Received: 9 February 2021 | Revised: 2 March 2021 | Accepted: 24 March 2021

DOI: 10.1002/jcb.29925

RESEARCH ARTICLE

Journal of Cellular Biochemistry WILEY

# On the origin of BAG(3) and its consequences for an expansion of BAG3's role in protein homeostasis

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**Funding information**
Corona-Stiftung; Deutsche Forschungsgemeinschaft, Grant/Award Number: 259130777-SFB 1177

**Abstract**

The B-cell CLL 2-associated athanogene (BAG) protein family in general and BAG3, in particular, are pivotal elements of cellular protein homeostasis, with BAG3 playing a major role in macroautophagy. In particular, in the contexts of senescence and degeneration, BAG3 has exhibited an essential role often related to its capabilities to organize and remove aggregated proteins. Exciting studies in different species ranging from human, murine, zebrafish, and plant samples have delivered vital insights into BAG3s' (and other BAG proteins') functions and their regulations. However, so far no studies have addressed neither BAG3's evolution nor its phylogenetic position in the BAG family.

**KEYWORDS**
autophagy, BAG3, LIR domain, proteostasis, WW domain

## 1 | INTRODUCTION

The intact homeostasis of cellular protein structures is fundamental for the cell's health and duties as part of a complex organism. This proteostasis is promoted through biogenesis of novel and degradation of damaged or dysfunctional proteins.[1] In particular, a decline in the cell's ability to eliminate defective or hazardous proteins is often observed as a consequence of senescence.[2] This causes proteins to accumulatively aggregate and may eventually lead to apoptotic and degenerative events.[3] Although multiple mechanisms exist that can clear aggregates, none of them are as efficient as their proper recycling through autophagy or the proteasome.

Members of the B-cell CLL 2-associated athanogene (BAG) protein family are major regulators of cellular proteostasis.[4–7] Most vertebrates evolved six different paralogues of the BAG family (BAG1–6), all of which have at least one C-terminal BAG domain. This domain allows the proteins to act as cochaperone to the chaperones 70-kDa heat shock protein and heat shock cognate 71-kDa protein.[8] Although all six vertebrate BAGs retained this particular property, they found their own functional niches, many of which are intertwined with proteostasis and the removal of disposable proteins.

BAG3 and BAG1 share a balancing relationship, in which BAG1 conveys protein degradation through the proteasome in younger cells, while BAG3-dependent macroautophagy (hereafter referred to as "autophagy") was more active in senescent cells.[5,9] This special relationship was recently also confirmed in a stroke model system.[10] Human BAG3 possesses modularly structured domains. Besides its C-terminal BAG domain, BAG3 has an N-terminal WW domain, which appears important for BAG3 mediated autophagy.[11] The third domain includes the proline-rich region, which facilitates aggresome formation through interaction with dynein leading to retrograde transport.[12] Furthermore, two small IPV-motifs exist that interact with 14-3-3 proteins and heat shock protein B6[13] (Figure 1). Although much has been done concerning BAG3's function and the regulation thereof, its origin and that of its domains

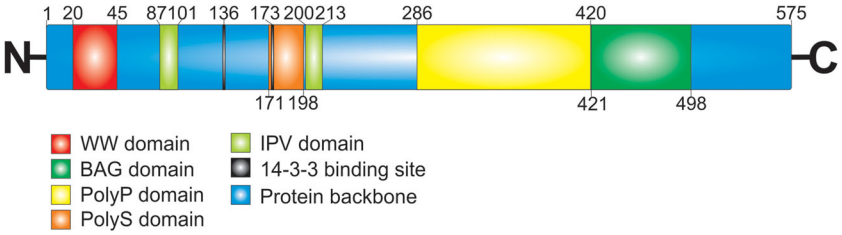


**FIGURE 1** B-cell CLL 2-associated athanogene 3 (BAG3) protein. Illustration of human BAG3's structure

remain obscure. Therefore, we were interested in how BAG3's protein structure developed throughout evolution. Here, we present novel insights that may help to better understand BAG3's position in the cellular proteome, and expand its role in autophagy.

## 2 | MATERIALS AND METHODS

### 2.1 | Phylogenetic reconstruction

BAG1–6 protein sequences of indicated species were obtained from NCBI and aligned using Clustal Omega from EMBL-EBI.[14] Phylogenetic trees were constructed with interactive tree of life.[15]

### 2.2 | BAG3 domain analysis

Protein domains, putative Casein Kinase 2, Protein Kinase C, and cAMP/cGMP-dependent protein kinase targets, as well as *N*-myristoylation, amidation, and N-glycosylation sites were extrapolated using ScanProsite.[16] LC3 interacting region (LIR)domains were annotated manually following the (W/Y/F)-X-X-(L/I/V) motif.[17] Lysine acetylation sites were predicted using GPS-PAIL.[18]

### 2.3 | Relative amino acid drift

The amino acid drift was established by calculating the relative amino acid composition inside a species' BAG3 and evaluated whether a linear regression was present using GraphPad Prism 7.03. Isoelectric points (IEPs) were calculated with IPC 2.0.[19]

## 3 | RESULTS

### 3.1 | The four classes of eukaryotic BAG proteins

First, to discern BAG3's origin, we explored the structural similarities and differences between BAG paralogues. Indeed, we could classify the BAG proteins into four groups (Figure 2). *Class I BAGs* encompassed *Animalia* BAG1 orthologues and BAG1–4 from *Arabidopsis thaliana* (Ath). Besides their BAG domain, they also carried an ubiquitin-like (UBL) domain. This is in concert with previous investigations of BAG1's evolution.[20]

Interestingly, *fungi* BAG1 orthologues (*Shizosaccharomyces pombe* BAG101 and *Amanita muscaria* (Amu) BAG1) appear as an outgroup of *class II BAGs*. Other *class II BAGs* only occur in *Vertebrata* and include orthologues of human BAG3, BAG4, and BAG5. Interestingly, *fungi* BAG1 orthologues are closely related to *Vertebrata* BAG5, yet BAG5 exhibited a multiplication of its BAG domain, but no remnants of an UBL domain. The primordial BAG5 of *Petromyzon marinus* (Pma), however, only presents one C-terminal BAG domain. The other three domains arose in *Callorhinchus milii* (Cmi). BAG3 and BAG4 are clustered with BAG5, while BAG4 is not represented in the genome of Pma, but in that of Cmi. Structurally, *class II BAGs* are diverse and retained individual domains. BAG3 obtained a WW domain, which also appears in BAG4 of Cmi. This may indicate BAG3 as BAG4's point of origin.

