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Review

Redundancy and divergence in the amyloid precursor protein family

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ABSTRACT

Gene duplication provides genetic material required for functional diversification. An interesting example is the amyloid precursor protein (APP) protein family. The APP gene family has experienced both expansion and contraction during evolution. The three mammalian members have been studied quite extensively in combined knock out models. The underlying assumption is that APP, amyloid precursor like protein 1 and 2 (APLP1, APLP2) are functionally redundant. This assumption is primarily supported by the similarities in biochemical processing of APP and APLPs and on the fact that the different APP genes appear to genetically interact at the level of the phenotype in combined knockout mice. However, unique features in each member of the APP family possibly contribute to specification of their function. In the current review, we discuss the evolution and the biology of the APP protein family with special attention to the distinct properties of each homologue. We propose that the functions of APP, APLP1 and APLP2 have diverged after duplication to contribute distinctly to different neuronal events. Our analysis reveals that APLP2 is significantly diverged from APP and APLP1.

1. Introduction

Amyloid β peptide is the main constituent of the amyloid plaques in Alzheimer patients. Amyloid precursor protein (APP) is the precursor protein from which the Aβ peptide is generated. This peptide is produced by endoproteolytic cleavages of APP, which in addition shed a larger soluble ectodomain in the extracellular environment and an intracellular domain into the cytoplasm [1,2]. The proteolytic processing of APP is a constitutive process, and explains in part the relative short half life (less than an hour) of full length APP [3]. Unbalanced proteolytic cleavage of APP or mutations in the Aβ sequence can result in increased production, and mainly in alterations of the biophysical properties of Aβ. Consequently, oligomerization and aggregation of Aβ can contribute to the brain pathology and neurodegeneration in familial and sporadic Alzheimer Disease [2]. In contrast, our knowledge of the physiological function of APP remains surprisingly incomplete. Although the loss of APP and its homologues were studied in several model organisms, no clear picture has yet emerged. Sometimes, the protein is called "All Purpose Protein" to indicate the many different signaling pathways and protein interactions in which APP has been implicated. The different proposed functions for APP are not always consistent. For instance both enhancement and inhibition of dendritic spine formation [4–6] or neuronal cell migration [7,8] have been proposed to be mediated by APP.

Next to APP, APP-like proteins are present in different species. Similar to APP, APP-like proteins (APLP) undergo proteolytic processing [9]. Furthermore, mutant mice lacking *Aplp2* combined with *App* or *Aplp1* display a lethal phenotype, with mice dying around birth [10]. The genetic interactions of the *App* and *App-like* genes and the similarity in proteolytic processing have been taken as evidence for functional redundancy of the three *App* paralogues. Therefore, experiments to deduce the biological function of APP are mainly based on the "redundancy model" which assumes that the *App* paralogues are functionally interchangeable. Such an approach pays too little attention to the unique properties of each *App* paralogue and might disregard the possibility that they are operating in different and independent pathways. In such a view, their combined mutations lead to a "synthetic phenotype" (lethality) by

Abbreviations: APP, amyloid precursor protein; APLP, amyloid precursor like protein; APBA1, precursor protein binding, family A; Arc, member 1, activity-regulated cytoskeleton-associated protein; ApoeR2, apolipoprotein E receptor 2; CR, Cajal-Retzius; CASK, calcium/calmodulin-dependent serine protein kinase; CP, cortical plate; DAB1, Disabled-1; dko, double knockout; GFAP, glial fibrillary acidic protein; FOS, FBJ murine osteosarcoma viral oncogene homolog; ko, Knockout; LTP, long term potentiation; MAP2, Microtubule-associated protein 2; PCP, planar polarity pathway; KCNH6, member 6; SVZ, Subventricular zone; VZ, ventricular zone; Vldlr, very low density lipoprotein receptor; WNT5A, Wingless-type MMTV integration site family, member 5A

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Table 1
The species and protein sequences used for functional divergence analysis.

