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Network analyses unveil ageing-associated pathways evolutionarily conserved from fungi to animals

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Abstract

The genetic roots of the diverse paces and shapes of ageing and of the large variations in longevity observed across the Tree of Life are poorly understood. Indeed, pathways associated with ageing/longevity are incompletely known, both in terms of their constitutive genes/proteins and of their molecular interactions. Moreover, there is limited overlap between the genes constituting these pathways across mammals. Yet, dedicated comparative analyses might still unravel evolutionarily conserved, important pathways associated with longevity or ageing. Here, we used an original strategy with a double evolutionary and systemic focus to analyse protein interactions associated with ageing or longevity during the evolution of five species of Opisthokonta. We ranked these proteins and interactions based on their evolutionary conservation and centrality in past and present protein-protein interaction networks (PPI), providing a big systemic picture of the evolution of ageing and longevity pathways, that identified which pathways emerged in which Opisthokonta lineages, were conserved and/or central. We confirmed that longevity/ageing associated proteins (LAPs), be they pro- or anti-longevity, are highly central in extant PPI, consistently with the Antagonistic Pleiotropy theory of ageing, and identified key antagonistic regulators of ageing/longevity, 52 of which with homologs in humans. While some highly central LAPs were evolutionarily conserved for over a billion years, we report a clear transition in the functionally important components of ageing/longevity within Bilaterians. We also predicted 487 novel evolutionarily conserved LAPs in humans, 54% of which are more central than mTOR, and 138 of which are druggable, defining new potential targets for anti-ageing treatments in humans.

Introduction

Ageing and longevity are critical components of organismal fitness, both characterised by their remarkable heterogeneity between, and sometimes even within, species across the Tree of Life. For instance, there is a 100-fold variation of longevity across mammals (Tacutu et al. 2018), and studies have revealed a diversity of paces and shapes of ageing across metazoans, even though all these taxa come from a last common ancestor (Baudisch and Vaupel 2012; Jones et al. 2014; Treaster et al. 2021; da Silva et al. 2022). Importantly, the genetic mechanisms that contribute to this heterogeneity are still poorly understood. While there is no doubt that ageing and longevity are in part genetically determined (Kenyon 2010), the pathways associated with ageing and longevity are incompletely known, both in terms of their constitutive genes/proteins and of their molecular

interactions. Indeed, genomic and transcriptomic analyses of organisms with high quality genomes, informed by careful considerations of molecular evolution, have uncovered sets of genes associated with ageing or longevity (Li and de Magalhães 2013; Gorbunova et al. 2014; Keane et al. 2015; Doherty and de Magalhães 2016; Foley et al. 2018; Sahm et al. 2018; Huang et al. 2019; Toren et al. 2020; Farré et al. 2021; Irving et al. 2021; Kacprzyk et al. 2021; Kolora et al. 2021; Orkin et al. 2021; Lu et al. 2022; Tejada-Martinez et al. 2022) with little overlap, even across mammalian species (Farré et al. 2021). This observation of a diversity of genetic bases of longevity and ageing across species is consistent with Darwinian theory, which predicts that longevity can be selected for, yet does not imply that homologous genes should be involved in longevity in different populations or species. Like extant organismal lineages, whose members thrive in very diverse environments and niches, members from ancestral organismal lineages from different clades and from different populations were not all exposed to the same ecological challenges during the course of evolution. For example, some organisms belong to lineages that can fly, while others can live in caves, or must face their predators on the ground (Lunghi and Bilandžija 2022). Similarly, some organisms are solitary, whereas others live in societies (Keller and Genoud 1997), and evolution of their longevities and ageing can thus be affected by social organisation and kin selection, etc. Such differences in past and present selective pressures likely explain the considerable variations in ageing and longevity observed throughout the Tree of Life, and since the effect of each gene variant on the longevity in individual species can be small, as is the case for humans (Singh et al. 2019) different combinations of different genes are possibly associated with the variability of lifespan within and between species.

More precisely, mainstream evolutionary theories of ageing, namely the Mutation Accumulation theory (MA) (Medawar 1952), the Antagonistic Pleiotropy theory (AP) (Williams 1957) and the Disposable Soma theory (DS) (Kirkwood and Holliday 1979), hold that ageing is not genetically programmed *per se* but occurs as a side-effect due to the existence of a selection shadow (Johnson et al. 2019). As a result, AP and MA do not make strong predictions regarding whether the same genes should be collaterally associated with ageing across populations and species, while DS predicts that the functions of the genes associated with ageing are likely related with repair/maintenance and energy allocation to trade-offs between reproduction and survival, mediated by a network of interacting, possibly synergistic processes, rather than by a single mechanism (Kirkwood 1997). In addition, reknown experts on aging consider that aging is a programmed process (Rando and Chang 2012). While more aging-associated genes or proteins than appreciated could be conserved (consistent with either AP theory or programmed theory), this is

not necessarily the case for longevity. Thus, although theoretically plausible, the limited overlap in genes associated with ageing and longevity across species nonetheless raises important conceptual and practical challenges (Farré et al. 2021). On the one hand, this limited overlap may reflect some limits of current methods of detection of longevity- and ageing-associated genes. On the other hand, this genetic heterogeneity does not preclude a form of functional unity at a higher level than genes: even though different gene families are associated with longevity or ageing across species, some belong to pathways (e.g. proteostasis, immune and inflammatory response, hemostasis, development, metabolism) that appear to be shared across species (Muntané et al. 2018; Farré et al. 2021; Treaster et al. 2021). Therefore, updated or enhanced comparative analyses may still unravel evolutionarily conserved pathways associated with longevity or ageing. To reach this goal, however, technical developments that enhance the predictive power of genomics and comparative genomics to detect longevity- and ageing-associated genes appear warranted.

Around twenty years ago, several pioneering ageing studies focusing on interactome data (e.g. (Promislow 2004; Ferrarini et al. 2005; Witten and Bonchev 2007; Fortney et al. 2010)) seeded an original path in this direction. They sought to identify not only the molecular components of pathways associated with ageing and longevity, but also the molecular interactions that compose these pathways. In brief, these approaches relied upon prior experimental evidence defining sets of genes associated with longevity or ageing, hereafter called longevity-associated genes (LAGs), such as those forming the mTOR nutrient-sensing signalling network (Kenyon 2010; Templeman and Murphy 2018; Papadopoli et al. 2019). mTOR is noteworthy because it has been shown to regulate many ageing-associated processes (including cellular senescence, immune responses, stem cell regulation, autophagy, mitochondrial function, and proteostasis) and to mediate caloric restriction-induced lifespan extension in model organisms. For some species, LAGs were further classified as pro- or anti-longevity (pro-LAG, anti-LAG), according to the altered lifespan phenotypes resulting from their genetic loss or gain of function in model organisms (Kenyon 2010; Tacutu et al. 2018). These phenotypic labels were then conjugated with additional, independent information from interactome databases (Szklarczyk et al. 2019) to perform protein-protein interaction networks (PPI) analyses with a focus on ageing and longevity associated pathways. Specifically, the topology of PPI of a few extant species was analysed to track the interactions between longevity-associated proteins (LAPs) (e.g. proteins encoded by LAGs) in order to determine the pathways to which these LAPs connected, and whether the patterns of connection of these LAPs presented distinctive topological features that could be exploited to predict additional components of longevity/ageing associated pathways using PPI. Owing to the paucity of the data,

these pioneering studies were generally conducted on one or two species, with a limited number of network metrics (Promislow 2004; Ferrarini et al. 2005; Budovsky et al. 2007; Bell et al. 2009; Wang et al. 2009; Zhang et al. 2016). Nonetheless, they made for stimulating findings. LAPs from model organisms (*Saccharomyces cerevisiae*, *Sc* and *Drosophila melanogaster*, *Dm*) were shown to be central, connected nodes in PPI networks. In particular, LAPs displayed higher node degree (the number of connections per node in the network) than other proteins not proposed to be associated with ageing or longevity (non-LAPs). Such a high connectivity was interpreted as a proxy for the functional pleiotropy of LAPs, in support of the AP theory (Promislow 2004), considering that, as more highly connected proteins tend to be more pleiotropic than expected by chance, these proteins will also be most likely to evolve an association with senescence. Indeed, by chance alone, more pleiotropic proteins are more likely to have some of their effects found at different ages, and that some of these hold opposite consequences on fitness. This idea that AP is a general principle of ageing was also brought forward in (Yanai et al. 2017). Moreover, the high centrality of LAPs in PPI was proposed as evidence that LAPs regulate fundamental biological processes (Fernandes et al. 2016; Tacutu et al. 2018). In addition, pro- and anti-longevity proteins were shown to be intertwined in the interactome of the worm *Caenorhabditis elegans* (*Ce*), with only few significant topological differences between their subnetworks, which represented two sets of interactions with opposing effects on longevity (Fernandes et al. 2016). The interactions between LAPs and non-LAPs in PPI were also a source of important findings. Interactions between LAPs and proteins encoded by age-related diseases (ARD) genes (Tacutu et al. 2011) and cancer genes (Budovsky et al. 2007; Bell et al. 2009; Budovsky et al. 2009; Wang et al. 2009; Zhang et al. 2016) unraveled connections between their respective pathways, hinting at mechanistical connections between some aspects of ageing and some diseases. Furthermore, using LAPs as reference nodes and mining PPI of a few extant species for non-LAP nodes with comparable topological properties than LAPs, allowed some authors to propose subsets of noteworthy non-LAP proteins that may contribute to presently unidentified ageing- or longevity-associated pathways in *Hs*, *Sc*, *Ce* and *Dm* (de Magalhães and Toussaint 2004; Managbanag et al. 2008; Tacutu et al. 2012; Wuttke et al. 2012; Avelar et al. 2020).

While powerful, the predictive approaches mentioned above did not systematically determine how critical for ageing/longevity their predicted candidate LAPs are, and did not fully exploit their comparative potential. Here, we developed evosystemics analyses of PPI (Watson et al. 2020) to uncover important, evolutionary conserved ageing associated pathways, i.e. pathways made of proteins with significant possible effects on ageing/longevity. We relied upon two non-

mutually exclusive criteria to rank proteins and protein interactions as critical to understanding ageing/longevity: their high evolutionary conservation across Opisthokonta, a one-billion-year-old clade, encompassing fungi and animals, and their high centrality in extant and/or ancestral protein interaction networks. Through systematic analyses of the topological properties of LAPs within such networks and through guilt-by-association analyses, we characterised central evolutionarily conserved pathways associated with ageing/longevity and predicted novel central, evolutionarily conserved LAPs in five Opisthokonta species (*Dm*, *Ce*, *Sc*, with *Mus musculus*, *Mm*, and *Homo sapiens*, *Hs*), some of these non-LAPs that we here proposed as new LAPs were supported by independent bibliographical validation. Promisingly, 265 of these 487 predicted additional LAPs are more central than mTOR in the human interactome and dominated by ribosomal proteins and ubiquitination pathways. Moreover, 28.3 % of the proteins predicted to hold important roles in longevity or ageing-associated pathways in humans are druggable, defining targets for potential novel anti-ageing treatments in humans in the short-term.