Interestingly, summarized as *class III BAGs*, Starvin and Amu BAGDC clustered with orthologues of human BAG6 and BAG2. Orthologues of human BAG6 were limited to *Chordata*, while orthologues of BAG2 were present in *Protostomia*. The UBL domain is conserved in BAG6, which may indicate a close relationship to BAG1. Orthologues of BAG2 demonstrated a decline in size with *Arthropoda* BAG2 exhibiting degenerated BAG domains.

Finally, we found a fourth class of BAG proteins (*class IV*). Here, Ath BAG5 and BAG6, and BAG7 and BAG8 were pairwise organized. Structurally, BAG5 and BAG6 carry an IQ-motif and the BAG domain, while the latter is degenerated in BAG7 and 8. Other *class IV BAGs* demonstrated no fixation of any domain outside the BAG domain and can thus be considered the most primordial.

### 3.2 | The evolution of BAG3

Next, we took a closer look at BAG3's evolution with Cmi BAG4 as an outgroup (Figure S1). The phylogeny

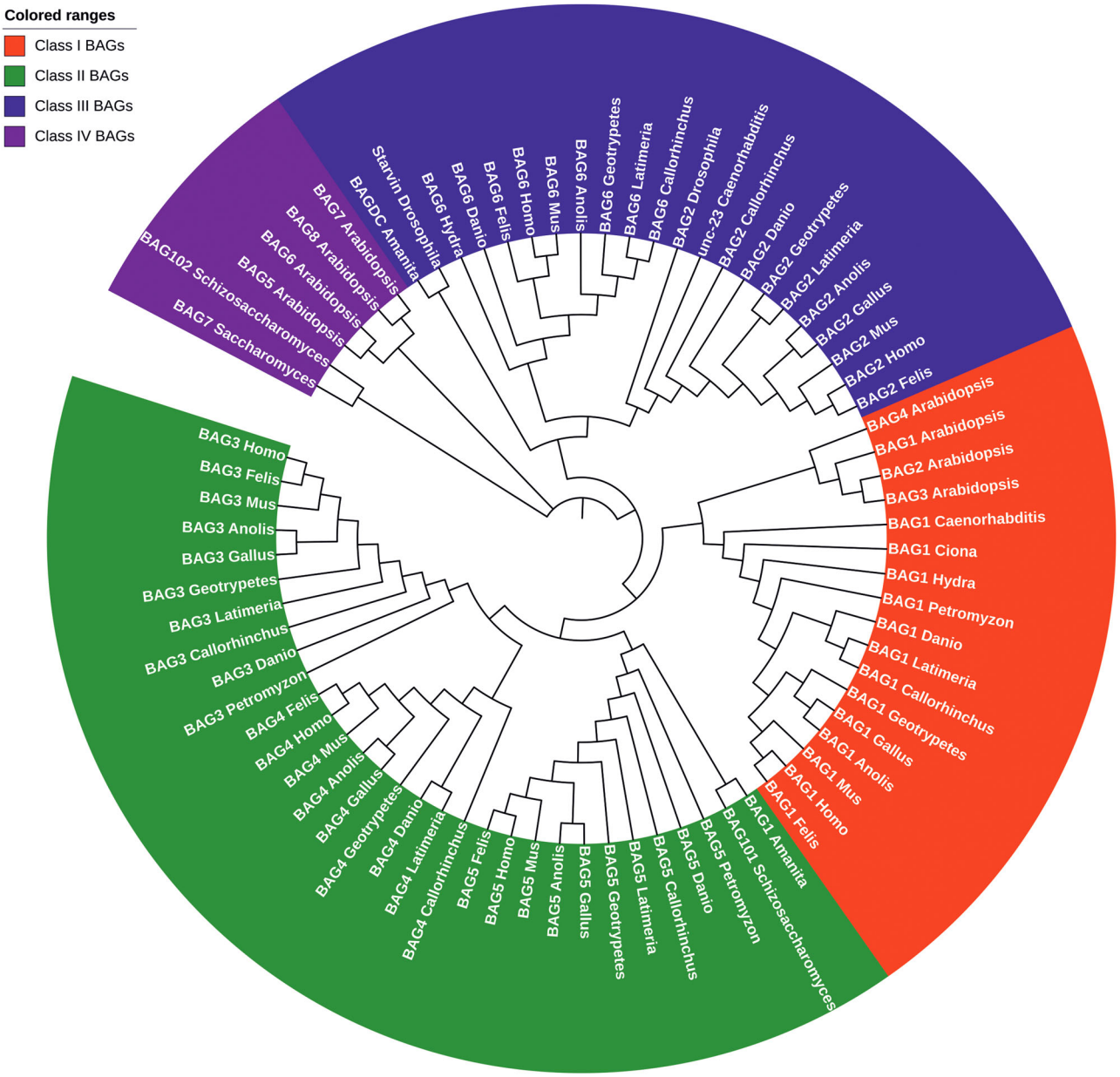


**FIGURE 2** The B-cell CLL 2-associated athanogene (BAG) protein family. Phylogenetic tree illustrating the phylogeny of the BAG protein family within the *Eukaryota*

of BAG3, in general, is similar to that of the *Vertebrata*.[21] However, *Actinopterygii* BAG3 orthologues were closer related to *Cyclostomata* than *Chondrichthyes*, which were closer to BAG3 orthologues of the *Sarcopterygii*. The phylogeny inside the *Actinopterygii* is close to published calculations.[22] First, *Cladistia, Chondrostei*, *Holostei, Osteoglossomoprha,* and *Elopomorpha* branch out from the main tree. Afterwards, the *Otocephala* branch out, followed by *Protacanthopterygii* and *Paracanthopterygii*. Finally, the *Percomorpha* monophylum is reconstructed. Here, however, BAG3's evolution differs from the published phylum.

