIP200* is embryonic lethal in mice [39,40].

The importance of ATG13 is also reflected in its role of organizing PAS formation, and the recruitment of Atg9/ATG9A vesicles, a putative seeding membrane. It has been demonstrated that the PAS displays liquid-like properties and is formed by liquid–liquid phase separation (LLPS) of proteins from the Atg1 complex [16,32]. The intrinsically disordered C-terminal part of Atg13 is the central mediator for a meshwork of site-specific interactions between Atg13 and two sites at Atg17 [known as the Atg17-binding region (17BR) and the Atg17-linking region (17LR)] [16,43]. Multiple repeats of the interactions would result in the higher assembly

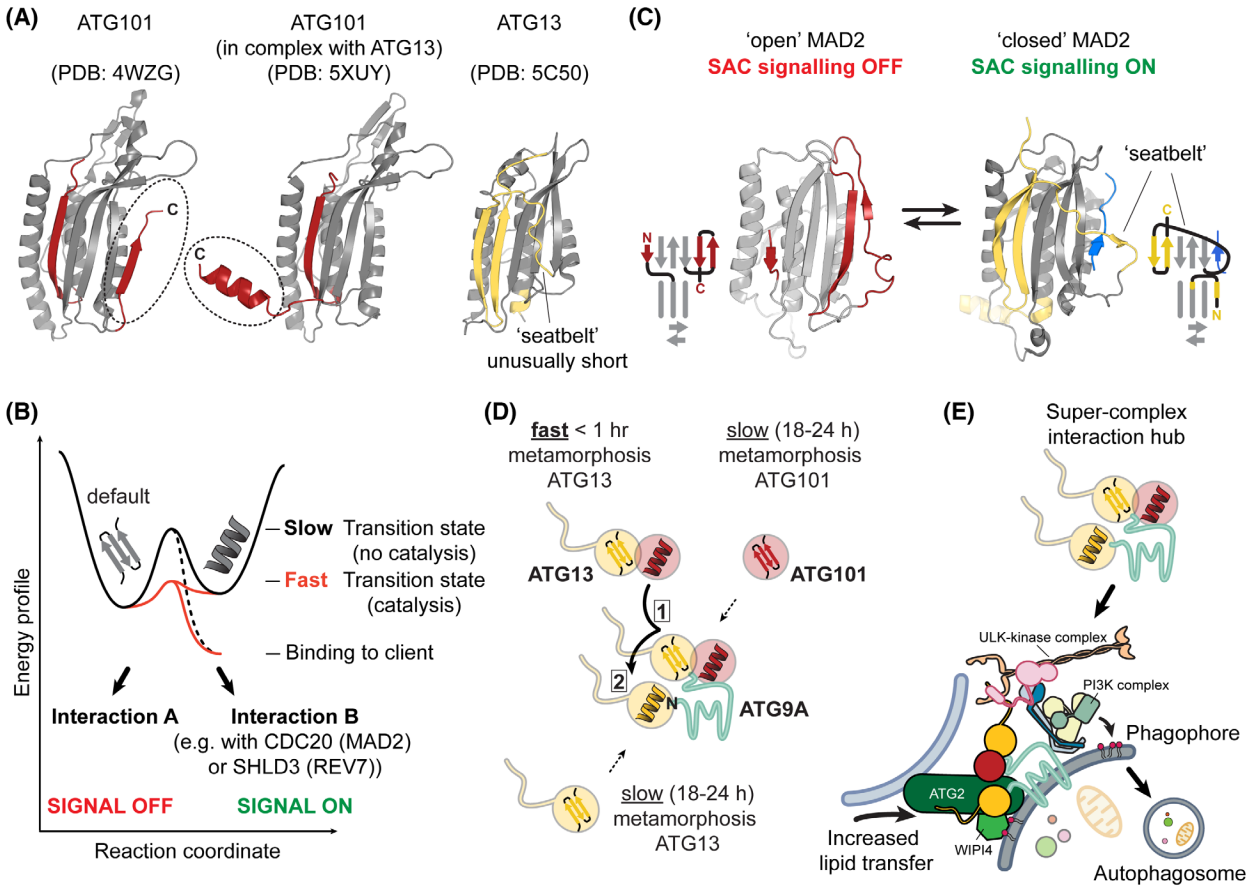

Fig. 1. HORMA metamorphosis. (A) Structures of human ATG13 and ATG101, with invariable part in grey and mobile elements highlighted in red (ATG101) and yellow (ATG13). Highlighting colours in ATG13 and ATG101 highlight the equivalent mobile elements compared to MAD2. When crystallized in the presence of ATG13 (middle), the C-terminal part of ATG101 is displaced, suggesting that dimerization might allosterically induce a change in secondary structure in this region. Crystal structures of ATG13 (right) show an unusually short seatbelt, suggesting it might not be able to capture client proteins in the 'closed' configuration typical for HORMA domains (as shown panel C). (B) The emerging paradigm for HORMA domain proteins is that they default to an inactive state, before converting to a second conformer that can interact with its client and allow signalling or effector complex assembly (dashed line). The spontaneous metamorphosis is slow; however, the transition can be accelerated by catalysts (red line). Aided by their unusual energy landscape, the purposely slow spontaneous metamorphosis of the HORMA domains serves as a regulatory switch that dictates the assembly rate of effector complexes. (C) Metamorphosis between the 'open' and 'closed' conformers of MAD2, where only the latter can interaction with the client protein CDC20, the key step in creating the mitotic arrest as part of the spindle assembly checkpoint (SAC). This interaction is embraced by the flexible C-terminus of the 'closed' MAD2, termed the 'seatbelt', around the client protein and associates with the opposite edge of the core β-sheet. Invariable part in grey and mobile elements