Results and Discussion

Theoretical pay-offs of evosystemic PPI analyses

Evosystemics jointly analyses evolutionary and topological signals (Watson et al. 2020). High evolutionary conservation across five species of Opisthokonta and high centrality in PPI (as defined in Figure S1A-D) provide two non-mutually exclusive criteria to rank proteins and protein interactions as critical to understanding ageing/longevity. On the one hand, highly conserved proteins and proteins interactions indicate homologous mechanisms, deeply rooted in the biology of Opisthokonta, which can therefore be experimentally studied in non-human species with potential translational payoffs. Likewise, highly central proteins contribute to interactions across many pathways and/or within large molecular machineries. Thus, changes in the genes coding such central proteins (or changes in their regulations) are likely to have strong impacts on many interconnected processes. Because evolutionarily conserved proteins are encoded by gene families with long time of residency within lineages, evolutionarily conserved proteins have had more opportunities to get involved into diverse functional partnerships, and are often expected to be more central in PPI than proteins from more recently evolved gene families (Baptiste and Huneman 2018). Consequently, uncovering i) highly central proteins and ii) evolutionarily conserved proteins

and interactions associated with ageing/longevity in PPI could identify critical pathways associated with longevity or ageing and suggest important targets to medically interfere with the ageing/longevity process, which could be validated across a broad range of species.

LAPs are highly central across PPI of five extant species of Opisthokonta

Our study used multiple stringency thresholds for PPI networks based on interaction confidence scores, a broader selection of Opisthokonta (*Sc*, *Ce*, *Dm*; *Mm* and *Hs*) and more network metrics (e.g. betweenness, closeness, degree, PageRank, Fig. S1A-D) than former PPI analysis on ageing/longevity (Promislow 2004; Ferrarini et al. 2005; Budovsky et al. 2007; Bell et al. 2009; Wang et al. 2009; Zhang et al. 2016). We used these different metrics, as, although often correlated in real networks, they could have captured slightly different aspects of network centrality. In our study, it turned out that irrespective of what specific network centrality was measured, the general conclusion regarding the centrality of LAPs is the same and generalises two previous findings derived from single species longevity networks. First, across the five tested species of Opisthokonta, LAPs are significantly more central in PPI than non-LAPs. This higher centrality was observed at various stringency thresholds, but limited by network size and the number of identified LAPs at high stringency for *Dm*, *Mm* and *Ce* (Fig. 1). This result, compatible with the AP theory (Promislow 2004; Ferrarini et al. 2005), is true for both pro-LAPs and for anti-LAPs (Fig. S2), with the same dependence on stringency, network size and number of identified LAPs; however, the imbalance between pro- and anti-longevity annotations in *Sc* (13% and 87% of the 400 *Sc* annotated ageing-associated proteins, respectively) may lead to a less robust signal of centrality for pro-longevity proteins. Accordingly, pro- and anti-longevity proteins appeared equally central in longevity networks across these five species of Opisthokonta (Fig S2). A few notable exceptions were found for experimental networks of *Sc*, possibly due to the imbalance mentioned above, and for *Ce*, where pro-longevity proteins displayed significantly higher centrality than anti-longevity proteins. The *Ce* observation is consistent with previously described higher clustering coefficient of pro-longevity proteins in the BioGrid PPI network (Fernandes et al. 2016).

Interestingly, pro-LAPs tend to interact significantly more with pro-LAPs whereas anti-LAPs tend to interact significantly more with anti-LAPs, both in entire interactomes (featuring both LAP and non-LAP nodes) and in longevity networks (featuring only LAPs as nodes) (Fig. 2, S3A), as determined by assortativity analyses and network permutation tests (Fig. S4). But this observation does not mean that there is a neat partition between pro-LAPs and anti-LAPs in the

PPI, since overall, the assortativity (Fig. S1E) values are only slightly positive (Fig. 2). In other words, interacting LAPs with antagonistic effects are widespread in PPI, suggesting that ageing and longevity associated pathways are commonly regulated by a diversity of checks and balances with opposite effects. However, remarkably, some pro-longevity and anti-longevity proteins entertain, in all analysed species, significantly more interactions with one another than expected by chance. It is interesting that our results identified differential correlations between pro-LAP and anti-LAP proteins, because this suggests that these two classes might indeed be biologically distinct, which was not necessarily obvious. Indeed, many of these pro-LAP and anti-LAP were identified by experiments that knock out genes and observe the effect on aging, whereas in nature more subtle variations in expression than a complete turn-off are likely to affect these genes, with effects that can both increase and decrease lifespan, depending on the specific expression changes for a given gene. Bearing this note of caution in mind, we propose that such LAPs, significantly strongly involved in interactions with proteins with opposite effects on longevity, correspond to key regulators in antagonistic regulatory mechanisms of longevity (ARMLs).

Three species harbor key antagonistic regulators of ageing/longevity

Using network permutation tests (Fig. S4), we found that three species displayed significantly stronger connections between pro-LAP and anti-LAP than what would be expected by chance, suggesting that such LAPs may be critically involved in the regulation of ageing/longevity (Fig. 3). Namely, we identified 13 LAPs involved in ARMLs in *Dm*; 38 in *Ce* and 40 in *Sc*. Several of these LAPs are homologs found in all three species and contribute to the mTOR signalling network, including mTOR itself (*Dm* Tor/*Ce* LET-363/*Sc* TOR1), but also the AGC kinases Akt (mTORC1 upstream activator and mTORC2 target: *Dm* Akt1, *Ce* AKT-1) and S6k (mTOR substrate: *Dm* S6k, *Sc* SCH9). As these LAPs are well-characterised for their key role in regulating ageing (Kenyon 2010; Templeman and Murphy 2018; Papadopoli et al. 2019), this observation provides proofs of concept that our approach can identify key antagonistic regulators. *Dm* and *Ce* also share the insulin/mTOR signalling network members PTEN phosphatase (*Dm* Pten/*Ce* DAF-18), Insulin receptor (*Dm* InR/*Ce* DAF-2), FOXO transcriptional regulator (*Dm* foxo/*Ce* DAF-16) and the NAD⁺-dependent protein acetylase sirtuin Sir2 (*Dm* Sir2/*Ce* SIR-2.1), whereas *Ce* and *Sc* share NAD⁺-reducing enzymes of the TCA cycle malate dehydrogenase and isocitrate dehydrogenase (*Ce* MDH-2/*Sc* MDH1/MDH2; *Ce* IDH-1/*Sc* IDH1). The existence of homologous key antagonistic regulators across Opisthokonta species supports the idea that

modulating lifespan has been evolutionarily important for at least a billion year and has occasionally relied on the same players, from fungi to animals.

In addition, we identified some LAPs (*Dm*: 3/13; *Ce*: 20/38; *Sc*: 23/40) involved in species-specific ARMLs with a comparable number of antagonistic interactions as emblematic mTOR network members, including, for example, the oxidative stress response protein Keap1, the cardiac-restricted actin-binding protein Vinc and the JNK phosphatase puc in *Dm* (Fig. 3A), which suggests that regulation by these LAPs may deserve careful investigations.

Overall, functions enriched in ARMLs in *Ce* and *Dm* correspond to functions described for proteins in the mTOR/Insulin network, such as stress response, regulation of translation, of regulated cell death and of growth and development (Fig. S5). Functions related to respiration were also enriched among *Ce* and *Sc* ARMLs: TCA cycle in *Ce* and *Sc*, ATP synthesis and mitochondrial electron transport chain in *Sc*. Finally, *Sc* ARMLs also displayed enrichment for chromatin regulation and DNA integrity checkpoint functions.

Strikingly, 52 ARML nodes and their associated interactions appear evolutionarily conserved at the taxonomic scale of Opisthokonta and can be found in humans, corresponding to 58% of these antagonistic regulators found either in *Ce*, *Dm* and *Sc*. These candidate antagonistic regulators of ageing/longevity in humans are associated with 5-14% of evolutionarily conserved interactions (depending on PPI stringency). Although genes are only seldom annotated as pro- or anti-longevity in humans, association with longevity can be inferred by genome-wide association studies (GWAS) as recorded in the LongevityMap database. Thus, among the 52 human homologs of ARMLs identified in *Dm*, *Ce* and *Sc*, we found 4 proteins (3 mTOR network members: AKT3, MTOR, RPS6KB1, and SOD2) that are significantly associated with long life in our species. Therefore, 48 other human genes may act as critical, evolutionarily conserved regulators of longevity/ageing in our species. Interestingly, 3 proteins (SOD2, ALDH2, PRKAA2) are also associated with ageing-related diseases (ARD) and can be targeted by drugs (Table 1).

Functional analyses of highly central LAPs

Unveiling highly central LAPs in ‘longevity PPI’, exclusively composed of LAPs, can point to especially critical components of ageing/longevity pathways. Indeed, by knowing these most central LAPs amongst the LAPs, one can focus on some ageing phenotypes that point to (the

expression of) important (central) proteins, at high risk of negatively affecting organismal or cellular homeostasy, or point to adaptations to longevity involving extremely deep structural components, i.e., the most highly central proteins of the interactome.

We analysed the functions of those LAPS by ranking LAPS by their centrality and by identifying high centrality outliers amongst LAPS in each extant longevity network, using network permutation tests (Fig. S4). Such high centrality outliers were found at all stringency thresholds, including in experimental networks, and their numbers unsurprisingly decreased with stringency (Fig. 4). As expected, many functions enriched among most central LAPS correspond to mTOR/Insulin signalling network-associated functions (Fig. S6), including maintenance/repair mechanisms, oxidative stress response, cell growth or autophagy (although Tor itself did not contribute to the top 20 most enriched functions among *Dm* centrality outliers).