After the *Chondrichthyes*, the *Actinistia* orthologue branched out. The exact *Tetrapoda* phylogeny is still disputed, but the distribution of *Amphibia* BAG3 orthologues fits the polyphyly hypothesis, as *Lysorophia* and *Temnospondyli* were not clustered together and the former appeared as a side-branch of the *Amniota*.[23] Then, the tree segregated *Mammalia* and *Sauropsida*. Mammalian BAG3 orthologues evolved similar to published phylogeny as *Monotremata* and *Maruspialia* branched off before the *Placentalia*.[24] Development of *Placentalia* BAG3 orthologues first separated the *Atlantogenata*, and then divided into *Euarchontoglires* and *Laurasitheria*. However, the *Atlantogenata* did not branch out in

unison. First, the *Paenungulata* appeared as outgroup of the remaining *Placentalia*, after which the *Afroinsectiphilia* branched off. Meanwhile, *Xenartha* orthologues demonstrated high levels of similarity with *Laurasiatheria* orthologues. The *Euarchontoglires* formed the last group of mammalian BAG3 orthologues, and gradually, first the *Lagomorpha*, then the *Rodentia* suborders, branched out leading to the *Euarchonta* and the *Primates*. Interestingly, human BAG3's sequence shared more similarities with *Dermoptera* than with *Strepsirrhini*.

In the *Sauropsida* phylum, the tree separated *Squamata* from *Archosauromorpha*. The *Squamata* then first branched off the *Gekkota*, and afterwards presented a phylum of *Laterta* and *Iguania*. The other branch moved towards the *Serpentes*. These relationships mimicked published phylogeny.[25] Finally, the phylum of the *Archosauromorpha* separated, surprisingly, into one phylum containing the *Testudines* and the *Suchia* and one including the *Aves*. However, the exact position of the *Testudines* in the *Sauropsida* phylum is still disputed.[26] Within the *Aves* clade, the *Palaeognathae* branched off first; with the *Struthioniformes* presenting as outgroup of the *Neognathae*.[27] Next, the *Galloanserae* branched off and separated into *Galliformes* and *Anseriformes*. Then the *Passeriformes* and a paraphyletic group of the remaining *Aves* are segregated. The former then formed the *Paridae* and *Tyrannidae*. The paraphyletic group first branched off two *Coraciiformes* and the *Falconiformes*. Afterwards, two phyla are presented, which represented the remaining *Afroaves*, *Gruimorphae* as well as the *Opisthocomiformes* and the *Ardae* with a *Gruimorpha* as an outgroup.

## 3.3 | Mammalian BAG3

BAG3 is known to serve different functions including antiapoptotic activities, and as an efficient player in proteostasis control and selective macroautophagy, in particular.[7,28] To analyze its development in the *Mammalia*, we chose Pma BAG3 as origin and *Homo sapiens* (Hsa) BAG3 as destination (Figures 3a,c and S2). Pma BAG3 only features three domains in total. The WW domain, the BAG domain and a putative LIR domain (YLAL) inside the BAG domain (BAGLIR); LIRs serve as a direct connector to the autophagosomal protein LC3.[29] Only three phosphorylation sites in Pma were predicted, although studies in human cells revealed BAG3 had multiple phosphorylation sites.[13]

BAG- and BAGLIR domain were conserved throughout mammalian evolution. Although the BAGLIR domain's sequence fluctuated (YLMI in *Rhincodon typus* (Rty), YLTL in *Latimeria chalumae* (Lch), YLIL in *Xenopus tropicalis* (Xtr), YLML in *Rhinatrema bivittatum*), its sequence was highly conserved in the *Mammalia* phylum (YLMI). The BAGLIR and its position within the BAG domain were also conserved in other *class II BAGs* (Hsa BAG4: YWLL; Rty BAG4: YRLL, Hsa BAG5: YIRL; Pma BAG5: YALL), and may thus be a structural similarity of *class II BAGs*. More LIR domains also arose throughout BAG3's evolution. Conspicuously, starting in Lch, retention of these domains occurred in tandem (YPQL/YIPI, YIS(P)I/Y(F)HK(R/S)I).

The WW domain, although generally well conserved, was lost in *Galeopterus variegatus* (Gva) and *Loxodonta africana* (Laf). A poly-proline (PolyP) domain close to the BAG domain firstly manifests in Xtr, but is established in mammalian evolution with the *Monotremata*. A poly-serine (PolyS) domain evolves in the *Theria*.

The number of potential phosphorylation sites increased in Rty and accumulated in the PolyP domain within the *Euarchonta* phylum. Inside the BAG domain, a conserved phosphorylation site appears in the *Mammalia*, and within the *Placentalia* a second arose (T447 and T489 in human). A phosphorylation site within the WW domain (T48 in humans) was conserved from Pma to Hsa. Amidation as a post-translational modification (PTM) caught our attention when we found a well-conserved site seven amino acids upstream of the BAGLIR domain. Outside of this area, only one more amidation site appeared in the C-terminus of the *Catarrhini*. Myristoylation can increase a protein's hydrophobicity and serve as an anchor for membrane binding. In the *Mammalia*, two myristoylation sites between the tandem LIR domains were conserved (G140 and G152 in humans). Within the *Placentalia*, a conserved phosphorylation site manifested between these myristoylation sites (T145 in humans). Lysine acetylation may change a protein's IEP and alter its hydrophobicity. Overall, we can report a gradual decrease of BAG3's acetylation potential throughout evolution. A high abundance of lysine acetylation occurred in PolyP domains of *Euarchontoglires* that did not show many phosphorylation sites. Furthermore, lysines between the amidation site and the BAGLIR domain carry a conserved acetylation potential. Finally, glycosylation may also affect the solubility of a protein. Interestingly, *Euarchontoglires* BAG3 gained a conserved glycosylation site between the second LIR domain tandem. In contrast, a glycosylation site within the WW domain is lost in the *Theria*.

Besides BAG3's structural development, we were also interested in whether its amino acid composition changed throughout mammalian evolution (Figure 3B). Indeed, the relative amount of proline gradually increased, while the glutamine ratio decreased. As glutamine is polar, while proline is hydrophobic, mammalian