highlighted in red ('open' MAD2) and yellow ('closed' MAD2). MAD2 interacting peptide of client protein is shown in blue. (D) Metamorphosis of ATG13 and ATG101 in human autophagy initiation. The assembly of the ATG9A–ATG13–ATG101 subcomplex requires the metamorphosis of ATG13 and ATG101. Metamorphosis is indicated as in (B) by a transition from a beta-sheet (the default conformer) to an alpha-helix. Since this metamorphosis is slow, this unusual mechanism introduces a rate-limiting step in the assembly of the ATG9–ATG13–ATG101 complex. Dimerization of ATG13–ATG101 accelerates the formation of the ATG9A–ATG13–ATG101 complex. (E) Once formed, the ATG9A–ATG13–ATG101 complex forms the interaction hub for the recruitment of all autophagy initiation subcomplexes to create a stable super-complex. The interaction of ATG2A with the ATG9A–ATG13–ATG101 and WIPI4 cooperatively enhances both its vesicle tethering and lipid transfer activities.

of supramolecular Atg1 complexes and thereby drive PAS formation [16,44]. Similar to budding yeast, the IDR of mammalian ATG13 also contains interaction sites for both FIP200 and ULK1; however, a similar assembly mechanism has not been observed [42,45]. Meanwhile, using its N-terminal HORMA domain, ATG13 interacts with the unstructured N-terminus of ATG9A in yeast and humans and is responsible for recruiting ATG9A [46–48]. This recruitment might be aided by a short phospholipid-binding motif located

at the very N-terminus of ATG13 [18,41]. It is postulated that the fusion of a small number of Golgi-derived Atg9/ATG9A-containing vesicles, ranging from approximately 30–60 nm in size, creates a seeding membrane for the phagophore [49–51]. Mice die within 1 day of delivery when ATG9A was knocked out, consistent with its essential role in autophagosome formation during (neonatal) starvation [52]. Indeed, ATG9A is a component of the autophagic membrane and can be isolated together with the autophagic membrane marker LC3II [53]. Isolated yeast Atg9 vesicles or reconstituted Atg9 proteoliposomes support local Atg8 lipidation upon the addition of PI3-kinase complex I and the components of the lipid-conjugation machinery (Atg21 and Atg12–Atg5–Atg16) and lipid transfer (Atg2–Atg18) [51]. Yeast Atg17 is thought to mediate the sequestration and fusion of Atg9 vesicles [47,54], but no such activity has been linked to its functional human homologue FIP200.