In Bilateria, other signalling pathways involved in development and homeostasis, crosstalking with the mTOR network, were also found within centrality outliers, including MAPK (*Ce*: LET-23, LET-60, SEM-5, MPK-1, JNK-1; *Dm*: p38b, bsk; *Hs*: ERBB2, EGFR, GRB2, HRAS, MAPK3, MAPK14 and various growth factors), TGFbeta (*Dm*: dpp), Wnt (*Ce*: BAR-1, *Hs*: GSK3B, CTNNB1), Notch (*Ce*: GLP-1), JAK/STAT (*Hs*: JAK2, STAT5B, STAT3, NFkB (*Hs*: RELA, NFKB1), AR (*Hs*: AR) and TP53 (*Hs*: TP53, MDM2, TP53BP1; *Mm*: Trp53, Trp53bp1) signalling pathways. Highly central proteins from these signalling pathways contributed to the enrichment in the same functions as the mTOR network, e.g. MAPK signalling through p38 and JNK was associated with response to stress functions. The above results are compatible with the idea that aspects of ageing and longevity are connected to developmental programs, as well as with claims that some developmental processes can provoke ageing when they are active late in life, executing detrimental quasi-programs due to selection shadow (Blagosklonny 2006; Gems 2022). Besides signalling-associated functions, most central LAPS also displayed enrichment for ribosome biogenesis/protein translation, cellular respiration (ATP synthesis/ mitochondrial electron transport chain/TCA cycle functions) in *Ce* and *Sc*, telomere regulation in *Hs* and *Sc*, and regulated cell death in *Mm* and *Hs* (Fig. S6).

Interestingly, some highly central LAPS belonged to the same orthology family and were consistently recovered as highly central across 3 or 4 different Opisthokonta species (Fig. 4). These conserved centrality outliers were less frequently found in experimental networks, which is likely explained by their small size compared to the thresholded networks (with the exception of *Sc*).

These highly central, evolutionarily conserved LAPs were mTOR, AKT/S6K AGC kinases and PTEN homologs, all acting in the mTOR/Insulin signalling network, as well as the homologs for the oxidative stress regulators superoxide dismutase 2 and catalase, and for the protein acetylase sirtuin2.

Progressive changes in enriched ageing/longevity associated functions during Opisthokonta history

We next turn towards a more systematic analysis of the evolutionary conservation of LAPs and their pathways by analysing ancestral PPI of five species of Opisthokonta. We inferred ancestral PPI networks throughout the phylogeny of these Opisthokonta (Fig. S7) to identify which pathways, implementing which functions, had emerged in which lineages. These ancestral longevity networks are comprised only of nodes corresponding to orthogroups (Fig. S3B) and edges associated with ageing or longevity in at least two extant species. Such edges are important, either because they represent convergences in longevity networks (hinting at their possible functional importance) or because they were inherited from a common ancestor. Considering that possibility, we could infer the ancestral longevity networks composed by such edges by comparing extant longevity networks featuring only orthoproteins as nodes (obtained for increasing levels of stringencies, from PPI edge score ≥ 500 to the use of experimental edges only) (M&M). Given the small number of species, and the very large number of potential protein-protein interactions, one might worry that the likelihood of both false-positive and false-negative claims for deep evolutionary conservation of PPIs (i.e., both presence of and absence of conserved interactions) could be elevated. We verify this was not the case: using resampling of input PPI networks with networks from sister species of *Ce*, *Dm* or *Sc*, we found that this approach had a false positive rate of 0 for all resampled species, and a false negative rate, depending on PPI score thresholds, comprised between 0 and 3.7 % for *Ce* or *Dm* replacement, and between 8 and 16% for *Sc* replacement, indicating that our inferences of ancestral edges are conservative and that *Sc* networks have the most weight on the inference of the common ancestral network (M&M).

Consequently, we first used these ancestral networks to estimate the proportion of LAPs and their interactions from extant longevity networks that had already evolved in the past. These numbers reflect the proportion of LAPs and their interactions inherited in extant interactomes. Thus, a third of human LAPs and 7 % of their interactions date from ancestral interactomes of

Opisthokonta (Fig. 5). Moreover, extant ARMLs, e. g. 8 to 43% of the human ARML orthologs (Table 1) and 1 to 10 % of their extant interactions, depending on PPI stringency thresholds, can also be found in ancestral networks. Therefore, a phylogenetically broad range of organisms could still be used to experimentally highlight numerous mechanistic aspects associated with human ageing and longevity.

Second, we assigned a GO-term to each node in these ancestral networks by using the most GO-annotated species for each lineage for ancestral networks (i.e. human proteins for the common, bilaterian and euarchontoglires ancestors, and *Ce* proteins for the ecdisozoan ancestor). We identified functionally enriched GO-terms (top 20) for each network with Metascape (M&M) (Fig. 6). To assess whether different GO-terms (and below different functions) had been associated with ageing or longevity as Opisthokonta diverged from their last common ancestor, we compared these top 20 GO functional annotations for ancestral longevity networks. 40 % of the enriched GO-terms were shared between the common and the bilaterian ancestral networks, but only 5% were shared between the bilaterian and the ecdisozoan ancestral networks and 5% were shared between the bilaterian and the euarchontoglires ancestral networks. Thus, functionally enriched GO-terms associated with ageing/longevity shifted over time, with some evolutionary conservation outside bilaterians. Next, we compared which gene families contributed to GO-terms enrichment between ancestral networks (Table S8): for each of the evolutionarily conserved GO-terms, we further checked whether they involved similar or different gene families in the successive ancestral networks, using a Jaccard index between pairs of sequential ancestral networks (M&M). We concluded that a significant proportion of both genes and pathways had been conserved between the common and the bilaterian ancestral networks (Jaccard indices ranging from 0.53 to 0.89), whereas there were less shared gene families between the bilaterian and the ecdisozoan ancestral networks (Jaccard index = 0.21 for their unique shared GO-term) and between the bilaterian and the euarchontoglires ancestral networks (Jaccard index = 0.29 for their unique shared GO-term). These results show a limited overlap between gene families from functionally enriched GO-terms associated with ageing/longevity, reinforcing the notion that important LAPs differ within lineages of Opisthokonta, especially since the last common ancestor of bilaterians, i.e., there are many lineage specific evolutionary roads to ageing and longevity.

Although gene families and functionally enriched GO-terms associated with ageing and longevity showed limited overlap between Opisthokonta, we still wanted to verify whether some convergence in their even more general functions could not be detected for these enriched GO-

terms. For this, we grouped functionally enriched GO-terms into common functions, e.g. autophagy, stress response, or protein translation, etc. Functions enriched in the common ancestral network are linked to protein translation, stress response, energy production, autophagy and apoptosis. Consistent with the above results, the bilaterian ancestral network shared most of these enriched functions found in the common ancestral network, to the exception of apoptosis and with the addition of response to nutrient levels, a function linked to the mTOR signalling network. Interestingly however, ecdisozoan and euarchontoglire ancestral networks that displayed different enrichment of GO terms were also functionally divergent. The ecdisozoan network was enriched in autophagy and cell death, whereas the euarchontoglire network was enriched in DNA repair, telomere, response to nutrient levels, glucose homeostasis, apoptosis, cell cycle regulation and cell senescence-related functions. This distinction may reflect genuine differences in biological processes, i.e. lineage-specific evolution of longevity/ageing processes, or reflect an experimental bias in the functions investigated by researchers for these different clades. Consistently, a Pubmed search for "cellular senescence" in combination with species names and ageing/longevity/lifespan as keywords returns 28 times more abstracts for euarchontoglire species (*Hs*: 1302; *Mm*: 346) than for ecdisozoan species (*Ce*: 41, *Dm*: 18), even though cellular senescence can occur in ecdisozoan at least in *Dm* (Ito and Igaki 2016), but a similar search for "autophagy/mitophagy" returns only 2.5 times more abstracts for euarchontoglire species (*Hs*: 899; *Mm*: 440) than ecdisozoan species (*Ce*: 313, *Dm*: 229). These patterns are indeed in accordance with the imbalance in numbers of studies on cellular senescence performed in humans and mice versus flies and nematodes (~40 times more studies in humans and mice), rather than reflecting a preferential link with ageing and longevity.

Overall, these results suggest a progressive transition in enriched functions associated with ageing/longevity along the Opisthokonta phylogeny, although this interpretation could still be dependent on experimental biases, and the limited species sampling that was available for our study. Indeed, this sampling included four highly lab-adapted species (for which labels relative to ageing/longevity had previously been gathered from experiments) and humans, which have an unusual longevity; and therefore future studies with a broader taxonomic sampling covering even more of the diversity of Opisthokonta will be useful to assess the generality of our conclusions across animals and fungi. Still, resampling-based estimations of false negative and positive rates suggest low rates of type I and moderate rates of type II errors and thus a good representativity of the results even with a limited number of input species.

Inferring new evolutionarily conserved LAPs

Finally, we expanded our analysis of ageing and longevity associated pathways beyond the borders of extant and ancestral longevity networks (composed only of LAPs) by connecting these networks to the rest of the PPI networks from which they constitute a subset (the global interactomes, also featuring non-LAPs). We mined the global PPI networks (comprised of all orthologous proteins from a given organism, or all the conserved orthologous protein families for a clade) and all significantly supported edges (for various stringency thresholds), both for extant interactomes and for inferred ancestral interactomes (Fig. S9), to identify non-LAPs with significantly similar sets of neighbours to LAPs (Fig. S10). We reasoned that when a non-LAP (or a non-LAP family) significantly interacts with the same sets of proteins than a LAP (or a LAP family), this non-LAP may perform biological functions closely related to that of the LAP with which it plays the same structural role in the PPI network.

In extant networks, depending on which species was investigated, hundreds to thousands of non-LAPs shared significantly similar sets of neighbours than LAPs. Achieving phenotypic assays for so many candidate components of longevity or ageing-associated pathways would be overwhelming. Fortunately, analyses of ancestral PPI networks returned a much more manageable number of candidates, which in addition are evolutionarily conserved. Predicted proteins from ancestral networks were enriched for functions such as translation/ribosomal biogenesis in all 5 species, ubiquitination/proteasomal degradation in bilaterians, neddylation, cell cycle progression and DNA repair in euarchontoglires.

Our strategy makes an asymmetric use of LAPs, as these proteins have been known for a long time to have some direct or indirect connections with ageing, and of non-LAPs, which are proteins that were not reported to have direct or indirect connections with ageing in GenAge. Therefore, an analysis of the scientific literature to validate our predicted for novel LAPs from non-LAPs should not capture information that was used, in the first place, to define longevity-associated proteins. Consistently, we validated a subset of these non-LAPs that we predicted to be associated with ageing or longevity by querying either publication records in PubMed database or phenotypic annotations in species-specific databases (M&M). Predictions with such external support amounted to 24% in *Ce*, 9% in *Dm*, 10% in *Hs*, 5% in *Mm* and 8% in *Sc* (Table S11). Although selecting *Dm* genes at random (1321 genes with ageing-related annotations among 13986 protein-coding genes:

9.4%) would lead to a similar number of ageing-related annotations as evosystemic predictions (9 out of 119 predictions: 7.5%, $P > 0.05$), we saw an enrichment for ageing-annotated *Ce* genes (17 out of 74 predictions: 23%) compared to random sampling (1171 genes with ageing-related annotations in Wormbase among 19886 protein-coding genes: 5.9%, $P < 0.05$), suggesting that evosystemic analysis can be used to predict new ageing-associated genes.