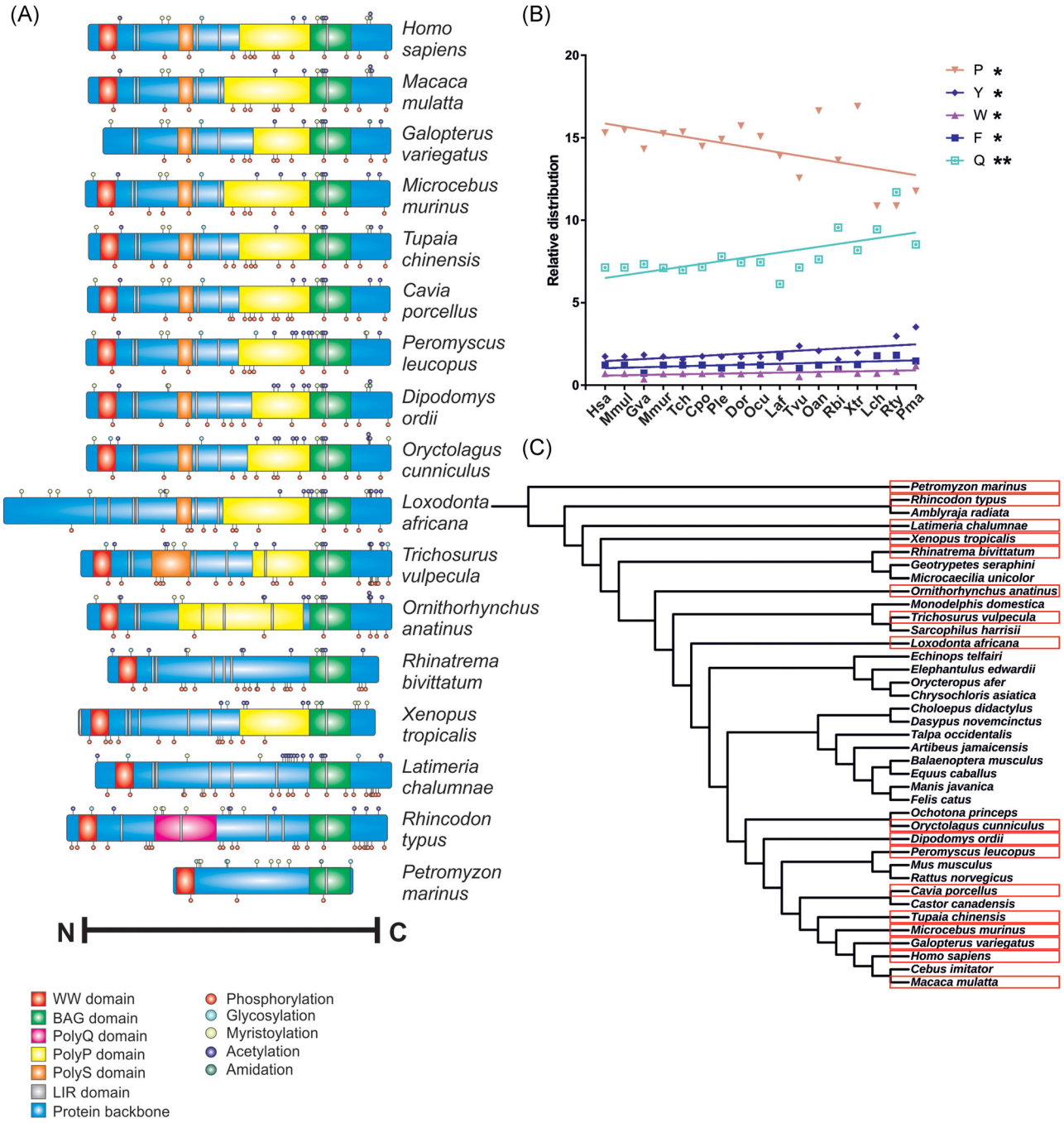


**FIGURE 3** Mammalian B-cell CLL 2-associated athanogene 3 (BAG3). (A) Predicted structures and post-translational modifications (PTMs) of BAG3 orthologues. (B) Amino acids that exhibit a significant gradual shift in their relative distribution using the species are shown in (A). Symbol number indicates the grade of significance with $*p < .05$ and $**p < .01$. (C) Phylogenetic tree of mammalian BAG3

evolution favored a BAG3 that would allow stronger hydrophobic protein-protein interactions. However, the gradual increase in proline does not correlate with an expansion of the PolyP domain, but with a stronger accumulation of the amino acid within the PolyP domain. The high abundance of polar PTMs within this domain could serve as a switch, which could uphold the protein's solubility while it is not bound to another protein. In parallel, all aromatic amino acids in BAG3 show a gradual depletion throughout mammalian evolution.

## 3.4 | Squamatian BAG3

Squamatian BAG3 evolved differently. We chose Pma as origin and *Pantherophis guttatus* as destination