ATG101 was the last component of the ULK1 kinase complex to be identified, likely because it is absent in *Saccharomyces cerevisiae* [22,34]. Mutating the HORMA dimer interface between ATG13 and ATG101 results in a strong inhibitory effect on autophagy [10,41], and the incorporation of ATG101 in the ULK1 kinase complex is believed to stabilize ATG13 [9,10]. Mammalian cells ATG101 knock-out cells or cells treated with ATG101 siRNA show reduced LC3-II puncta formation and increased endogenous LC3-I, suggesting that LC3 conjugation is impaired [10,22]. In *Caenorhabditis elegans*, loss of *epg-9* (homologous gene of mammalian ATG101) function causes defects in autophagy similar to those in UNC-51/ATG1 and EPG-1/ATG13 mutants [55], whereas both starvation-induced and basal autophagy in *Drosophila melanogaster* were impaired by the depletion of ATG101 [56]. A quantitative BioID proteomics approach in mammalian cells showed that the ATG13–ATG101 complex recruited ATG9A to promote p62/SQSTM1-dependent autophagy and that deleting *ATG13* or *ATG101* resulted in an accumulation of p62 aggregates resembling the phenotype observed in the *ATG9A* knockout [57]. Indeed, human ATG13 and ATG101 together interact weakly to the very C-terminal HDIR (HORMA dimer-interacting region) of ATG9A [46,58]. A recent crystal structure shows the ATG9A HDIR peptide draped on top of the ATG13–ATG101 dimer, where the majority of contacts are made by ATG13 (Fig. 2B, middle) [58].

Besides their involvement in Atg9/ATG9A trafficking and localization, ATG13 and ATG101 are also involved in the recruitment of the PI3-kinase complex I [7,11,46,59]. After its recruitment, the PI3-kinase complex I produces PI3P on the isolation membrane. PI3P serves as a signalling lipid that helps recruit downstream factors involved in autophagosome formation and maturation and is the receptor for adaptors of the lipid transfer protein ATG2A and the LC3 conjugation machinery. ATG13 also harbours a MAP1LC3/LC3-interacting region (LIR) motif and can form complexes with the GABARAP subfamily of Atg8 proteins [60,61]. ATG101 contains a Trp-Phe (WF) finger motif that is responsible for direct or indirect recruitment of downstream factors such as LC3, WIPI1 and ZFYVE1/DFCP1 to the autophagosome formation site [10,41].

Collectively, these observations suggest that ATG13 and ATG101 act as a central scaffolding unit to mediate a network of interactions between the autophagy initiation subcomplexes. Recent purifications of recombinant full-length versions of almost all proteins of the human initiation subcomplexes provided a first indication that the initiation site does indeed include a stable super-complex built on specific interactions between (at least) the initiation complexes. The resulting super-complex does not assemble in the absence of the ATG9A–ATG13–ATG101 complex, highlighting its central role [46]. Indeed, stable interactions of defined stoichiometry between the ATG13–ATG101 dimer with all autophagy initiation subcomplexes were observed: with ULK1–FIP200, ATG9A, as well as ATG14–BECN1. This work also showed that the ATG9A–ATG13–ATG101 complex directly interacts with the lipid transfer protein ATG2A and its adaptor protein WIPI4, compatible with previous reports on the direct interaction between ATG9A and ATG2A/WIPI4 (Atg2/Atg18) [62–64]. The vesicle tethering and lipid transfer activities by ATG2A alone are weak, but both activities are progressively enhanced with the addition of WIPI4 (when PI3P was present in the vesicle), ATG9A and ATG13–ATG101. This suggests that ATG2A serves as a functional ‘co-incidence sensor’ for the successful assembly of the super-complex, as its enhanced activity requires the cooperative association and activity from all canonical autophagy initiation subcomplexes. This could provide a way of ‘sensing’ the co-incidence of early autophagy initiation factors as a mature membrane contact site before committing to autophagosome formation.

The HORMA domain proteins are among the oldest conserved proteins in autophagy initiation [65]. Collectively, these observations show that HORMA domain proteins ATG13 and ATG101, in close collaboration with ATG9A, have evolved as an essential hub at the early stages of autophagy initiation. A question that remains however is whether a minimal ‘machine’ can