Organism	Accession number	Gene name
Homo sapiens (Human)	NP_000475.1	APP
	NP_001019978.1	APLP1
	NP_001135748.1	APLP2
Pan troglodytes (Chimps)	NP_001013036.1	APP
	XP_003316372.1	APLP1
	XP_001155401.1	APLP2
Canis lupus familiaris (Dog)	NP_001006601.1	APP
	XP_533688.4	APLP1
	XP_536530.2	APLP2
Mus musculus (Mouse)	NP_001185752.1	APP
	NP_031493.2	APLP1
	NP_001095925.1	APLP2
Gallus gallus (Chicken)	NP_989639.1	APP
	NP_001006317.2	APLP2
Danio rerio (Fish)	NP_571639.1	APPa
	NP_690842.1	APPb
	XP_001342921.4	APLP
	NP_690842.1	APLP2
Xenopus laevis (Frog)	NP_001082098.1	APP
	NP_001089419.1	APLP1
	NP_001094408.2	APLP2a
	NP_001094407.1	APLP2b
Drosophila melanogaster (Fly)	NP_001245451.1	APPL
Caenorhabditis elegans (Worm)	NP_508871.1	APL1

affecting distinct pathways. This also implies that *App* paralogues are not simply extra copies but have evolved to perform specialized function.

We structure our review on the divergence of APP function by asking following questions: What are the possible evolutionary fates of duplicated APP homologues? What does the loss of function studies tell us about the specialization of APP family proteins? What are the similarities and differences in processing of APP and APLPs? How does transcriptional and interaction network divergences contribute to the evolution of the APP family? Finally, we provide support for the "divergence" idea by using a computational method to predict critical amino acid and sub-domains that potentially contribute to the divergence of the APP protein family. Based on this comparison, it will become clear that the different APP genes do not simply encode duplicated proteins with interchangeable function. This should bring the focus back on the unique properties of each member of the APP family and might help to explain some of the discrepancies in the field.

2. The APP family

Genes encoding for the APP protein family have experienced several twists and turns during evolution (Table 1 lists all the species and proteins discussed in this review). APP-like proteins have not been identified in prokaryotes, yeasts and plants (Fig. 1). The simplest and earliest branches of the evolutionary tree in which *APP*-like genes have been identified contain insects such as the fruit fly (*Drosophila melanogaster*) and roundworms (*Caenorhabditis elegans*) each carrying one gene encoding for an APP-like protein. It is intriguing that APP-like proteins first emerge in *Bilaterians* with an early nervous system with functional synapses [11,12]. Indeed, the extracellular domains of APP molecules have cell adhesion properties and can promote cell-cell adhesion [13]. Such intercellular interaction is important in early evolution for the generation of the synaptic junction [12,14]. Strikingly, when overexpressed in HEK cells, APP can potently induce synaptogenesis in the contacting axon and this activity requires the extracellular domain as well as the intracellular part of APP. The later associates with presynaptic molecules such as APP binding family A (APBA1) and Calcium/calmodulin-dependent serine protein kinase (CASK) [15]. Interestingly, APP is required both at pre- and postsynaptic compartments to induce synaptogenesis [15] which suggests that ancestral APP indeed might be a transmembrane protein responsible for homophilic interactions at the synaptic junction early in evolution.

Five nodes of duplications are observed in the phylogenetic tree of the APP protein family when using Ensemble comparative genomics tools (schematically represented in Fig. 1). For example, fishes (*Danio rerio*) have in total four genes encoding APP proteins: two homologues for the human *APP* gene (*appa* and *appb*) plus *aplp1* and *aplp2* (Fig. 1). Similar to fishes, amphibians (*Xenopus laevis*) carry four *app* genes in their genome but they have two homologues for the human *APLP2* gene: *aplp2a*, *aplp2b* plus *app* and *aplp1* (Fig. 1). Instead, birds (*Gallus gallus*) have lost the *APLP1* gene leaving them with *APP* and *APLP2* genes (Fig. 1). The paradoxical expansion and contraction of the APP family suggest that the duplications of the encoding genes have been the subject of highly selective evolutionary forces. The complicated trajectory of the evolution of the APP protein family ends with the three best-studied members in mammals: *APP*, *APLP1* and *APLP2* (Fig. 1) [16].