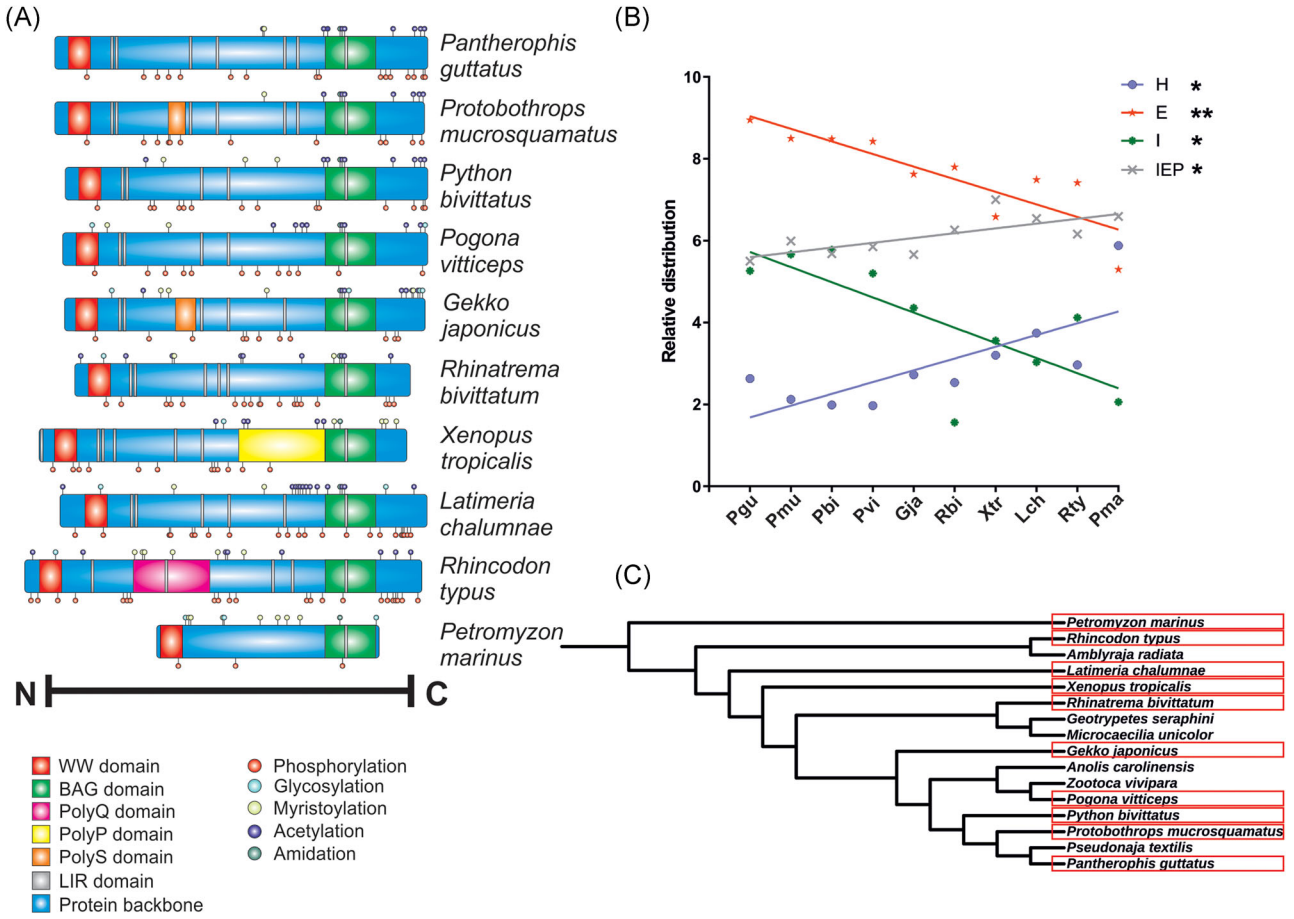


**FIGURE 4** Squamatian B-cell CLL 2-associated athanogene 3 (BAG3). (A) Predicted structures and post-translational modifications (PTMs) of BAG3 orthologues. (B) Amino acids that exhibit a significant gradual shift in their relative distribution and their isoelectric point (IEP) using the species shown in (A). Symbol number indicates the grade of significance with $*p < .05$ and $**p < .01$. (C) Phylogenetic tree of squamatian BAG3

(Figure 4A,C). Mammalian LIR domains were also conserved in the *Squamata*, including the BAGLIR domain (YLM(W)I). Likewise, the sequences of the tandem LIR domains were mostly unaltered (YPKL/YIPI, YIPI/Y(F)HK(Q)I). Interestingly, within the *Proteroglypha* a third LIR tandem came to be (FSPI/FPTV). *Gekko japonicus* (Gja) and *Protobothrops mucrosquamatus* developed a PolyS domain, but it was neither conserved nor did it share similarities with the mammalian homopolymeric PolyS domain.

Although the amidation site in the BAG domain was conserved, phosphorylation sites occurred only occasionally. The phosphorylation site within the WW domain was conserved. Interestingly, phosphorylation sites moved from the protein's center towards its termini. Acetylation sites within the BAG domain were also conserved. Myristoylation sites moved from their original position between the first LIR tandems to a position between the second and third tandem in the *Proteroglypha*.

We could not observe a gradual proline increase (Figure 4B). However, the relative abundance of glutamate and isoleucine increased, while the histidine ratio declined. This caused a net gain of negatively charged amino acids, thus decreasing the IEP.

## 3.5 | Avian BAG3

Here, we chose Pma(r) BAG3 as origin and *Parus major* BAG3 as destination (Figure 5A,C). The BAGLIR domain was identical to mammalian (YLMI). Another LIR domain, which also appeared in Gja, manifested within the *Archosauromorpha* (YIPI). A C-terminal LIR domain (FTNL) was conserved, as were the two tandem LIR domains (YPK(Q)L/YIPI, YI(S)PI(V)/F(Y)HKI). Within the *Archosauria,* BAG3's protein structure changed fundamentally. Many specimens lost their WW domain, which was reintroduced in some *Passeriformes*. However, the presence or absence of this domain can differ