Remarkably, 265 non-LAPs that we predicted to be associated with ageing/longevity are more central than MTOR in the human interactome (Table S12), forming a list of novel evolutionarily conserved super-central candidate proteins associated with ageing or longevity, dominated by ribosomal proteins and ubiquitination pathways. This list also includes KEAP1, a candidate regulator of ageing or longevity, since KEAP1 is also homologous to a key antagonistic regulator that we identified in *Dm*. Consistently with our prediction, KEAP1 has been suggested to regulate the ageing of human aortic endothelial cells (EC) in culture, as a repressor of Nrf2 transcription factor, the critical modulator of cellular stress-response (Kopacz et al. 2020). In addition, 138 out of the 487 new LAPs we predicted are potentially druggable, based on documented drug-protein interactions (M&M), and KEAP1 is one of those (Fig. 7, Table S11). Most interestingly for translational purposes, 28/138 of these druggable proteins are more central than MTOR (Table S12) and 1 amongst those is associated with ageing-related diseases (the proto-oncogene small GTPase superfamily member KRAS). Consequently, these candidate LAPs figure as possible targets for potential novel anti-ageing treatments in humans.

Conclusion

We presented a network-based approach to make some progresses in the analysis of ageing and longevity genes. This approach is surely not the only strategy to reach this goal, given that protein interaction networks lack direct information on mutations associated with longevity located in non-coding/regulatory regions of the genomes, however, we showed that evosystemics has some potential for ageing studies and for proposing novel candidate ageing or longevity related genes. Namely, we tracked the evolutionary history of protein interactions associated with ageing or longevity throughout the phylogeny of five species of Opisthokonta, to identify which pathways known to be associated with ageing or longevity had emerged in which lineages, were conserved and central. To do this, we conjugated two non-mutually exclusive criteria to rank proteins and protein interactions by their high evolutionary conservation across these Opisthokonta and their high centrality in extant and in inferred ancestral PPI. We applied our approach to five species of Opisthokonta, a broader taxonomic selection than used thus far in any previous PPI analyses on

ageing and longevity, mapped extant and ancestral networks with LAP, pro- and anti-longevity labels, and analysed their connections with a large range of network metrics. We confirmed that LAPs, be they pro- or anti-longevity, are highly central in extant PPI, and identified key antagonistic regulators of ageing/longevity. While some highly central proteins are evolutionarily conserved, we observed a transition in functionally important components of ageing or longevity along these different Opisthokonta lineages. Still, a third of the human LAPs and 7 % of their interactions date from ancestral interactomes of Opisthokonta, indicating that a phylogenetically broad range of model organisms could be investigated to understand central mechanistic aspects associated with human ageing and longevity. We also predicted new central, evolutionarily conserved LAPs, of which some could be validated by published independent experimental support. Importantly, we propose that a set of 487 LAPs should be included in the human longevity network. About half of them, largely associated with ribosomal proteins and ubiquitination pathways, are more central than mTOR in the human PPI. In addition, 28.3% of the proteins we predict to hold important roles in longevity or ageing-associated pathways in humans are druggable, defining potential targets for novel anti-ageing treatments in humans. While we feel this approach is promising, it is worth keeping in mind that our analysis could at the time being only rely upon five species for which *a priori* knowledge on ageing- and longevity-associated proteins were already available. Although this was the most phylogenetically diverse set of species we could currently analyze by this approach, two of the five species (mice and humans) have diverged less than a hundred million years ago, and as such represent a fraction of the genetic diversity of animals. Consequently, we hope that future evosystemic studies, benefiting from an enlarged taxonomic dataset from fungi to animals, will determine to what extent our conclusions generalize to all Opisthokonta species, beyond the five species carefully investigated here.

Materials and Methods

Protein-protein interaction stringency

Protein-protein interaction (PPI) networks were built from the STRING database (<https://string-db.org/>)(Szkłarczyk et al. 2019) for five species with longevity-related annotations in the GenAge database (<http://genomics.senescence.info/genes/>, Build 20)(Tacutu et al. 2018): *S. cerevisiae* (*Sc*, txid4932), *C. elegans* (*Ce*, txid6239), *D. melanogaster* (*Dm*, txid7227), *M. musculus* (*Mm*, txid10090) and *H. sapiens* (*Hs*, txid9606). STRING-recorded interactions between pairs of

proteins are weighted by confidence scores ranked from 0 to 1000. STRING PPI networks were filtered at different PPI stringencies either based on interaction score thresholds (scores above 500, 600, 700, 800 or 900), or solely based on interactions experimentally supported (hereafter referred to as experimental networks).

Label-based subnetwork induction

Nodes in PPI networks were labelled as longevity/ageing-associated proteins (LAPs) in accordance with the annotations in the GenAge database. Pro- (genes whose decreased expression by knockout, mutations or RNA interference reduces lifespan and/or whose overexpression extends lifespan) or anti-longevity (genes whose decreased expression extends lifespan and/or whose overexpression decreases it) labelling was also used for *Ce*, *Dm*, *Mm* and *Sc* proteins; genes associated with ‘unclear’ or ‘unannotated’ effects on longevity were labelled as ‘unclear’ in our dataset. LAPs, subdivided into pro-longevity, anti-longevity and ‘unclear’ labelled nodes and the edges connecting them (at the indicated PPI network threshold) defined the species-specific longevity networks used in this study (Fig S3A).

Orthology relationships

Orthology criteria were those of the Alliance of Genome Resources Portal (Alliance Database Version: 4.1.0, <http://www.alliancegenome.org>), which focuses on aggregating and curating orthology relationships between model organisms from a diversity of databases (The Alliance of Genome Resources Consortium et al. 2020) to annotate homologs among ARML and centrality outliers (using a ‘--HH + number’ arbitrary code (Table S13). For ancestral PPI network inference, orthology relationships were based on the more stringent OMA orthogroups from the OMA database (Orthologous MAtrix, <https://omabrowser.org/oma/home/>, OMA All.Jan2020 release)(Altenhoff et al. 2018). Orthology-labelled nodes were used to derive networks of orthologs, sharing the same OMA orthogroup identifiers (Fig. S3B), for each species and at each PPI stringency threshold.

Centrality analysis and outlier detection

Four metrics distributions were computed to determine the centrality of LAPs in PPI networks (Figure S2A-D). Three metrics (betweenness, closeness, and degree) were calculated using the NetworkAnalyser plugin in Cytoscape (Doncheva et al. 2012) and the PageRank (Page

et al. 1999) of each node was calculated using the algorithm implemented in the networkx Python package (<https://networkx.org>) with default parameters. Metrics distribution for pro-, anti-longevity proteins (model organisms) or only LAPs (for all five species), were compared to proteins not associated with longevity (non-LAPs) using the Mann-Whitney U-test (unilateral or bilateral, as indicated). P-values were adjusted for multiple testing using the Bonferroni method. To detect centrality outliers with statistically significant high values for centrality metrics in longevity networks (Figure S2A-D), normalized betweenness, degree, PageRank and closeness were computed using the networkx Python package at all PPI stringency thresholds. Statistically significant high values were determined by node rewiring permutation tests (see Network permutation tests Methods section). Because network permutation tests scale poorly with increasing network size, we compared node centrality in entire PPI networks by their average degree rank across all PPI stringency threshold.

Homophily analysis and candidate ARML detection

Homophily between labelled nodes in networks, i.e. the preferential connection with the same labels, was measured by computing assortativity coefficients (Figure S2E) as defined in equation II from Newman, 2003 (Newman 2003), in order to quantify the extent to which nodes with the same label connect with each other rather than with differently labelled nodes. Assortativity coefficients were calculated using the `attribute_assortativity_coefficient` function from the networkx Python package either for LAPs in entire networks or for pro- and anti-longevity labelled nodes in entire networks and longevity networks. Statistically significant assortativity coefficient values were determined by label permutation tests (see Network permutation tests Methods section). To detect candidate key regulators of ARMLs among pro- or anti-longevity labelled nodes in longevity networks, statistically significant high numbers of neighbors with the opposite label were determined by node rewiring permutation tests (see Network permutation tests Methods section).

Inference of ancestral PPI networks

To infer ancestral PPI networks, species-specific networks of orthologs (Fig. S3B) derived from longevity networks (Fig. S3A) at all PPI stringency thresholds and the reference phylogenetic tree associated with these taxa (in the bracketed format: (*Sc*, (*Ce*, *Dm*), (*Mm*, *Hs*))) were used as input for an in-house script (available on GitHub:

https://github.com/TeamAIRE/ancestral_interactome_inference), to detect conserved protein-protein interaction (edges between the same pair of orthologous proteins, present in different species) and map these edges on the phylogeny of five species of Opisthokonta. For any given edge, all mapped extant taxa in which the protein-protein interaction is observed were given as input to the 'get_common_ancestor' function of the ete3 Python package version 3.1.1 (Huerta-Cepas et al. 2016) to conduct a parsimony analysis and identify the last common ancestor (returned as an intermediate node of the phylogenetic tree) in which the protein-protein interaction was likely present (Fig. S7). Next, this last common ancestor was used to define the root of a subtree, subsequently explored to define which of its children intermediate nodes have likely conserved the edge (at least one descendant extant taxon possessing the edge) or lost the edge (none of its descendant extant taxa possessing the edge). For each intermediate node of the phylogeny, all the edges inferred to be present were then used to reconstruct the corresponding ancestral PPI network. To estimate the false negative/positive rates associated to ancestral edge inferences, we used a resampling approach to construct alternative ancestral networks from thresholded entire networks, by replacing the input of either *Sc*, *Ce* and *Dm* networks by the PPI network of a species with the same taxonomic rank (family) in the NCBI reference phylogeny, with more than 85% of protein sequences being 100% identical between the OMA and STRING databases. Alternative species were, for *Ce*: *C. briggsae* (txid6238), *C. remanei* (txid31234); for *Dm*: *D. ananassae* (txid7217), *D. erecta* (txid7220), *D. grimshawi* (txid7222), *D. persimilis* (txid7234), *D. sechellia* (txid7238), *D. simulans* (txid7240), *D. virilis* (txid7244), *D. yakuba* (txid7245), *D. willistoni* (txid7260); for *Sc*: *K. lactis* (txid284590), *C. glabrata* (txid284593), *E. gossypii* (txid284811), *V. polyspora* (txid436907), *L. thermotolerans* (txid559295), *K. naganishii* (txid1071383). False negatives were defined as edges present in all alternative but absent from reference ancestral networks, and false positives as edges present in reference and absent in all alternative ancestral networks. False positive rates were 0 for all three resampled species, and false negative rates were comprised between *Sc*: 12 and 20%; *Ce*: 0.5 and 4%; *Dm*: 0 and 1.5%.