The evolutionary maintenance of a duplicated gene in the genome is influenced by the accumulation of genetic mutations affecting the function of the descendant duplicates. Three possible

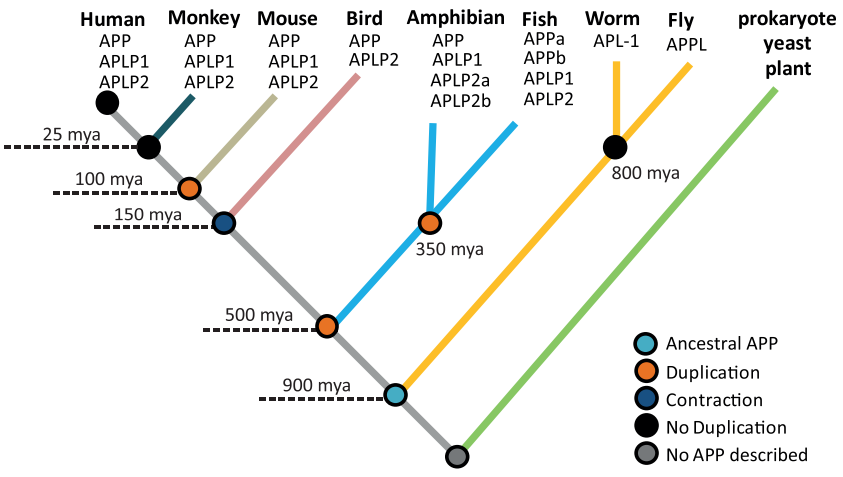

Fig. 1. A simplified dendrogram based on APP protein family tree of Ensemble illustrates the important events in the evolution of APP gene family. The duplication and contraction nodes are color coded. The lengths of the lines are not proportional to the evolutionary distance of species. The scientific names of species are listed in the Table 1. For details of APP protein family evolution see the text. Mya: million years ago.

outcomes of duplication have been proposed: non-functionalization, neo(sub)functionalization or increased gene dosage [17]. The non-functionalization scenario is the result of the accumulation of deleterious mutations leading to pseudogenization [18]. In case of neo-functionalization, mutations confer new features to the duplicate which leads to the acquisition of new functions distinct from the ancestral ones [19]. Sub-functionalization is a modified version of neo-functionalization in which the function of the ancestral gene becomes subdivided into sub-functions for each duplicate providing cells with proteins with more specialized functions [17]. Finally without any functional innovation, duplication can provide cells with genetic robustness and redundancy by increasing the gene dosage for dosage sensitive genes [20].

Which model of gene duplication and evolution can be applied to the APP family? While the prevalent vision stresses the "increased gene dosage", more in depth interpretation of the data provide supporting evidence for neo- or sub-functionalization of APP and APLPs as well. It should be noted that differential splicing of APP can contribute to functional diversity as well by for example changing the adhesion properties of APP or APLPs [21]. Discussion on the role of alternative splicing in the functional divergence of the APP protein family is however beyond the scope of this review.

3. Various roles of the APP members in the nervous system of different species

Loss of function studies are still the standard approach to deduce the physiological role of a gene. The APP family has been covered rather well in that regard with gene inactivation or knock outs (ko) in *D. melanogaster*, *C. elegans* and several combinations of gene ko in *Mus musculus*. The data are somewhat divergent, but overall they suggest strongly a role for the *APP* family in the central nervous system.

3.1. The importance of the extracellular APP domain in the development of C. elegans

In *C. elegans,* a single gene encodes for a member of the *APP* family which is called *apl-1*. The encoded protein is very similar to the mammalian counterpart with a large extracellular and a small intracellular domain, while the Aβ sequence as such is lacking in APL-1 [22]. Loss of APL-1 leads to a molting defect resulting in developmental lethality [23]. In addition, the a*pl-1* null mutant worms are hypersensitive to the acetylcholinesterase inhibitor aldicarb supporting a role for APL-1 protein at the synaptic junctions [24]. Many reports stress the importance of the conserved intracellular domains of the APP family for its function, but, unexpectedly, the conserved carboxyl terminus fragment of APL-1 is not involved in the phenotype as demonstrated by rescuing the lethality of the *apl-1* null mutant by the c-terminus truncated version of APL-1 [23]. In contrast, the extracellular domain of APL-1 is sufficient to rescue both the lethality and hypersensitivity phenotypes. As this domain is soluble, these data suggest a receptor for APL-1 ectodomain, and indicate the importance of this domain in development.