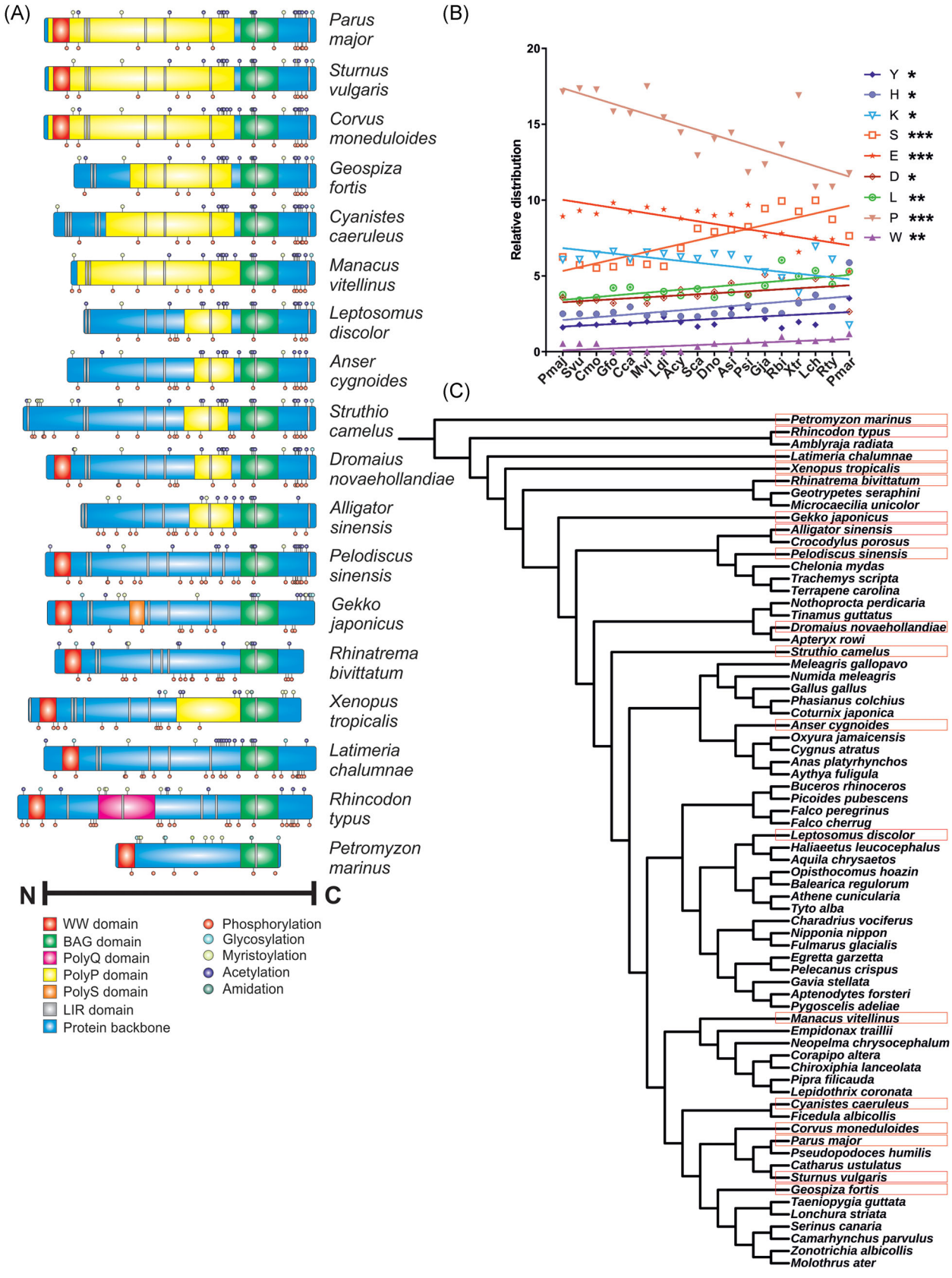


**FIGURE 5** Avian B-cell CLL 2-associated athanogene 3 (BAG3). (A) Predicted structures and post-translational modifications (PTMs) of BAG3 orthologues. (B) Amino acids that exhibit a significant gradual shift in their relative distribution using the species shown in A. Symbol number indicates grade of significance with $*p < .05$, $**p < .01$ and $***p < .001$. (C) Phylogenetic tree of avian BAG3

between two genera in this order. Besides its resurgence, only the *Palaeognathae* minus the *Struthioniformes* kept the WW domain. In parallel to mammalian evolution, BAG3 orthologues of the *Archosauria* also developed a PolyP domain, which experienced a massive extension in the *Passeriformes*.

Amidation and acetylation sites within the BAG domain were conserved throughout the *Archosauromoprha*. Parallel to *Mammalia*, the *Aves* developed a phosphorylation site within the BAG domain, which was augmented by a second site in the *Passeriformes*. Lysine acetylation sites accumulated in the C-termini of PolyP domain and protein. In contrast to mammalian, avian BAG3 exhibited a C-terminal concentration of phosphorylation sites. Potential myristoylation sites were rare, but one between the LIR tandems was conserved within the *Archosauromorpha*. Interestingly, *Archosauria* BAG3 developed a C-terminal glycosylation site.

Throughout avian evolution, BAG3 accumulated more prolines (Figure 5B). Considering the parallel expansion of the PolyP domain in the *Aves*, the increase was not as focused as in the *Mammalia*. The lysine ratio increased, while the serine ratio decreased. Indeed, within the phylum of the *Neoaves*, the lysine ratio exceeded the serine ratio, as did the number of potential acetylation the phosphorylation sites. Simultaneously, the glutamate ratio increased. Within the *Neognathae*, specimens demonstrated similar variance in glutamate and lysine ratios. As lysine normally is positively charged, an increase in glutamate may stabilize the protein's IEP. In parallel, the ratios of aspartate, histidine, tyrosine, leucine, and tryptophan decreased.

## 3.6 | Actinopterygian BAG3

The most drastic differences to mammalian BAG3 occurred in the *Actinopterygii*. Again, we chose Pma BAG3 as origin and *Amphiprion oceallaris* as destination (Figure 6A,C). The BAGLIR domain was present in every specimen and its sequence well conserved (YLL(V)L). *Acipenser ruthenus* and *Lepisosteus oculatus* exhibited an influx of LIR domains, of which two were retained (YI(V)P(S)I, YIPI). With the *Clupeocephala*, a first tandem LIR motif formed (Y(F)SYI). Then, the *Percomorpha* developed a second tandem LIR to their YIPI LIR domain (FHRL(I)). Some *Otocephala* and all *Euteleostei* obtained a variably sized PolyQ domain. Surrounding this PolyQ domain, in the *Neoteleostei*, some families developed a PolyP domain, while others did not. The WW domain is present in every *Actinopterygii*.

Although the potential amidation site in front of the BAGLIR domain is conserved, its acetylation potential is reduced in many *Percomorpha*. Potential PTMs are scarce and concentrated at the termini of the protein, with the exception of myristoylation sites near the second LIR tandem or its predecessor. Furthermore, all *Actinopterygii* retained a potential glycosylation site in their WW domain.

The actinopterygian orthologues gradually increased their glutamine ratio (Figure 6B). Simultaneously, serine and leucine ratios decreased, while threonine and valine ratios increased. Furthermore, histidine and arginine were exchanged for lysine. Glycine and tyrosine ratios also decreased, while the general protein size increased, likely due to an expansion of the PolyQ domain.

## 3.7 | BAG3 LIR domains evolve towards high variability

Based on a previous study, we tried to estimate which human autophagy-related protein 8 (ATG8) paralogue would have the highest probability to interact with the evolved LIR domains based on their amino acid composition. Thus, we composed consensus sequences of conserved LIR domains for each *Vertebrata* class. Amino acids in brackets symbolize changes that occurred at least two times consecutively but were not fixed throughout the entire tree (Figure 7).

Humans have evolved seven different ATG8 paralogues (LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, and GABARAPL2).[30] However, LC3B2 is only present in the *Catarrhini*, GABARAPL1 only in the *Tetrapoda* and LC3B in the *Osteichthyes*. Notably, LIR domains in all classes have high likelihoods to interact with LC3A, LC3C, or GABARAPL2. Increased preference for LC3B developed with the *Tetrapoda* likely caused by LC3B's novelty over the elder LIR domains. Interestingly, within the *Tetrapoda* only the *Simiiformes* demonstrated an active adjustment of their interaction partner likelihood with a transition of LC3A towards GABARAP.