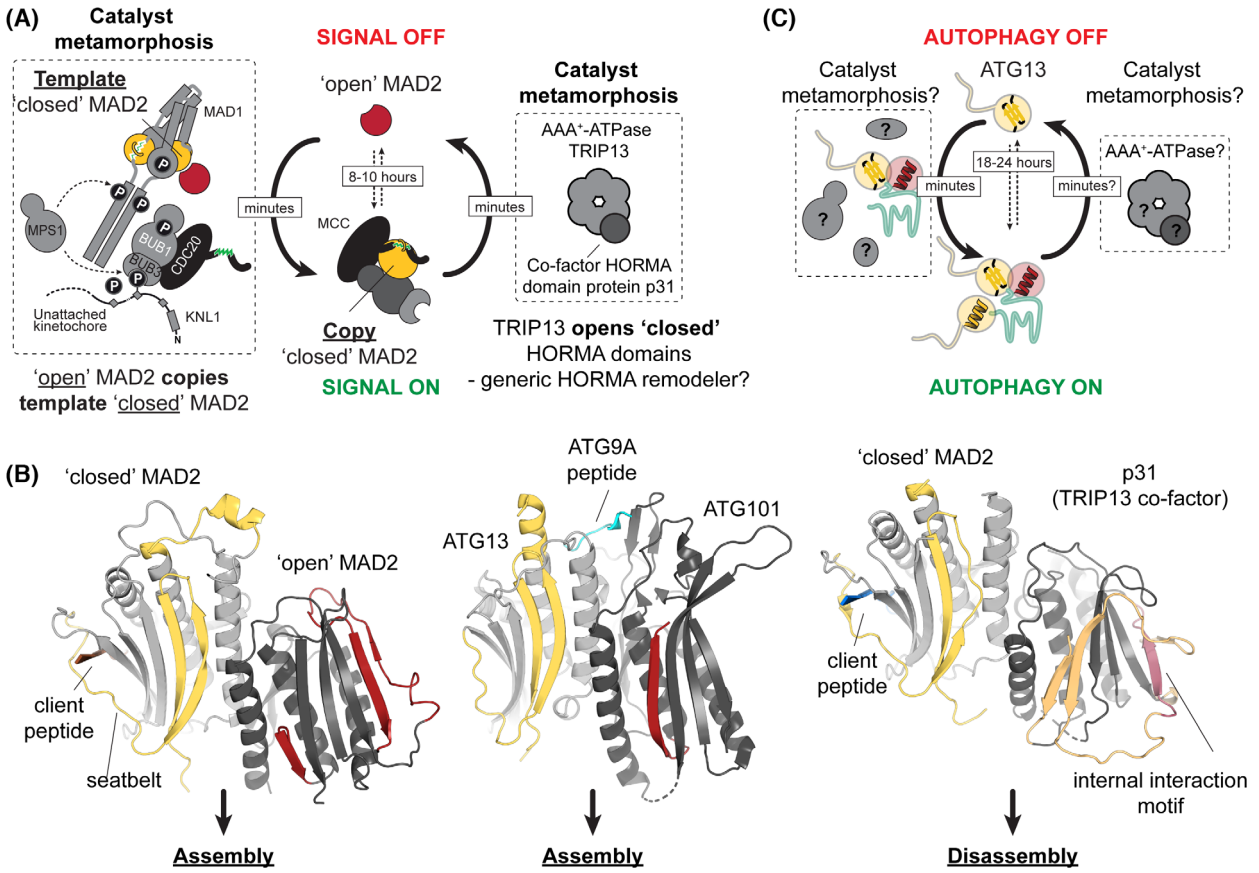

Fig. 2. Catalysed metamorphosis as a regulatory switch. (A) Slow spontaneous MAD2 metamorphosis is catalysed by specialized protein machinery, allowing for dynamic control of complex assembly and disassembly. Both the catalysed assembly and disassembly require conformer-sensitive dimerization ['open' MAD2- 'closed' MAD2 (assembly) and 'closed' MAD2-p31 (disassembly)]. Additional abbreviations: MPS1, monopolar spindle 1; BUB1, budding uninhibited by benzimidazoles 1; BUBR1, BUB1-related protein kinase; CDC20, cell division cycle protein 20; KNL1, kinetochore scaffold 1. (B) Structure of asymmetric homo-dimer of MAD2 (left, PDB: 2V64), ATG13–ATG101 (middle, PDB: 8DO8) and p31–MAD2 (right, PDB: 2QYF), with invariable part in grey and mobile elements highlighted in red ('open' conformers: MAD2, ATG101) and yellow ('closed' conformers: 'closed MAD2, ATG13 and p31). Highlighting colours in ATG13, ATG101 and p31 show the equivalent mobile elements compared to MAD2, with the internal interaction motif in p31 in pink. The MAD2-interacting peptide of client protein is shown in blue and ATG9 HDIR peptide in cyan. (C) Hypothetical catalysed metamorphosis of ATG13 (and ATG101) would regulate autophagy initiation by controlling the assembly of the initiation super-complex.

be defined that, by means of regulated assembly and disassembly, can integrate signals to initiate autophagosome biogenesis at the right place and time. This assembled machine might then be repeated multiple times to give rise to 'regional' contact sites that support autophagosome growth.