LAP prediction based on ancestral networks

To predict ancestral proteins with similar topological properties as inferred ancestral LAPs, inferred ancestral PPI networks were mined for orthogroups with similar roles. This prediction is of course a first step. We do not consider that when a non-LAP shares the centrality and conservation properties of one or several ageing- or longevity- associated proteins, this topological proximity is in itself a sufficient evidence that the non-LAP is also an ageing- or longevity-

associated protein, e.g. it may not be sufficient to share topological properties with an ageing-associated protein to be another, undetected, ageing-associated protein. Further validation of the prediction is also necessary. However, it is worth noting here that our predictions were very stringent.

Precisely, node label permutation tests were used to identify significantly high Jaccard indices combined with significantly high numbers of common direct neighbors, with a Jaccard index minimal threshold of 0.5 and at least 1 common neighbor. Species-specific LAP predictions were obtained from the non-LAPs with significant LAP neighborhood in at least one of the ancestral networks from the lineage of the focal species. As expected, this approach identified non-LAPs in a species that are homologous to LAPs in another species and figured by construction as non-LAPs in the ancestral networks, which reassuringly supported our guilt-by-association approach. These already documented LAPs, representing from 0 to 28 % of all predictions, depending on the species, were filtered out.

To further validate our approach of functional prediction by guilt-by-association within a PPI network, independently of the ageing-associated labels, we verified that the functional distributions of these protein families and that of the protein families to which they were structurally equivalent matched with one another. To compute the functional similarity between predicting and predicted proteins, each predicting and associated predicted orthogroups were translated to species-specific representative Ensembl identifiers, and the biomaRt R package was used to retrieve species-specific associated GO-terms from the Ensembl database. Semantic similarity analysis was performed using GOGO on pairs of proteins (predictor-predicted) and semantic similarity scores for each GO category were computed as described (Zhao and Wang 2018). Median semantic similarity for molecular function was found superior to 0.5, indicating good functional correspondence between predictors and predicted proteins and supporting the potential involvement of predicted proteins in regulating ageing and longevity (Figs. S14, S15).

Supporting information for a role as a novel candidate LAP in extant species was gathered using systematic queries to the Pubmed database, following the template: {*symbol of the predicted LAG*} AND {*species-specific keywords*} AND (ageing OR aging OR longevity OR lifespan OR "life span" OR senescence). Species-specific keywords were 'elegans' for *Ce*, 'drosophila' for *Dm*, 'human OR sapiens' for *Hs*, 'mouse OR musculus' for *Mm* and 'yeast OR cerevisiae' for *Sc*. Curated abstracts with mention of a potential link between the predicted LAP and longevity were

retained as supporting data. Further, predicted LAPs for the three species *Sc*, *Dm* and *Ce* were compared with the list of genes associated to lifespan and ageing alterations in genetic experiments recorded in the corresponding species-specific databases *Saccharomyces* Genome Database (yeastgenome.org), Flybase (flybase.org) and Wormbase (wormbase.org). *Sc* phenotypes queried were ‘aging’, ‘lifespan decreased’ and ‘lifespan increased’; *Dm* phenotypes queried were ‘aging’, ‘lifespan’, ‘abnormal aging’, ‘delayed aging’, ‘premature aging’, ‘short lived’ and ‘long lived’; *Ce* phenotypes queried were ‘dauer lifespan variant’ (WBPhenotype:0001540), ‘extended life span’ (WBPhenotype:0000061), ‘shortened life span’ (WBPhenotype:0001171) and ‘aging variant’ (WBPhenotype:0001739). To calculate enrichments in ageing-related annotations in our predictions relative to random sampling, the number of all known protein-coding genes was derived from the number of genes associated with and Uniprot reference identifier in Wormbase for *Ce*, and from Flybase statistics for *Dm*, and a chi-square test was used. *Hs* predicted LAPs were additionally probed for genes associated with human cell senescence in the CellAge database (<https://genomics.senescence.info/cells/>, (Avelar et al. 2020)), genes with alleles associated with exceptional human longevity in the LongevityMap database (<https://genomics.senescence.info/longevity/>, (Budovsky et al. 2013)), Aging-Related Disease (ARD) genes (Fernandes et al. 2016) and druggable proteins recorded in the DGIdb database (Griffith et al. 2013). We also determined from DGIdb the number of drugs, the number of US Food and Drug Administration (FDA)-approved drugs, and the maximum DGIdb interaction score associated with each predicted LAP in human.

Network permutation tests

To detect centrality outlier nodes or candidate key antagonistic regulators, node rewiring permutation tests (Fig. S4) were performed by 1000 random network permutations rewiring the nodes but preserving the total number of edges, without preserving the degree distribution. To detect statistically significant high values of assortativity, and significantly high values of Jaccard index and numbers of common direct neighbors, node label permutation tests (Fig. S4) were performed by randomly shuffling node labels 1000 times. For each node and each metric, a counter was incremented each time the random value was greater than the reference value. P-values were then calculated by the ratio counter/number of permutations, and adjusted for multiple testing using the Bonferroni method.

Functional enrichment analysis

Functional enrichment analysis was performed using the Metascape online tool (<https://metascape.org/gp/index.html>) (Zhou et al. 2019) with customized Enrichment tab settings to retrieve enriched Biological Processes GO terms only. For inferred ancestral networks, orthogroups were translated to their extant protein representative in the most annotated species for each lineage, according to the Gene Ontology statistics (<http://current.geneontology.org/products/pages/downloads.html>): *Hs* proteins for the common, bilaterian and euarchontoglires ancestors, and *Ce* proteins for the excidisozoan ancestor. Metascape analysis files were parsed to retrieve the genes annotated with the top 20 enriched GO terms and a Jaccard index was computed to compare gene sets for shared GO terms between networks.

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Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

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Figure legends

Figure 1. Analysis of the centrality of LAPs in PPI networks.

Matrix displaying pairwise comparisons (LAPs VS non-LAPs) of node centrality metrics distributions in the PPI networks from five Opisthokonta species (*S. cerevisiae*, *D. melanogaster*, *C. elegans*, *M. musculus* and *H. sapiens*) for six PPI stringency thresholds (from 500: less stringent networks to exp: more stringent networks). Stars in red cells indicate significantly higher centrality values for LAPs. Significance was determined using the unilateral Mann-Whitney U-test: **: $P < 0.001$; *: $P < 0.05$; NS: not significant (blue cells). P-values were adjusted for multiple testing using the Bonferroni method. This analysis shows that LAPs are more central than non-LAPs in PPI networks.

Figure 2. Homophily of LAPs in entire PPI networks and in longevity networks.

Homophily represents preferential interactions between similar kinds of nodes, and is estimated by assortativity coefficients. The distributions of assortativity coefficients were computed at six PPI stringency thresholds: (A) for LAPs and non-LAPs in the entire PPI networks of *S. cerevisiae*, *D. melanogaster*, *C. elegans*, *M. musculus* and *H. sapiens*; (B) for pro-longevity LAPs (pro-LAPs) and anti-longevity LAPs (anti-LAPs) in the entire PPI networks of *S. cerevisiae*, *D. melanogaster*, *C. elegans* and *M. musculus*; (C) for pro-LAPs and anti-LAPs in the longevity networks of *S. cerevisiae*, *D. melanogaster*, *C. elegans* and *M. musculus*. Assortativity coefficients were all significantly positive in node label permutation tests ($P < 0.05$), except in the *C. elegans* experimental longevity network (NS: not significant), indicating that proteins belonging to the same kind of LAPs (hence with the same effects on longevity) preferentially interact together.

Figure 3. Identification of candidate antagonistic regulatory mechanisms of longevity in longevity networks.

In longevity networks, some LAPs display a significantly high proportion of direct neighbours with an opposite effect on longevity (e.g. a pro-LAP being connected to significantly more anti-LAPs than expected by chance) and are therefore candidate regulators of longevity. Matrices of nodes with such a property for one or more PPI stringencies are indicated for three species: (A) *D. melanogaster*, (B) *C. elegans* and (C) *S. cerevisiae*. For each protein, the proportion of its neighbours with opposite effect on longevity is color-coded in cells, from blue: 0% to red: 100%.

Black cells indicate that the corresponding protein is absent from the network at the corresponding PPI stringency thresholds. The numbers of neighbors with opposite effects are indicated in the relevant cells only when significantly higher than by chance according to a node rewiring permutation test. Homolog outlier LAPs, i.e. LAPs found in multiple species, are indicated by a ‘-H + number’ homology code (as defined in Table S13) to the right of the protein names, highlighted in blue (when shared by two species) or yellow (when shared by three species), whereas species-specific outlier LAPs are indicated in bold. A star labels LAPs which were not found among centrality outliers in Fig. 4, indicating candidate regulators of longevity that are not highly central in the network. The white, light grey and dark grey bars on the left of each matrix further classify proteins depending on their outlier status, respectively, in both thresholded and experimentally-supported (exp) networks, in thresholded networks only, or in exp networks only. This figure shows that several species host evolutionary conserved LAPs highly connected to proteins with opposite effects on longevity/ageing.

Figure 4. Identification of the most central LAPs in longevity networks.

Some LAPs display a significantly high centrality in longevity networks, defined as possessing a significantly high value for at least two centrality metrics among betweenness, closeness, degree and PageRank. Matrices of nodes with such a property for one or more PPI stringencies are indicated for five species: (A) *S. cerevisiae*, (B) *C. elegans*, (C) *D. melanogaster*, (D) *M. musculus* and (E) *H. sapiens*. For each protein, the number of significantly high centrality metrics is color-coded in cells, from blue: 0-1 to red: 4. Homolog outlier LAPs found in multiple species are indicated by a ‘--H + number’ homology code (as defined in Table S13) to the right of the protein names, highlighted in blue (when present in four species) or yellow (when present in five species), whereas species-specific outlier LAPs are indicated in bold. The white, light grey and dark grey bars on the left of each matrix further classify proteins depending on their outlier status, respectively, in both thresholded and experimentally-supported (exp) networks, in thresholded networks only, or in exp networks only. This figure shows that several species host evolutionary conserved highly central LAPs in their longevity networks.

Figure 5. Inference of ancestral interactions based on shared LAP-LAP interactions between longevity networks.