The first LIR domain in *Mammalia* (YPQL) slightly increased its preference for LC3B in the *Sauropsida* (YPKL). The *Proteroglypha* (YLWI) exhibit a non-consequential alteration in their BAGLIR domain. The *Aves* (FHKI) experienced a substitution in the second LIR domain of their second tandem (originally YHKI). This might increase the likelihood of this LIR domain's interaction with LC3C, as LC3C has a higher affinity towards phenylalanine than other ATG8 paralogues. The *Archosauromorpha* developed two additional LIR domains, which have the highest probability to interact with LC3A (YIPI) or LC3B (FTNL). Although throughout *Actinopterygii* evolution two BAG3 LIR domains slightly adjusted (YVPI, FHRL), it did not provoke a change in

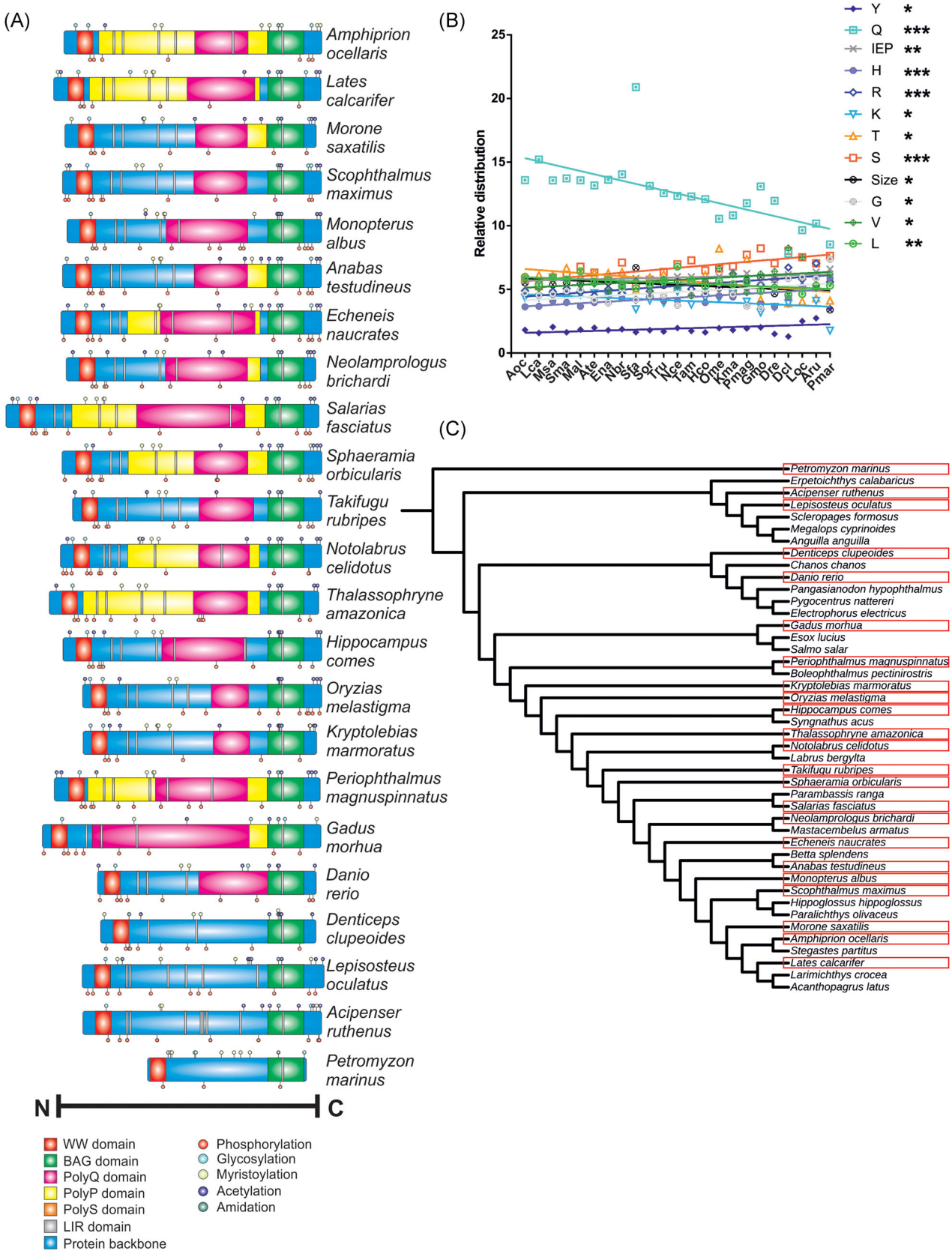


**FIGURE 6** Actinopterygiian B-cell CLL 2-associated athanogene 3 (BAG3). (A) Predicted structures and post-translational modifications (PTMs) of BAG3 orthologues. (B) Amino acids that exhibit a significant gradual shift in their relative distribution using the species shown in (A). Symbol number indicates grade of significance with $*p < .05$, $**p < .01$ and $***p < .001$. (C) Phylogenetic tree of actinopterygian BAG3

(A)

| *Mam.*† | | *Squam.*‡ | | *Aves* | | *Actin.*§ | |
|---|---|---|---|---|---|---|---|
| YPQL | LC3B | YPKL | LC3B | YP(H)KL(I) | LC3B | YI(V)PI | LC3A |
| YIPI | LC3A | YIPI | LC3A | YIPI | LC3A | YSYI | GABARAP |
| YIP(S)I | LC3A/ GABARAP | YIPI | LC3A | YIPI | LC3A | YIPI | LC3A |
| YHKI | LC3C | YHKI | LC3C | Y(F)HKI | LC3C | FHRI(L) | LC3C |
| YLMI | GABARAPL2 | YLM(W)I | GABARAPL2 | YIPI | LC3A | YLLL | No specificity |
| | | | | YLMI | GABARAPL2 | | |
| | | | | FTNL | LC3B | | |

† *Mammalia*, ‡ *Squamata*, § *Actinopterygii*.

(B)

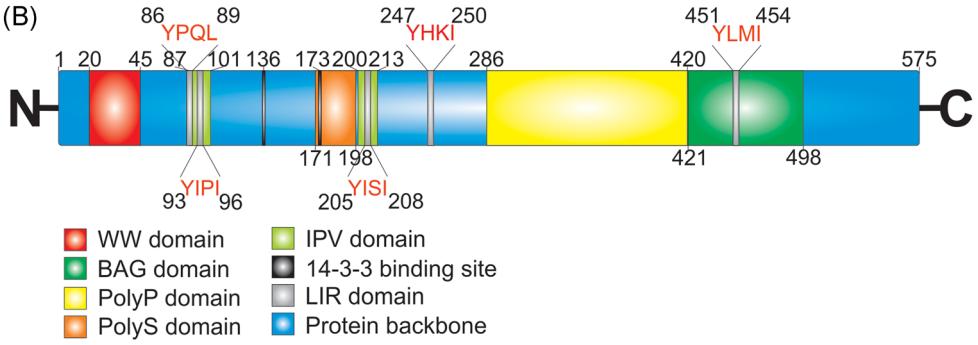


**FIGURE 7** B-cell CLL 2-associated athanogene 3 (BAG3) LC3 interacting region (LIR) interacting autophagy-related protein 8 (ATG8) paralogues. (A) Comparison of the most preferential interacting ATG8 paralogues for conserved LIR domains within BAG3 orthologues. (B) Illustration of human BAG3's structure with its LIR domains

their likeliest interaction partner (LC3A, LC3C). A noteworthy adjustment occurred in their BAGLIR domain (YLLL), which did not exhibit a strong preference to any ATG8 paralogue.