HORMA metamorphosis is rate-limiting for assembly complexes

This autophagosome biogenesis machinery is assembled within minutes after autophagy is induced by starvation [66]. However, the mechanism that makes the different functional subgroups suddenly co-localise is unclear. This raises the question about the identity of the triggering event (or rate-limiting step) of the nucleation of the assembly of the PAS. It seems likely that its foundation lies in a network of interactions between the functional subcomplexes that cooperatively coordinates the co-incidence at the contact site. But which interaction is the rate-limiting step that would prevent self-assembly until required? This 'missing' obligatory intermediate interaction would engage upon the arrival of the proper signal, thereby triggering the self-assembly of the initiation site. Indeed, the spontaneous self-assembly of complexes observed *in vitro* is in apparent contrast with the regulated assembly of the PAS in cells after autophagy

induction, suggesting it is missing or already overcame the inhibitory regulation.

The recent structures and biochemical reconstitutions have placed a new spotlight on the HORMA domains of ATG13 and ATG101 as potential regulators of the assembly of larger assemblies. As discussed above, the recruitment of ATG13 and ATG101 is essential for autophagy initiation and recruitment of most of the components of the initiation machinery [7,20,41]. HORMA domain proteins are a class of signalling proteins that dictate the assembly rate of effector complexes. They achieve this through their unusual ability to switch between two topologically distinct folds (conformers) under physiological conditions, as explained below in more detail (Fig. 1B). Each conformer is capable of engaging in a separate set of interactions, where typically only one conformer can interact with the client protein and trigger the assembly of effector complexes. However, considerable activation energy needs to be invested in the structural changes in the HORMA topology to allow effector complex formation. Since the spontaneous metamorphosis is typically slow, this introduces a rate-limiting step in the assembly and disassembly mechanism and therefore controls the rate of signalling [67]. This obligatory metamorphosis therefore creates a unique requirement to allow cells to tightly regulate assembly and disassembly of HORMA domain effector complexes in order to achieve highly specific signalling in space and time (Fig. 1B). HORMA domain proteins share no functional overlap, but use this unusual mechanism to serve as infection sensors in a bacterial immune system and playing central roles in eukaryotic cell cycle, genome stability, sexual reproduction and cellular homeostasis pathways.

The emerging paradigm for HORMA domain proteins is that they default to an inactive state, before converting to a partner-bound active state. Crystal structures of HORMA domains show a compact domain of about 200 amino acids whose core contains a three-stranded β-sheet. The two edges of the core β-sheet are interaction sites for binding partners and for the domain's topologically mobile N- and C-terminal regions (Fig. 1C). The metamorphosis of the HORMA domains involves the unfolding and refolding of these mobile regions to the static β-sheet core. The repositioning of the mobile region thereby creates or obstructs interaction sites for client proteins. This topological plasticity allows the HORMA domain protein to adopt its typical, but highly unusual, partner-bound 'closed' conformer. In this state, a 6–10-amino-acid region of a binding partner forms a short β-strand that binds along the C-terminal edge of the HORMA domain's core β-sheet. This interaction is embraced by the flexible C-terminus of the HORMA domain, termed the 'seatbelt', around the client protein and associates with the opposite edge of the core β-sheet (Fig. 1C). This creates a topological union between the HORMA domain and client protein, such that metamorphosis requires at least a partial unfolding of the HORMA domain to break this unusually strong binding mode. In nearly all HORMA-mediated signalling pathways, specifically this 'closed' HORMA domain conformer interacts with the client protein. This association subsequently triggers the assembly of larger effector complexes, such as the Mitotic Checkpoint Complex ('closed' MAD2–CDC20) or the Shieldin complex ('closed' REV7–SHLD3) (Fig. 1B). Conversely, the MAD2 'open' state represents an autoinhibited initial state where metamorphosis is obligatory before interaction can be established [68]. Intermediate 'unbuckled' states might allow access to the binding site by (partially) disengaging the seatbelt [69,70]. A partially opened seatbelt requires the client protein the protein to thread through the created opening, which is slow and only possible for N- or C-terminally positioned disordered interaction motifs [71].