The longevity networks of orthologs from *S. cerevisiae* (*Sc*), *C. elegans* (*Ce*), *D. melanogaster* (*Dm*), *M. musculus* (*Mm*) and *H. sapiens* (*Hs*) were used to infer ancestral networks at inner branches of the species phylogeny (as described in Figure S7) from ortholog-ortholog interactions shared between species. The resulting ancestral networks are shown here for PPI stringency threshold = 500. In these ancestral networks, node and edge colours indicate inferred presence in the last common ancestor of all 5 species (blue, in the last common ancestor of Opisthokonta), of all 4 bilaterian species (pink, in the bilaterian ancestor), of *Hs* and *Mm* (yellow, in the euarchontoglires ancestor) or of *Dm* and *Ce* (green, in the ecdisozoan ancestor). Extant longevity networks and proportions of ancestral nodes and edges in these networks are displayed to the right of the species phylogeny. These extant networks represent the interactions between LAPs belonging to shared orthogroups for each species. Nodes and edges in extant networks are coloured based on their inferred phylogenetic date of appearance, or grey if only present in the corresponding species. This figure shows that each extant longevity network contains evolutionarily conserved interactions, some of which as old as the last common ancestor of Opisthokonta.

Figure 6. Functional enrichment analysis of ancestral longevity networks.

To perform functional enrichment analysis of the proteic interactions inferred to be present in ancestral networks, ancestral orthogroups identified at each PPI stringency were analyzed using Metascape. *H. sapiens* protein identifiers were used as Metascape input to represent the functions of orthogroups found in common, bilaterian and euarchontoglires ancestral networks, and *C. elegans* protein identifiers were used as Metascape input to represent the functions of orthogroups found in ecdisozoan ancestral networks. On the species phylogeny, the top 20 enriched GO-terms identified by Metascape for ancestral proteins are positioned at the inner branches, and the top 20 enriched GO-terms for extant proteins (orthogroups found in extant longevity networks) are positioned at the leaves. Identical enriched GO-terms between ancestors or between ancestor and extant species are highlighted with the color corresponding to the oldest ancestor implementing the function (blue: for the last common ancestor of Opisthokonta; pink: for the bilaterian ancestor; yellow: for the euarchontoglires ancestor; green: for the ecdisozoan ancestor). Colored boxes surround GO-terms absent from ancestral networks but identical between sister species, suggesting that the same functions are used by closely related species to regulate longevity. This figure shows that the main enriched functions associated with longevity/ageing regulation have changed with the history of species.

Figure 7. Druggable human predicted LAPs.

We predicted 138 novel human LAPs, most with known drug interactions recorded in the DGIdb database. Predicted LAPs are ordered from left to right by their inferred age of first appearance (as old as the last common ancestor of Opisthokonta, as old as the Bilaterian ancestor, as old as the euarchontoglires ancestor) and by decreasing average centrality (average degree rank) in the corresponding ancestral longevity networks. A bar plot indicates the number of drugs linked to each of the 138 predicted human LAPs, with the number of FDA-approved drugs, when available, on top of each bar. Below the bar plot, a matrix displays support (purple cells) for a LAP function from (P) the literature in Pubmed abstracts, (C) the cellular senescence database CellAge or (L) GWAS data providing an association with longevity from the LongevityMap database. (A) indicates whether the LAP is associated to an Aging-Related Disease (ARD). This figure reports predicted evolutionary conserved human longevity-associated proteins, currently known to be targeted by drugs.

Figure 1

		LAPs compared to non-LAPs					
		PPI stringency					
		500	600	700	800	900	exp
<i>S. cerevisiae</i>	Betweenness	**	**	**	**	**	**
	Closeness	**	**	**	**	**	**
	Degree	**	**	**	**	**	**
	PageRank	**	**	**	**	**	**
<i>D. melanogaster</i>	Betweenness	**	**	**	**	**	NS
	Closeness	**	**	**	**	NS	NS
	Degree	**	**	**	**	NS	NS
	PageRank	**	**	**	*	NS	NS
<i>C. elegans</i>	Betweenness	**	**	**	**	**	**
	Closeness	**	**	**	**	*	NS
	Degree	**	**	**	**	**	NS
	PageRank	**	**	**	**	**	**
<i>M. musculus</i>	Betweenness	**	**	**	**	**	**
	Closeness	**	**	**	**	**	**
	Degree	**	**	**	**	*	**
	PageRank	**	**	**	**	**	**
<i>H. sapiens</i>	Betweenness	**	**	**	**	**	**
	Closeness	**	**	**	**	**	**
	Degree	**	**	**	**	**	**
	PageRank	**	**	**	**	**	**

Figure 2

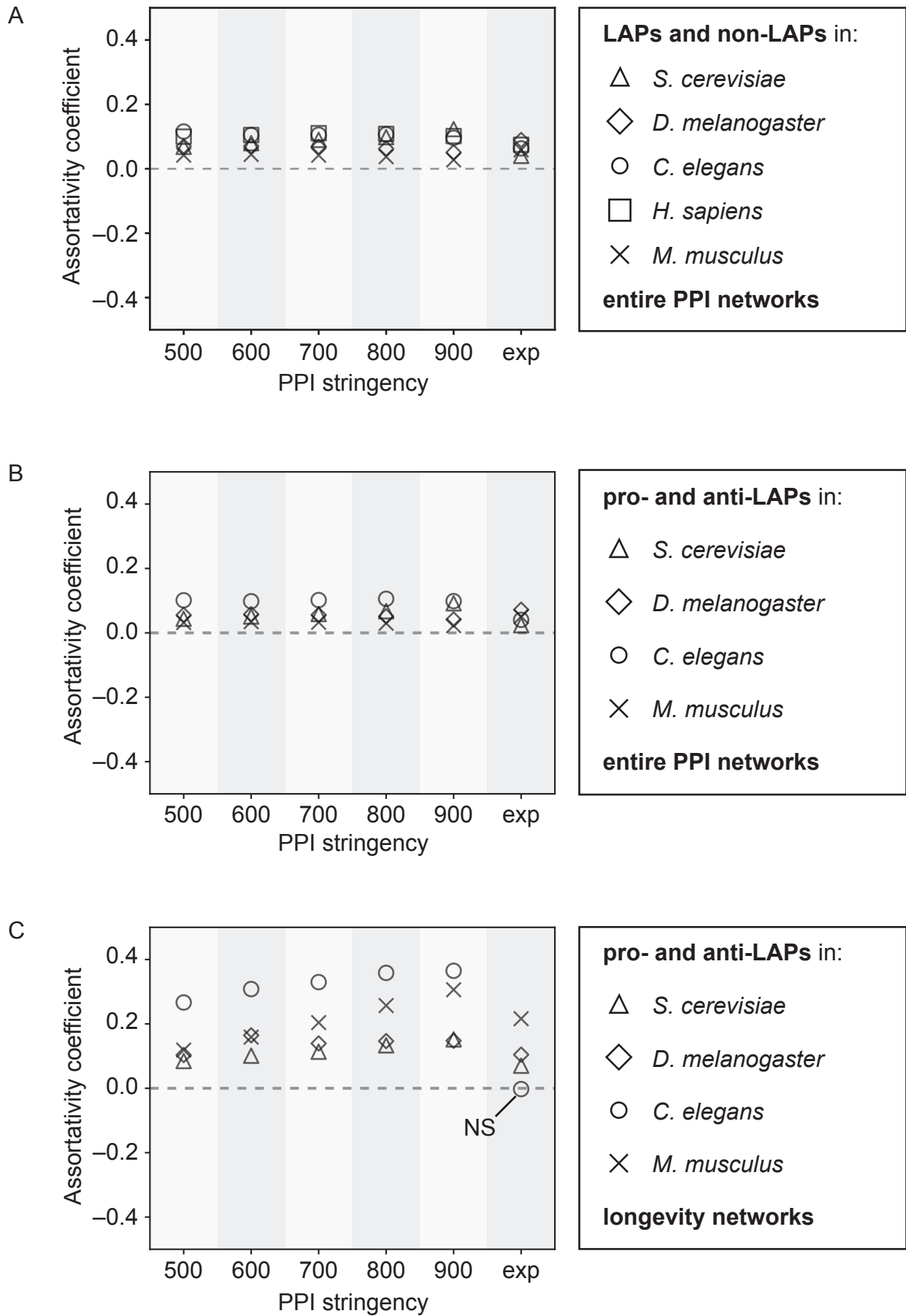
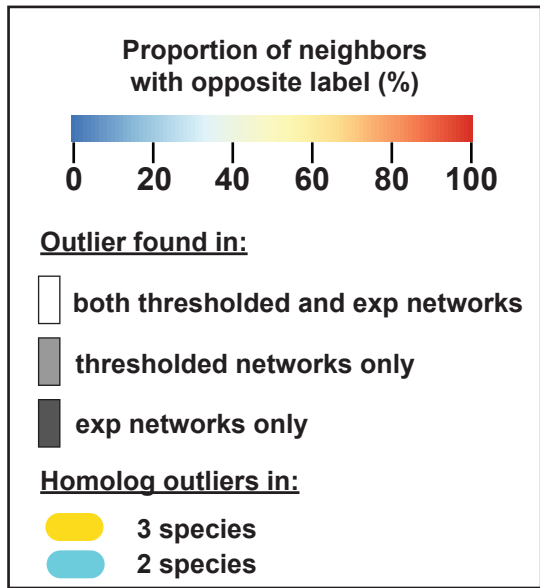
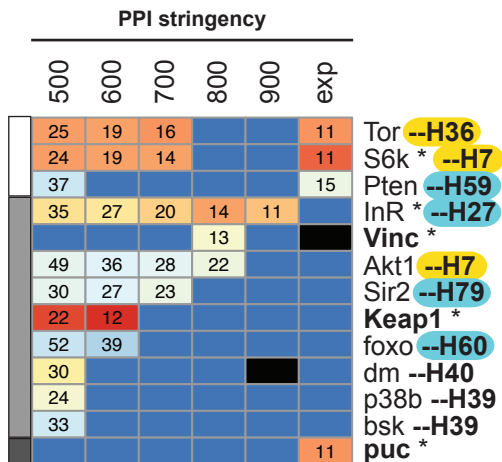