3.2. The single Appl gene in D. melanogaster is involved in axonal wiring and synaptic function

Like *C. elegans, D. melanogaster* also carries one homologue for the *APP* gene that is called *Appl*. *Appl* expression is first seen in developing neurons during axogenesis [25]. Flies with an *Appl* null mutation are viable and fertile, but show subtle phenotypes. For instance at the neuromuscular junction *Appl* null mutant flies have a reduced number of neuromuscular buttons, whereas larvae overexpressing APPL show an increased number of buttons [26]. Interestingly, Torroja et al. showed that APPL is transported to synaptic buttons and a highly conserved cytoplasmic YENPTY motif of APPL is required for promoting synapse formation [26]. This synaptogenic property might be mediated through interaction of Fasciclin II with APPL, while APPL is binding via its conserved cytoplasmic domain to dX11/Mint at the synapse [27]. APPL has also been implicated in regulation of neurite arborization [28]. Leyssen et al [28] showed that both APPL and its human homologue APP can promote post-developmental neurite arborization. Similar to its synaptogenic role, APPL requires the conserved cytoplasmic YENPTY for its effects on neurite arborization, but this time the signal is transduced through the Abelson tyrosine kinase (Abl) pathway [28]. These data suggest a role for APP in the structural plasticity of neurons, whereas in pathological conditions such as brain injury, APPL might promote neurite arborization [28]. Recently, loss of APPL was shown to induce a developmental defect in axonal outgrowth in mushroom bodies, a *D. melanogaster* center for learning and memory. Heterozygosity for *Abl kinase* significantly enhanced the axonal phenotype of *Appl* mutant flies. Mechanistically, APPL turned out to interact with core components of the planar polarity pathway (PCP) mediating the Wingless-type MMTV integration site family, member 5A (WNT5a) induced phosphorylation of Disheveled. Thus, it was suggested that modulation of the PCP pathway by neuronal APPL might regulate developmental axonal wiring in mushroom bodies [29]. Overall, the loss of function experiments in flies and worms suggest that the ancestral *App*-like gene has evolved to serve in the nervous system, in particular in axonal outgrowth and synapse formation. While the *Drosophila* counterpart is really a nervous system protein, the situation in worms is not completely clear. Absence of APL-1 results in multiple developmental defects, for instance decreased body size and egg-laying rate [30]. It is uncertain whether these phenotypes are the result of defects in the neuronal system or indicate that APL-1 also operates in other cells, and that its function is therefore cellular context-dependent.

3.3. The APP family in M. musculus

The situation in mammals is even more complex. The different functions proposed for APP and its paralogues, are not converging to a concrete model for APP family function. The single *App* KO mice are viable but show various subtle phenotypes such as 15–20% reduced body weight, disturbed forelimb strength and reduced locomotor activity [31]. The interpretation that *App* ko mice show subtle phenotypes because of compensation by other App members, is not supported by expression studies of the other members of the App family: compensatory up-regulation of *Aplp1* and *Aplp2* were not observed in these mice [31].

The alterations in muscular strength and decreased locomotor activity in the *App* null mutant mice might reflect the synaptic role of App in the central nervous system. Immunocytochemical analysis of *App* null mice revealed age-dependent reduced staining for synaptic markers such as synaptophysin, synapsin and microtubule-associated protein 2 (MAP2) and increased glial fibrillary acidic protein (Gfap) immunoreactivity indicating gliosis [32]. In addition, the mice showed impaired long term potentiation (LTP) recordings which was highly correlated with gliosis [32]. Consistent with a defect in LTP, *App* null mutants mice spend more time finding the hidden platform in the Morris water maze test [32] further suggesting a role for App in spatial learning. The defect in LTP was associated with attenuation of GABA-mediated inhibitory post-synaptic currents [33]. Increased expression of calcium channel, Cav1.2, was suggested as a potential mechanism regulating GABAergic synaptic activity in inhibitory neurons [34]. Further

experiments will clarify the role of App in synaptic plasticity of excitatory vs. inhibitory neurons.