ATG13 and ATG101 metamorphosis in human autophagy initiation

Like other HORMA domain proteins, ATG13 and ATG101 are ideally placed to function as a 'switch' to trigger the assembly of super-complexes. The structures of ATG13 and ATG101 showed surprising strong similarities with the 'closed' and 'open' conformers of MAD2, respectively (Fig. 1A,C) [7–11]. This provided the first strong hint that they might have a regulatory role. However, the hypothesis that ATG13 and ATG101 could use metamorphosis to nucleate the assembly of effector complexes had remained untested in autophagy until recently. A series of experiments aimed at deconvoluting the details of ATG9A–ATG13–ATG101 complex assembly showed that both ATG13 and ATG101 alone can interact with $ATG9A^{N}$ and $ATG9A^{C}$, respectively (Fig. 1D) [46]. However, this interaction formed extremely slowly (~ 18–24 h). This shows that both ATG13 and ATG101 default to an inactive non-ATG9A-binding state, and that metamorphosis is obligatory before interacting with ATG9A. In contrast, the default ATG13 conformer binds ATG101, and together they create the composite interface that allows for a fast engagement with $ATG9A^{C}$. The interaction of ATG13–ATG101 with $ATG9A^{C}$ is relatively weak (the dissociation constant is 2.4 μM), which explains why

the interaction between ATG13 and ATG9A^{C} does not readily form at concentrations found in cells [58]. Therefore, the metamorphosis of ATG101 is required to recruit ATG13 to ATG9A^{C}. Once recruited to ATG9A^{C}, ATG13 can switch conformer state and ATG101 loses the ability to bind ATG13, which 'hands ATG13 over' to ATG9A^{N}. The increased local concentration and proximity might aid in this process, as binding of ATG13 to ATG9A^{N} is dramatically accelerated from 18–24 h to 30 min after a prior interaction of ATG13–ATG101 with ATG9A^{C}. Next, ATG101 can recruit another ATG13 to saturate all ATG9A molecules in the trimer. This yields the ATG9A–ATG13–ATG101 complex in a 3 : 6 : 3 stoichiometry, which allows for the formation of the initiation super-complex [46]. Therefore, as previously observed with other HORMA domain proteins, ATG13 and ATG101 introduce a rate-limiting step in the assembly of the effector complex. This is likely part of regulating the first steps in initiation of autophagy in cells, as introducing mutants that lack specific metamorphic elements showed that autophagic flux was abolished in MEFs [46]. Additionally, these mutants prevented the co-localization of ATG16L, FIP200, ATG14 and WIPI2 under autophagy-inducing conditions [46]. Collectively, these observations suggest that the metamorphosis of ATG13 and ATG101 is involved in the regulation of the early steps of autophagy initiation.

The structural details of what defines the ATG13 and ATG101 metamorphoses are currently unclear. However, there are hints that they differ from the canonical HORMA domain metamorphosis. The structure of human ATG101 shows that the C-terminal mobile seatbelt region can adopt a β-strand bound to the core β-sheet of the HORMA fold (Fig. 1A, left) [8]. However, when in complex with ATG13, this region folds to anα-helix that no longer interacts with the rest of the molecule (Fig. 1A, middle) [11]. The deletion of this region shows a significant defect in the interaction with PI3-kinase complex I and impaired autophagosome formation [11]. This topologically flexible part of ATG101 docks on a symmetry-related molecule in the crystal. It is therefore currently unclear if this reflects the true metamorphic event, where heterodimerization with ATG13 induces the switch in structure and exposes an interaction interface for the PI3-kinase complex. In *S. cerevisiae*, which lacks ATG101, Atg13 has an extra cap that is proposed to help in the stability of the protein [7]. This observed stability could be related to the dynamics of metamorphosis, but it might also allow for interactions otherwise mediated by ATG101.