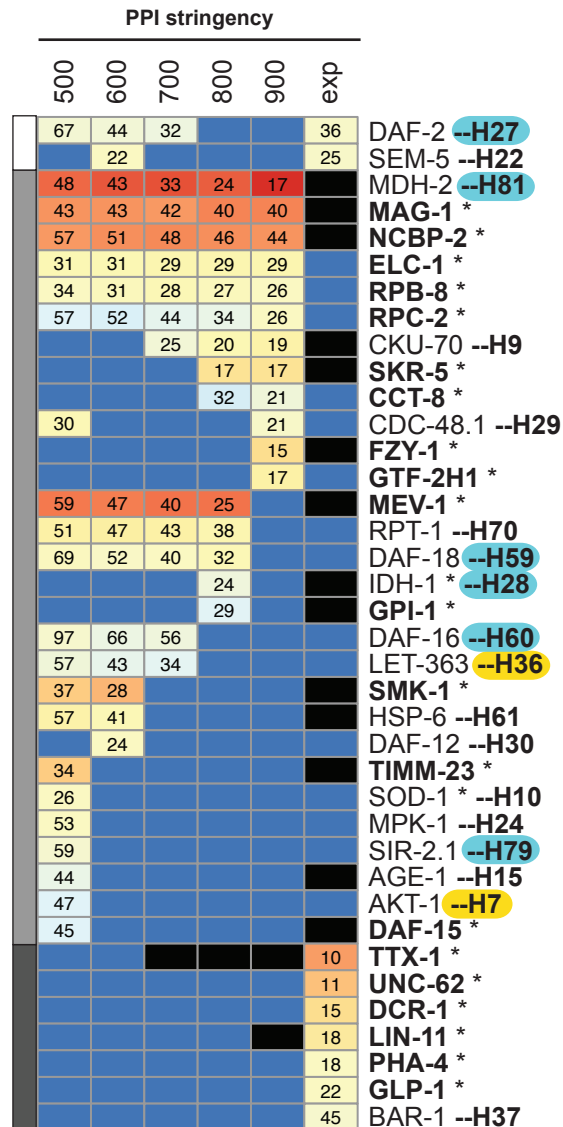
Figure 3



A



B



C

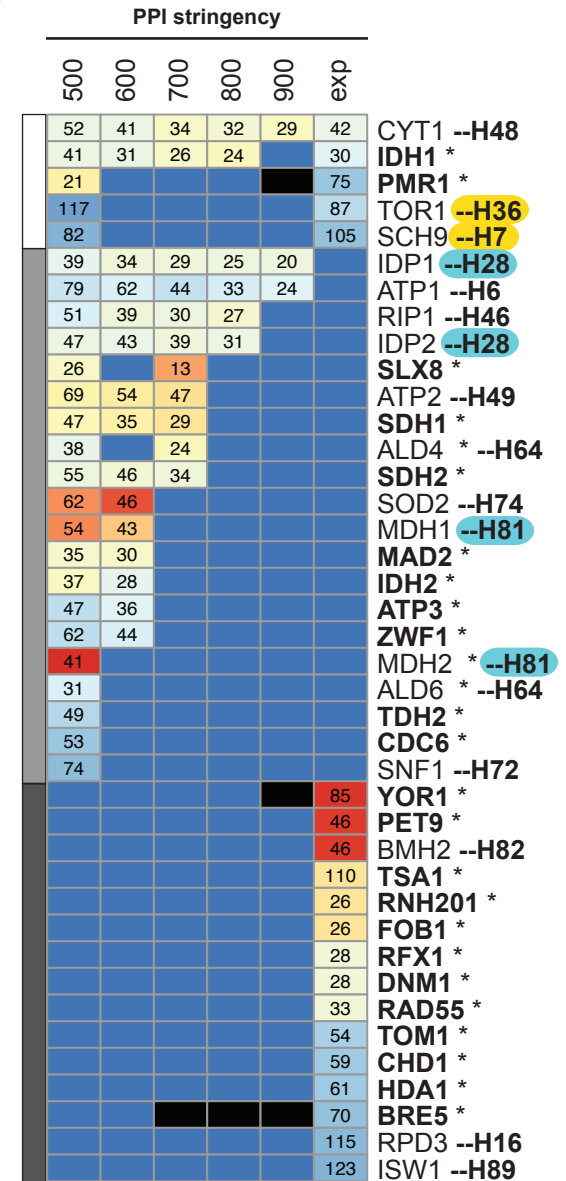
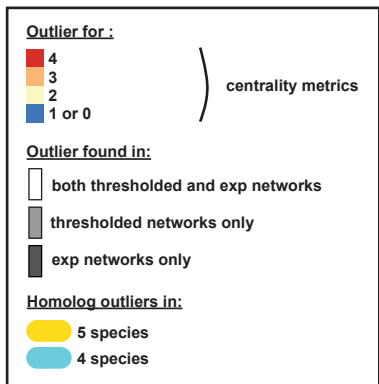
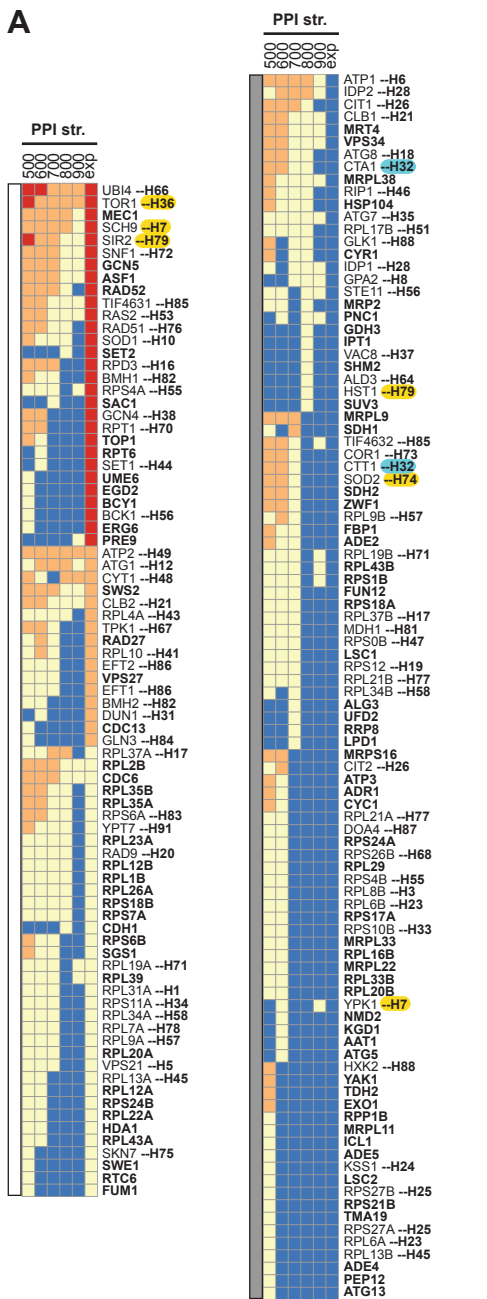


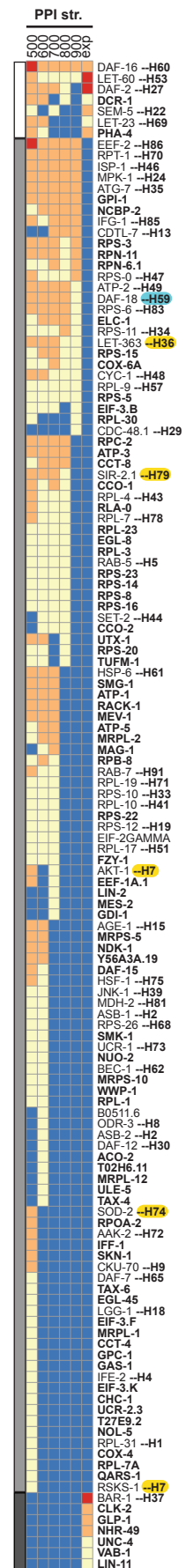
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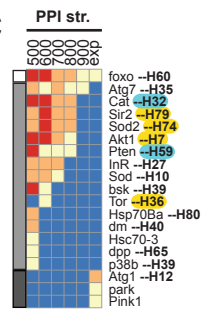
A