Mixed results were obtained on the role of APP in the formation of dendritic spines [5,6,35]. Bitnner et al [6] used in vivo two photon imaging to show that γ-secretase inhibition reduced spine density in an APP dependent manner. γ-secretase is one of the proteases that mediate the second cleavage of APP after first cleavage by α or β-secretase (for a review see [2]). In their study layer III and Layer V cortical neurons of *App* ko mice showed a two fold increase in the number of dendritic spines [6]. In contrast, Lee et al. [5] used primary rat hippocampal neurons to show that down-regulation of App decreases the number of spines, whereas overexpression of App had the opposite effect. App needs both its extracellular and intracellular domains to mediate these effects. *Golgi* staining of spines of CA1 pyramidal neurons and layer II/III cortical neurons revealed a significant decrease in density and length of spines in *App* null mutants [5]. More recently, Tyan et al. [4] reported decreased spine density in primary neuronal culture of *App* null mutant mice confirming further the role of App in promoting spine formation. The discrepancies may arise from different methodologies used to image the spines (*Golgi* staining vs. in vivo two photon imaging) or analyzing different types of neurons, i.e., deep layer vs. upper layer pyramidal neurons of cortex or CA1 neurons of hippocampus. One can speculate that the effect of App is cell type specific and age dependent, which remains intellectually an unsatisfying explanation, as it brings little insight into the real function of App. It is interesting however that the role of App in the regulation of neurite formation is reminiscent of the role of *D. melanogaster* APPL in the arborization of neurites. Clearly, there is some conservation of this role in evolution [28,29].

If App-like proteins are functionally redundant, one would expect that *Aplp1* or *Aplp2* ko mice would display similar phenotypes as the *App* knock out. Although both mice are much less extensively analyzed than the *App* knock out, several features of their phenotypes do not overlap [10]. *Aplp1* ko mice appear normal both in forelimb strength and in reduced locomotor activity [10]. Similar, analysis of dendritic spines of *Aplp2* null mutant neurons did not reveal alterations of spines in those mice [36]. A very different example of non-conserved function is the feroxidase activity in APP which is mediated by the REXXE motif in the extracellular domain of APP and is not found in APLP1 and APLP2 [37]. Thus, although further work is needed, the different phenotypes of the single ko mice support the idea that App is specialized in its functions at the synaptic junction, which are likely not fully compensated by Aplp1 and Aplp2. Lack of overt phenotypes in *Aplp1* and *Aplp2* single KO mice does not exclude phenotypes that have escaped scrutiny at this moment.

Combinations of the genetic deletions of *App/Aplps* have been generated [10,38]. Expression of *Aplp2* alone is sufficient for survival of the mice meaning that double deletion of *App* and *Aplp1* is viable. However, in the absence of *Aplp2*, mice can survive only if they express both *App* and *Aplp1*. The viability of *Aplp2* single ko might indicate that App and Aplp1 can work together to compensate for a function that is dominated by Aplp2. At first glance, compensation by *App/Aplp1* together for *Aplp2* deficiency is a possibility, however, no compensatory up-regulation of *App* or *Aplp1* was detected after deletion of *Aplp2* [10,39]. Alternatively, it is equally possible that App family independent mechanisms are compensating for the lack of *Aplp2*. It is noteworthy that combination of *Aplp1* ko with *App ko* or with *Aplp2* ko leads to different outcomes (survival or lethality respectively) showing specificity in the function of App and Aplp2 [10].

Several studies support a role for App at the neuromuscular junction. A phenotype emerges only when *App* deletion is analyzed in an *Aplp2* ko background (*App/Aplp2* double knockout (dko)). These mice show reduced vesicle density in the presynaptic active zone, excessive nerve terminal sprouting and aberrant apposition of presynaptic and postsynaptic markers indicating a key role for App in the proper formation of synaptic structures at the neuromuscular junction [40,41]. Interestingly, proper development of the neuromuscular junction requires App and Aplp2 in the presynaptic motor neurons and the post-synaptic muscles suggesting a transynaptic homophilic or heterophilic interaction between App and Aplp2 [15]. In contrast to the prominent role for soluble APP in *C. elegans*, expression of soluble APP β (sAPPβ) in the *App/Aplp2* dko mice (*App/Aplp2 sAPP*$\beta^{ki/ki}$) did not rescue the lethality or neuromuscular defects of *App/Aplp2* dko mice [42]. Strikingly, expression of soluble APP α (sAPPα) rescued the lethality of the *App/Aplp2* dko mice (*App/Aplp2 sAPP*$\alpha^{ki/ki}$) [43] indicating that a few amino acid between α and β cleavage are instrumental in the biological function of sAPP. How only a few amino acid residues at the carboxyterminus of APPs make such a difference needs clearly further investigation. However, *App/Aplp2 sAPP*$\alpha^{ki/ki}$ showed a widened end plate, impaired neuromuscular transmitter release, and structural abnormalities at the neuromuscular synapses correlating with decreased grip strength. In the central nervous system, the mice showed impaired LTP accompanied with impaired spatial memory [43]. Thus, sAPPα was not able to rescue several neurological phenotypes, implying that full length APP is needed. Interestingly, *App/Aplp2 sAPP*$\alpha^{ki/ki}$ mice did not have any spine or morphological defects in cortical or hippocampal neurons.