The role of the seatbelt in ATG13 might also be atypical. Removing or mutating the mobile elements in HORMA domains affects metamorphosis, presumably by changing the relative stability of the conformers [72]. Although the structures of ATG13 show that it can adopt the typical 'closed' HORMA domain fold, the position and length of the seatbelt suggest it cannot engage with a client protein in a seatbelt-mediated manner (Fig. 1A, right). Removal of the seatbelt typically prevents the interaction between HORMA domains and their client proteins [73–77], but surprisingly does not seem to affect the ability of ATG13 to bind to ATG101 nor ATG9A. This raises the possibility that something else could be captured by the seatbelt or that the seatbelt mechanism is rather used allosterically by stabilizing different conformers. Regardless, the change in structure and surface charge due to metamorphosis allows conformers to be separated using anion exchange chromatography [46,68,73,76,78]. Wildtype ATG13 elutes as a single (default) conformer, which indeed promptly binds ATG101 but requires a long incubation with ATG9A^{N} before interacting. In contrast, removing the seatbelt of ATG13 yields a distinct second conformer that shows an inverted binding behaviour: it readily binds to ATG9A^{N}, but requires hours to interact with ATG101 [46]. This shows that, in contrast to other HORMA domain proteins, the seatbelt is only indirectly involved by stabilizing different conformer structures. The seatbelt thereby ensures that the wild-type protein defaults to a distinct (inhibited) conformer, that needs to switch when an as-of-yet unknown right signal arrives.

Could metamorphosis be regulated for an on-demand assembly?

The interaction of ATG13 and ATG9A is strongly enhanced after autophagy induction by rapamycin in yeast [48]. This confirms that the larger complexes are assembled on-demand and suggests that ATG13 and ATG9A are not associated at the start of autophagy. Moreover, it has been established that the PAS is assembled within seconds to minutes after the induction of autophagy [66,79,80]. However, this could not be observed when mixing ATG9A, ATG13 and ATG101 *in vitro*. This raises a question: what regulates or accelerates the initial assembly? Could there be inhibitory interactions or post-translational modifications that prevent assembly until it is necessary? The identification of ATG13 and ATG101 metamorphoses as rate-limiting, presents an enticing possibility to solve this paradox. This hypothesis is inspired by work

on HORMA domain protein MAD2. In short, MAD2 is recruited to kinetochores in specifically the default 'open' conformer state (Fig. 2A). Until the kinetochores are properly attached to the mitotic spindle, the Spindle Assembly Checkpoint (SAC) is tasked with creating the inhibitory Mitotic Checkpoint Complex (MCC). Spontaneous assembly of the MCC requires over 8 h at physiological concentration [81–83]. Regulatory SAC proteins are recruited to unattached kinetochores and act as catalysts in the production of the MCC by accelerating the rate-limiting metamorphosis of MAD2 to the 'closed' conformer (Fig. 2A, left) [81,82,84–86]. Indeed, adding the regulatory SAC proteins dramatically accelerates MAD2 metamorphosis (completed within a few minutes) which reproduces signalling kinetics previously observed in cells [81]. When signalling needs to be silenced, the resulting 'closed' MCC complex is disassembled by converting MAD2 back to its 'open' conformer. This task is performed by the dedicated AAA^+-ATPase Thyroid hormone receptor interactor 13 (TRIP13), a generic HORMA remodeller (Fig. 2A, right) [13,87,88].

Dimerization of metamorphic proteins, including HORMA domains, is usually required to catalyse metamorphosis. HORMA domains dimerize through a canonical interface composed of the edge of the domain's β-sheet and a neighbouring α-helix (Fig. 2B). The dimerization surface changes upon metamorphosis, and dimer formation is therefore conformer specific. For example, MAD2 can only dimerize asymmetrically between an 'open' and 'closed' form: the ability to dimerize is lost when the 'open' MAD2 converts to the 'closed' partner-bound conformer [68]. Importantly, dimerization introduces strain in the 'open' MAD2 conformer, lowering the activation energy for conformational conversion to the 'closed' state [69]. Indeed, the asymmetric dimerization of MAD2 (between specifically only an 'open' and 'closed' conformer) is essential in accelerating and regulating metamorphosis (Fig. 2A, left) [81]. The asymmetric dimerization between an 'open' and 'closed' HORMA is conserved in ATG13 and ATG101 and is essential in cells (Fig. 2B) [9,11,41,48]. Like with MAD2, dimerization of ATG13 and ATG101 indeed does strongly increase complex formation (to about 30 min compared to 18–24 h with individual proteins) and strongly affect autophagy in cells (Fig. 1D) [41,46]. However, further accelerating factors are likely missing, as the observed ATG9A–ATG13–ATG101 assembly kinetics are insufficient to assemble the complex in a few minutes as observed in cells [48]. To create the MCC, the two molecules in the MAD2 dimer have a distinct role. The 'template' closed MAD2 is presented by specifically unattached kinetochores, where it acts as an enzyme to convert multiple open MAD2 molecules to 'copy' the closed conformer and assemble the effector complex (Fig. 2A) [89]. In human autophagy, the 'templating' role might be taken up by ATG101 to guide (or 'hand over') potentially multiple molecules of ATG13 to engage in the functional interaction with ATG9A (Fig. 2C). Species that lack ATG101, could use the MAD2 'template' mechanism more faithfully by having ATG13 serve in both roles. Future work will be needed to deconvolute the regulation of this intricate assembly mechanism.