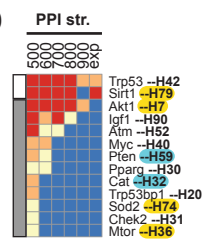
B



C



D



E

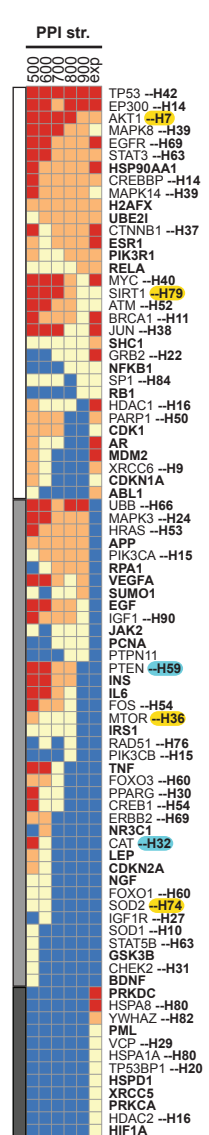


Figure 5

Proportion of ancestral
nodes / edges

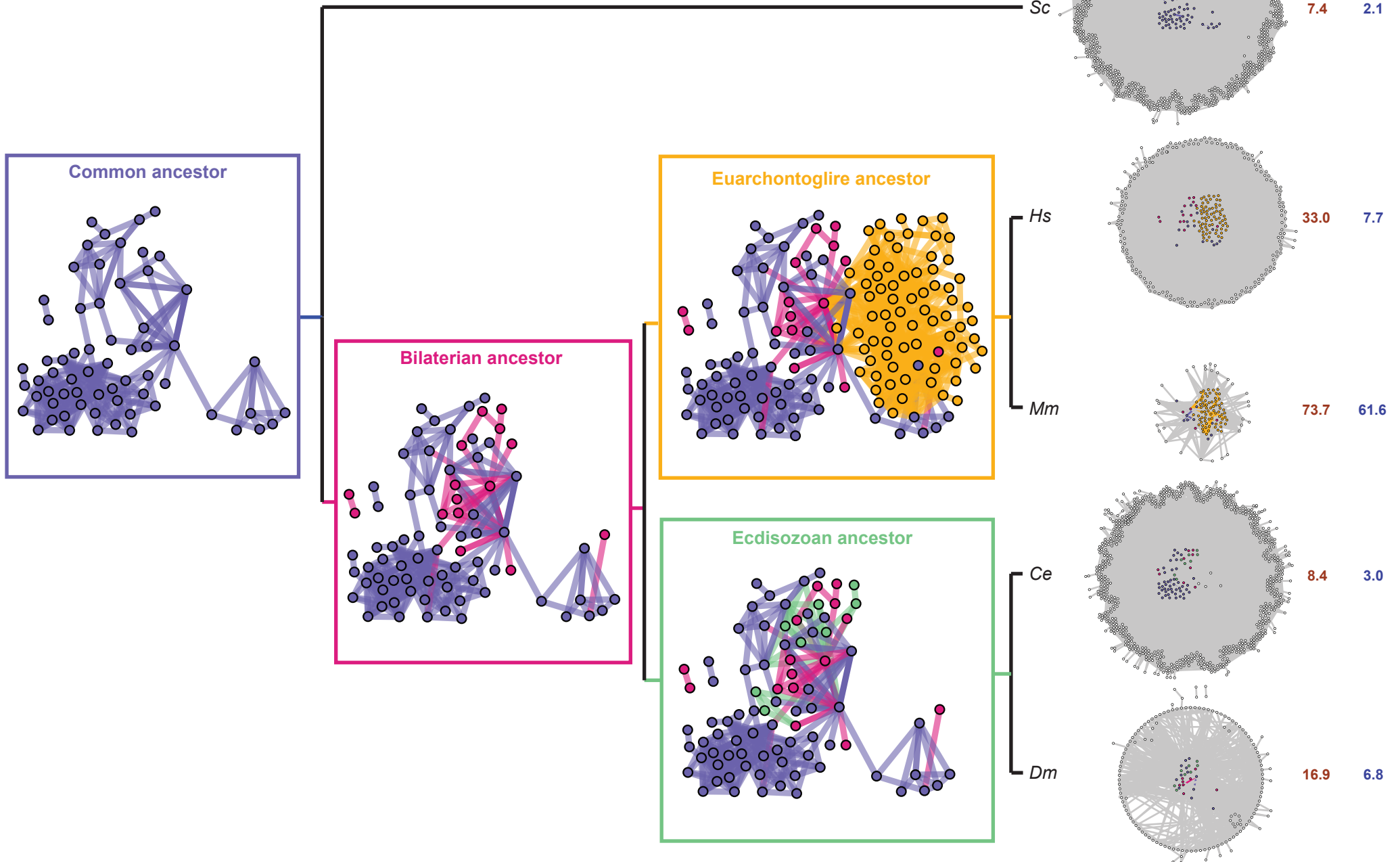


Figure 6

- Common ancestor**
- GO:0006091: generation of precursor metabolites and energy
 - GO:0043603: cellular amide metabolic process
 - GO:0062197: cellular response to chemical stress
 - GO:0007005: mitochondrion organization
 - GO:0006790: sulfur compound metabolic process
 - GO:0042743: hydrogen peroxide metabolic process
 - GO:0005975: carbohydrate metabolic process
 - GO:0002262: myeloid cell homeostasis
 - GO:0010506: regulation of autophagy
 - GO:0010332: response to gamma radiation
 - GO:0016570: histone modification
 - GO:0009725: response to hormone
 - GO:0001649: osteoblast differentiation
 - GO:0002639: positive regulation of immunoglobulin production
 - GO:0097190: apoptotic signaling pathway
 - GO:1901699: cellular response to nitrogen compound
 - GO:0021766: hippocampus development
 - GO:0019216: regulation of lipid metabolic process
 - GO:0048660: regulation of smooth muscle cell proliferation
 - GO:1903169: regulation of calcium ion transmembrane transport

- Bilaterian ancestor**
- GO:0015980: energy derivation by oxidation of organic compounds
 - GO:0062197: cellular response to chemical stress
 - GO:0043603: cellular amide metabolic process
 - GO:0006099: tricarboxylic acid cycle
 - GO:0009725: response to hormone
 - GO:0031667: response to nutrient levels
 - GO:0006790: sulfur compound metabolic process
 - GO:0007005: mitochondrion organization
 - GO:0010212: response to ionizing radiation
 - GO:0018105: peptidyl-serine phosphorylation
 - GO:0019725: cellular homeostasis
 - GO:2000377: regulation of reactive oxygen species metabolic process
 - GO:0010506: regulation of autophagy
 - GO:0055093: response to hyperoxia
 - GO:0005975: carbohydrate metabolic process
 - GO:0009408: response to heat
 - GO:0001775: cell activation
 - GO:0009896: positive regulation of catabolic process
 - GO:0031400: negative regulation of protein modification process
 - GO:0016570: histone modification

- Euarchoptoglires ancestor**
- GO:0006974: cellular response to DNA damage stimulus
 - GO:0071417: cellular response to organonitrogen compound
 - GO:0031667: response to nutrient levels
 - GO:2001233: regulation of apoptotic signaling pathway
 - GO:0010564: regulation of cell cycle process
 - GO:0006979: response to oxidative stress
 - GO:0043549: regulation of kinase activity
 - GO:1901214: regulation of neuron death
 - GO:0009411: response to UV
 - GO:0031331: positive regulation of cellular catabolic process
 - GO:0040008: regulation of growth
 - GO:0090398: cellular senescence
 - GO:0048732: gland development
 - GO:0097193: intrinsic apoptotic signaling pathway
 - GO:0046324: regulation of glucose import
 - GO:0006468: protein phosphorylation
 - GO:0042593: glucose homeostasis
 - GO:0032200: telomere organization
 - GO:0019216: regulation of lipid metabolic process
 - GO:0010821: regulation of mitochondrion organization

- Ecdisozoan ancestor**
- GO:0008340: determination of adult lifespan
 - GO:0009060: aerobic respiration
 - GO:0044271: cellular nitrogen compound biosynthetic process
 - GO:0000422: autophagy of mitochondrion
 - GO:0046034: ATP metabolic process
 - GO:0006979: response to oxidative stress
 - GO:0002119: nematode larval development
 - GO:0009792: embryo development ending in birth or egg hatching
 - GO:0050793: regulation of developmental process
 - GO:0051128: regulation of cellular component organization
 - GO:0009605: response to external stimulus
 - GO:0033554: cellular response to stress
 - GO:0032879: regulation of localization
 - GO:0019725: cellular homeostasis
 - GO:0009893: positive regulation of metabolic process
 - GO:0010942: positive regulation of cell death

- Sc**
- GO:0034727: piecemeal microautophagy of the nucleus
 - GO:0048519: negative regulation of biological process
 - GO:0031497: chromatin assembly
 - GO:0006099: tricarboxylic acid cycle
 - GO:0006464: cellular protein modification process
 - GO:0051128: regulation of cellular component organization
 - GO:0007154: cell communication
 - GO:1901293: nucleoside phosphate biosynthetic process
 - GO:0051726: regulation of cell cycle
 - GO:0002181: cytoplasmic translation
 - GO:0010737: protein kinase A signaling
 - GO:0072350: tricarboxylic acid metabolic process
 - GO:0031399: regulation of protein modification process
 - GO:0044087: regulation of cellular component biogenesis
 - GO:0051053: negative regulation of DNA metabolic process
 - GO:0051090: regulation of DNA-binding transcription factor activity
 - GO:0010906: regulation of glucose metabolic process
 - GO:0140053: mitochondrial gene expression
 - GO:0090033: positive regulation of filamentous growth
 - GO:0007005: mitochondrion organization

- Hs**
- GO:0006979: response to oxidative stress
 - GO:0009725: response to hormone
 - GO:0006974: cellular response to DNA damage stimulus
 - GO:0080135: regulation of cellular response to stress
 - GO:0009314: response to radiation
 - GO:2001233: regulation of apoptotic signaling pathway
 - GO:0010035: response to inorganic substance
 - GO:0001934: positive regulation of protein phosphorylation
 - GO:0002520: immune system development
 - GO:0009991: response to extracellular stimulus
 - GO:00010942: positive regulation of cell death
 - GO:0007167: enzyme-linked receptor protein signaling pathway
 - GO:0097190: apoptotic signaling pathway
 - GO:0048732: gland development
 - GO:0040008: regulation of growth
 - GO:1901214: regulation of neuron death
 - GO:0010564: regulation of cell cycle process
 - GO:0006468: protein phosphorylation
 - GO:0031331: positive regulation of cellular catabolic process
 - GO:0051098: regulation of binding

- Mm**
- GO:0006974: cellular response to DNA damage stimulus
 - GO:1901653: cellular response to peptide
 - GO:0008630: intrinsic apoptotic signaling pathway in response to DNA damage
 - GO:0048638: regulation of developmental growth
 - GO:0062012: regulation of small molecule metabolic process
 - GO:2001233: regulation of apoptotic signaling pathway
 - GO:0048660: regulation of smooth muscle cell proliferation
 - GO:0007568: aging
 - GO:0007346: regulation of mitotic cell cycle
 - GO:0001934: positive regulation of protein phosphorylation
 - GO:0043086: negative regulation of catalytic activity
 - GO:0006979: response to oxidative stress
 - GO:00010942: positive regulation of cell death
 - GO:0010817: regulation of hormone levels
 - GO:0048145: regulation of fibroblast proliferation
 - GO:0010638: positive regulation of organelle organization
 - GO:0014070: response to organic cyclic compound
 - GO:0071363: cellular response to growth factor stimulus
 - GO:0048732: gland development
 - GO:0032204: regulation of telomere maintenance

- Ce**
- GO:0008340: determination of adult lifespan
 - GO:0050793: regulation of developmental process
 - GO:0002119: nematode larval development
 - GO:0009893: positive regulation of metabolic process
 - GO:0044271: cellular nitrogen compound biosynthetic process
 - GO:0051128: regulation of cellular component organization
 - GO:0061062: regulation of nematode larval development
 - GO:0009628: response to abiotic stimulus
 - GO:0048609: multicellular organismal reproductive process
 - GO:0022611: dormancy process
 - GO:0070887: cellular response to chemical stimulus
 - GO:0009792: embryo development ending in birth or egg hatching
 - GO:0009605: response to external stimulus
 - GO:0046034: ATP metabolic process
 - GO:0048583: regulation of response to stimulus
 - GO:0048523: negative regulation of cellular process
 - GO:0010506: regulation of autophagy
 - GO:0061063: positive regulation of nematode larval development
 - GO:0065008: regulation of biological quality
 - GO:0071495: cellular response to endogenous stimulus

- Dm**
- GO:0008340: determination of adult lifespan
 - GO:0006979: response to oxidative stress
 - GO:1901700: response to oxygen-containing compound
 - GO:0031667: response to nutrient levels
 - GO:0010506: regulation of autophagy
 - GO:0051128: regulation of cellular component organization
 - GO:0034539: cellular response to oxidative stress
 - GO:0043467: regulation of generation of precursor metabolites and energy
 - GO:0009628: response to abiotic stimulus
 - GO:0033500: carbohydrate homeostasis
 - GO:0007610: behavior
 - GO:0007552: metamorphosis
 - GO:0031175: neuron projection development
 - GO:0031399: regulation of protein modification process
 - GO:0006091: generation of precursor metabolites and energy
 - GO:0002682: regulation of immune system process
 - GO:0042981: regulation of apoptotic process
 - GO:006796: phosphate-containing compound metabolic process
 - GO:1902531: regulation of intracellular signal transduction
 - GO:0040007: growth

Figure 7