It is very likely that the developmental function of App at the neuromuscular junction is mediated through its highly conserved YENPTY motif in its carboxyl terminus domain. App knock in mice with a single Tyr(682) to Gly(682), Y682G, mutation (*APP*$^{Y682G/Y682G}$) in an *Aplp2* null background, display lethality and neuromuscular defects similar to *App/Aplp2* dko mice [44]. This conserved Tyr(682) residue is both a docking site for several cytoplasmic partners and a regulator of APP processing. For example, a significant 15 fold increase in sAPP-α production together with a 3.5 fold decrease in sAPP-β was detected in brain tissue from *APP*$^{Y682G/Y682G}$ mice, highlighting the importance of this residue in regulating APP processing [45]. NGF-TrkA signaling was proposed to regulate the phosphorylation of Tyr(682) of APP. Tyrosine phosphorylation of App was induced after NGF treatment of primary hippocampal neurons. The tyrosine kinase activity of TrkA receptor may mediate the NGF induced tyrosine phosphorylation of App [46]. It was further shown that Y682G mutant neurons are insensitive to trophic activity of NGF, suggesting that phosphorylation of Tyr(682) can work downstream of NGF-TrkA signaling to mediate the trophic effect of NGF. A nearby conserved phosphorylated Thr(668), part of a pSer/Thr-Pro motif, is a docking site for Pin1 and this interaction can down regulate production of Aβ peptide from APP [47]. Pin1 is a unique peptidyl–prolyl *cis*/*trans* isomerase that can catalyze cis/trans isomerization of pSer/Thr-Pro motifs [48]. Binding of Pin1 to the pThr 668-Pro motif in the c-terminus of APP was shown to accelerate its isomerization leading to conformational changes in the c-terminus of APP [49]. In contrast to the instrumental role of Tyr(682) for survival of the mice during development, mutation in Thr(668) of APP (T668A) in an *Aplp2* null background, does not cause lethality or neuromuscular defects [50] highlighting further the importance of tyrosine phosphorylation of APP during development.

The fact that several *App* loss of function phenotypes emerge in an *Aplp2* null background is evidence for genetic interaction but does not directly address the question whether this reflects really functional redundancy between these two paralogues. Further thorough analyses of the single mutant mice should be more informative to identify pathways in which the specific effects of *Aplps* knock out become apparent. Indeed the question remains whether double mutants generate more severe phenotypes either because of complete loss of a redundant function or because of disruption of multiple independent pathways leading to similar phenotypes. For example, transcriptional profiling of the *App* and *Aplp2* single