After sufficient autophagosomes have been generated, the initiation machinery is inactivated, which presumably involves its disassembly. Again, as with other HORMA domain-based signalling, metamorphosis could be a rate-limiting step for disassembly as it requires the opening of the 'closed' conformers. This activity is typically performed by AAA^+-ATPase TRIP13, which has co-evolved with almost every HORMA domain protein [67]. The interaction with TRIP13 requires the dimerization of HORMA domains (Fig. 2A,B); thus, dimerization is critical for both assembly and disassembly of signalling complexes [87,90,91]. The ring-shaped TRIP13 hexamer targets the HORMA protein's N-terminus to its central pore, after which ATP hydrolysis coordinates conformational changes to partially unfold the HORMA domain [90–92]. The HORMA domain will revert to the 'open' conformer, leading to the disassembly of the effector complex and inactivation of the signal. TRIP13 can also convert (prematurely switched) 'empty' HORMA domains to the default conformer. TRIP13 can therefore have both activating and inactivating functions, depending on the context. So far, TRIP13 has not been linked to autophagy yet; therefore, ATG13 and ATG101 might be remodelled by an as-of-yet unidentified factor. Future work is required to confirm if the emerging concept of regulated complex assembly and disassembly through accelerated metamorphosis is conserved in autophagy.

Conclusions and perspectives

Since its discovery in 1998, the HORMA domain has emerged as an unusual scaffolding protein. Aided by their unusual energy landscape, their purposely slow spontaneous metamorphosis serves as a regulatory switch that dictates the assembly and disassembly rates of effector complexes. Conformational sensors (for example conformer sensitive dimerization) provide a way for cells to achieve conditional signal activation and inactivation. Co-evolved specialized protein machinery can accelerate metamorphosis, allowing for

dynamic control of complex assembly and disassembly. This likely defines a broader paradigm for HORMA domain signalling, as core properties (e.g. an inherently unstable topology, the capture of peptides in a closed conformation, dimerization and co-evolution with proteins that catalyse metamorphosis) are conserved in the majority of HORMA domain proteins, including ATG13 and ATG101.

In this Review, we have explored how this hypothetical mechanism would provide an alternative mechanism to regulate the assembly of the autophagy initiation machinery. While many requisite features of HORMA-based signalling have been identified in ATG13 and ATG101, we are only at the beginning to understand if and how these are involved in regulating the initiation of autophagy. The near future will require the structural analysis of the metamorphic changes in ATG13 and ATG101 and the elucidation of their role in super-complex formation, organization and function. Many exciting questions will need to be explored and answered, both in cells and *in vitro*. What is the mechanism of metamorphosis? What are the putative factors that accelerate metamorphosis? How would a minimal machinery look like and how would it function? Do the current biochemical reconstitutions miss components or modifications? How do the (accelerated) interactions measured *in vitro* compare to the assembly kinetics in cells? How are the super-complexes disassembled and is this ATP-hydrolysis dependent in cells? In the past decade, we have seen that fundamental genetic and cell biological experiments have increasingly been complemented by biochemical reconstitution of growing complexity. This complementary and intrinsically reductionist approach will allow for the careful analysis of individual interaction strengths and kinetics. The resulting quantitative and comprehensive mechanistic models will explain the complex assembly of the autophagy initiation site and clarify how the controlled co-incidence of the functional units supports the initiation of autophagosome growth. Deep mechanistic knowledge of the unusual ATG13–ATG101 HORMA domain-based signalling module would set the stage for alternative interventions and the development of therapeutics that may interfere with autophagy in cells.

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