Overall, BAG3's evolution within the *Vertebrata* phylum developed towards high accessibility to different ATG8 paralogues with only GABARAPL1 left out. This progression was maximized within the *Simiiformes*, whose BAG3 may bind five out of their six ATG8 paralogues.[29] Combined, our data may suggest that BAG3 may be capable to directly interact with autophagosomal ATG8s and recruit phagophores to aggresomes independent of external receptors, adaptors, or modifications (Figure 8).

## 4 | DISCUSSION

BAG3's contribution to macroautophagy, which is especially important in senescent cells, was studied extensively.[5,7,31,32] However, peculiarities observed in previous studies left room for an extension of BAG3's role in autophagy. In particular, the formation and conservation of possible LIR domains, as well as a diversification towards their interacting ATG8 paralogue, allow us to create novel hypotheses of BAG3's involvement in autophagy. First insights of the crystal structure of the conserved BAGLIR domain reveal an inward orientation, which would oppose possible interactions with ATG8 paralogues.[33] However, the well-conserved amidation

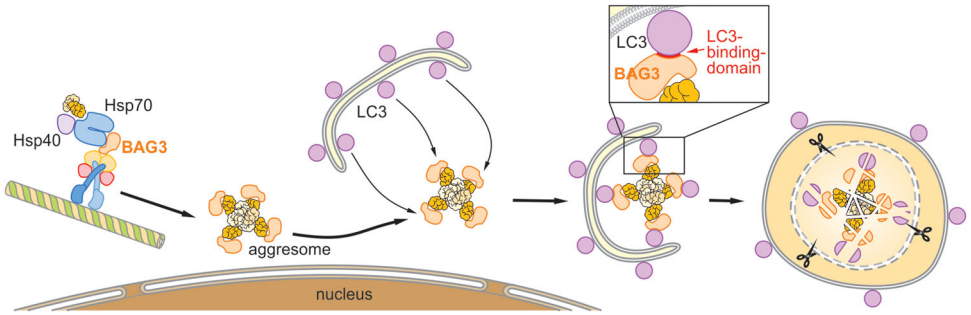


**FIGURE 8** Novel implication of B-cell CLL 2-associated athanogene 3 (BAG3) in autophagy. Illustration of a proposed hypothetical model of a ubiquitin independent autophagy mediated by BAG3 as an anchor for autophagosomal structures

site (E463 in human) could cause a turn in the second helix, thus presenting the BAGLIR domain outward.

Formation and conservation of the detected tandem LIRs could also add new roles for BAG3 in autophagy. BAG3 delivers proteins to aggresomes and remains on their deposition site.[5,7] As previously reported, BAG3-driven autophagy also clears degradation-prone proteins that are not ubiquitinated.[12] Such non-ubiquitinated autophagy substrates could be directly transferred to autophagosomes via the discovered BAG3 LIR domains. Here, its LIR domains, combined with the conserved myristoylation sites in close proximity as membrane anchors, may be able to guide the phagophore across the aggresome like a blanket (Figure 8). As aggresomes can come in different shapes and sizes, this mechanism could optimize their autophagic degradation. The high level of diversification towards the interacting ATG8 paralogues could support this hypothesis, as it would enable BAG3 to recruit various types of autophagosomes. However, membrane anchors facilitated by myristoylation often fluctuate, but a protein-protein interaction between the myristoylated protein and another membrane-bound protein (like LC3) can establish long-term anchorage.[34] An ion or phosphorylation-dependent electrostatic switch can regulate myristoylated groups. Indeed, in *Placentalia* BAG3 a phosphorylation site between the myristoylation sites is conserved, while many other *Vertebrata* show potential phosphorylation sites in close proximity. Overall, early formation and conservation of LIR domains and myristoylation sites throughout all vertebrates may suggest an early specialization event of BAG3 to gauge aggresomes.

Furthermore, our data reveal an interesting evolution of BAG3 within the *Archosauria*. The loss and resurgence of the WW domain are likely caused by mutations on splicing sites. As some *Mammalia* have also lost the WW domain, its apodictical importance for a key process like autophagy may be in need of reevaluation.[11] Although organisms lacking this domain could have evolved a mechanism to compensate for this loss, verification is required. Aside from its possible involvement in autophagy, the WW domain was associated with processes of cell adhesion.[35] The asparagine-glycosylation sites within the WW domain may support its significance in this regard.[36] Interestingly, within the *Aves* another C-terminal asparagine-glycosylation site emerged, which could compensate for the loss of the WW domain. The *Mammalia* lacking a WW domain either obtained many N-terminal myristoylation sites (Laf), or another C-terminal asparagine-glycosylation site (Gva).

Considering the *Aves* have an unusual lifespan/weight ratio, which is highest within the *Passeriformes*, BAG3 may influence their longevity.[37,38] Although theories of avian longevity often revolve around oxidative stress, an improved proteostasis system may also be of relevance. The massive expansion of the PolyP domain in the *Passeriformes* could improve the aggresome clearance of the organism and thus increase its lifespan. Finally, we observed within the *Actinopterygii* strong conservation of a PolyQ-, but not a PolyP-domain. Thus, although studies investigated BAG3-dependent pathologies in *Danio rerio*, and show the protein's importance for correct muscle function, drawing cross-species deductions could turn into a slippery slope.[39] The high divergence of BAG3's structures between the *Vertebrata* classes would advise being wary of cross-class conclusions in general.

## ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) Project-ID 259130777- SFB 1177 and the Corona Foundation of the Deutsche Stifterverband. We thank Hagen Körschgen for his helpful assistance during finalization of the manuscript. Open access funding enabled and organized by Projekt DEAL.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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**How to cite this article:** Baeken MW, Behl C. On the origin of BAG(3) and its consequences for an expansion of BAG3's role in protein homeostasis. *J Cell Biochem*. 2022;123:102-114. https://doi.org/10.1002/jcb.29925