ko mice reveal different sets of genes [39]. From the 1061 genes that are up or down regulated after deletion of *Aplp2*, only 181 are also found altered in *App* mutant mice [39,51]. For instance, signalling molecules that regulate early response to synaptic activity such as member 6 (KCNH6) (Erg2), FBJ murine osteosarcoma viral oncogene homolog (FOS) and member 1, activity-regulated cytoskeleton-associated protein (Arc) are significantly down-regulated in *App* mutant mice, but not in *Aplp2* mutant mice, strengthening the evidence for App function in synaptic plasticity. P21 is an example of a protein that is down regulated in both *App* and *Aplp2* ko mice cortices. P21 is a cyclin-dependent kinase inhibitor that regulates cell-cycle progression during G1 phase. Deficiency of p21 decreases cell cycle exit and enhances proliferation of neural stem cells by regulating the expression of pluripotency factor Sox2 [52,53]. Thus, down-regulation of p21 in both *App* and *Aplp2* null mutants may enhance the proliferation of neural stem cells. This is consistent with data from Lopez-Sanchez et al. [54] demonstrating that *Aplp2* transcripts are predominantly enriched in the proliferative zone of the developing cortex while *App* shows a partial overlapping expression with *Aplp2* in this area. Aplp2 appears indeed to play a central role in promoting cell cycle exit during developmental neurogenesis in the ventricular zone, and this function is likely shared with App [55]. Soluble fragments of APP and APLP2, also, can promote proliferation of Egf expressing progenitors in the subventricular zone [56]. Thus, overall it appears that App and Aplp2 indeed are partially redundant in neurogenic niches. Nevertheless, it should be stressed that significant differences in the transcriptional response of *App* or *Aplp2* deletion suggest that there are distinct pathways regulated by either App or Aplp2. It remains unclear whether changes in expression of genes in *App* and *Aplp2* ko mice are due to direct transcriptional activity of the intracellular domains of these proteins or to an indirect effect of loss of those proteins [39,57,58].

4. A role of App and Aplps in cortical development

The mammalian cortex has expanded rapidly across different species and this is associated with the evolution of neocortical related behaviour such as perception and cognition [59]. The expansion of the cortical surface is believed to underlie the transition from a smooth cortex (lissencephalic) to a highly folded cortex (gyrencephalic). Indeed, changes in the proliferative pattern of ventricular zone resident neural stem cells have been titled as "a giant leap for mankind" referring to the expanded surface of the human cortex as a consequence of the more proliferative radial glia cell units in the developing human brain [60]. Neural progenitors outside the ventricular zone such as the intermediate progenitors in the subventricular zone might be as well important contributors to the evolutionary expansion of the cortex [59,61]. In mice, *App* and *Aplp2* are expressed in both ventricular and subventricular neurogenic niches of the developing cortex, making these two proteins interesting candidates for an important role in the development and evolution of the cortex [54,62].

Two major neurogenic niches exist during cortical development: the ventricular zone (VZ) largely populated by the radial glia cells and the subventricular zone (SVZ) populated by intermediate progenitors. More recently an additional class of progenitors called Outer SVZ (OSVZ), populated by radial glia like cells that are not attached to the ventricular zone, were described in human, ferret and mouse [61,63,64]. Post-mitotic neurons born in those neurogenic niches migrate towards the cortical plate (CP) using the fibers provided by the radial glia cells. This mode of migration is called glia guided migration. The glia guided migration stops at a cellular layer populated by Cajal-Retzius (CR) cells. CR cells produce Reelin which binds to apolipoprotein E receptor 2 (ApoER2) and Very low density lipoprotein receptor (Vldlr) receptor to signal via disabled-1 (Dab1) to control the end stage of neuronal migration by promoting glia independent somal translocation [65,66]. Triple deletion of *App*, *Aplp1* and *Aplp2* results in a reduced number of CR cells and accumulation of neurons that over migrate to the marginal zones of the developing cortex [7]. Likewise, deficiency of presenilin-1 decreases the number of CR cells and ectopic accumulation of neurons in the marginal zones of the developing cortex [67], suggesting the importance of γ-secretase processing of APP or perhaps other substrates in CR cell function. In contrast to this over-migration phenotype, Young-Pearse et al. [8] showed that single knockdown of *App* in wild type mouse brain inhibited migration of cortical neurons, while APP over-expression promoted the migration of neurons. The effects depended on the conserved YENPTY in the carboxyl terminus. It is noteworthy that the endogenous expression of *Aplp1* and *Aplp2* was not able to compensate for the App shRNA effect, but co-electroporation of *Aplp1* or *Aplp2* could rescue the *App* deficient migration phenotype indicating that, when overexpressed, the proteins can be redundant. Thus the regulation of expression levels of App and Aplps is critical for their function [8]. Down-stream, Disc1 is an interactor of App with key roles in neuronal progenitor proliferation and neuronal migration [68]. Over-expression of Disc1 can significantly rescue the migration effect observed after Dab1 or App down-regulation [69]. Upstream, binding of different isoforms of pancortins mediate different effects on processing of APP. Using an unbiased assay for identification of ectodomain binders of APP, Rice et al showed that pancortins can bind to APP and that binding of pancortin isoform 1 and 2 (B-domain containing pancortins) can significantly decrease β-secretase processing of APP [70]. Down-regulation of pancortin 1 or over-expression of pancortin 4 resulted in similar migration defects as observed after down-regulation of App in developing cortex suggesting opposed roles for different isoforms of the pancortins [70]. Expression of pancortin 1 or APP could rescue the retarded migration of pancortin 4 over-expressing migratory neurons [70]. Similar to pancortins, Reelin interacts with the extracellular domain of APP in primary hippocampal neurons [71] and Dab1 interacts with the highly conserved YENPTY motif in the carboxyterminus of App. This interaction most likely depends on the phosphorylation of tyrosine, highlighting further the importance of this residue for the developmental function of App proteins [72]. Extracellular interaction of App with Reelin and intracellular binding to Dab1 shows that App might work together with ApoER2/Vldlr as (co)receptor to mediate the Reelin effect during migration of neurons. Indeed, Dab1 over-expression could rescue the blocked migration induced by *App* shRNA in the developing cortex. From these findings, a model emerges in which the ectodomain of App binds to Reelin or Pancortins at the cell surface, which leads to signal transduction through down-stream effectors such as Disc1 and Dab1 to regulate neuronal migration. It is very likely that phosphorylation of the YENPTY motif at the c-terminus plays a central role in this process. However, this model does not explain the reduction of CR cells in triple ko mice [7], unless a cell autonomous effect of Reelin and App on for instance the survival of CR cells is postulated.

It might be that Reelin has a dual site of action in the developing cortex. For example, similar to what happens in the post-mitotic migratory neurons, Reelin-Dab1 could directly signal to radial glial cells regulating their morphology and rate of neurogenesis [73–75]. Therefore, it is likely that during cortical development App and Aplp2 can regulate Reelin signalling in both migrating neurons and proliferating neural stem cells and, hypothetically, in CR cells. Moreover, the YENPTY motif is present in the App-like proteins raising the possibility that Reelin can also signal through Aplps to control migration and differentiation of cortical precursor and progenitors.

To our surprise, we did not observe overt migration phenotypes in App/Aplp1 dko neurons or dko neurons expressing Aplp2 shRNA or triple ko neurons [55,76]. Instead, we showed that down-regulation of *Aplp2* in *App/Aplp1* dko progenitors decreased cell cycle exit and delayed differentiation of progenitors [55] which matched very well with its expression profile in the VZ/SVZ [54].

Regarding the discrepancy in the migration phenotypes caused by alterations of App expression in different experimental paradigms, it is possible that the genomic deletion of *App* and/or *Aplp1* triggers adaptive responses that are not activated when App is acutely knocked-down using shRNA. Altogether, the available findings suggest that the different App paralogues have both specialized and redundant functions in different sub-regions of the developing cortex. Aplp2 is important in the regulation of differentiating neuronal precursor cells, App in migratory neurons. Further understanding of the roles of App and Aplps during cortical development would emerge by identification of their specific interaction network in different sub-regions of the developing cortex. Although the current model proposes App as a cell surface (co)receptor, it is likely that soluble ectodomains of App and Aplps work as ligands to regulate migration and differentiation in a non-autonomous fashion. In flies soluble APPL function non-autonomously to regulate the axonal outgrowth in developing mushroom bodies. The developmental defect in outgrowth of α lobe axons in mushroom bodies of *appl* mutant flies was rescued by soluble APPL while β lobe defects were rescued only with full-length APPL, suggesting a non-autonomous effect of soluble APPL on α lobe development [29]. Moreover, it has been shown that sAPP can induce proliferation of Egf positive neural stem cells in the lateral ventricle suggesting the possibility of ligand-receptor model for sAPP function in neurogenesis [56]. Therefore, identification of sAPP and sAPLPs putative receptors would be a major step in understanding the biology of App and Aplps.

5. Processing of the APP family is conserved over evolution

A common feature of APP and APP-like proteins is their processing by membrane secretases [49,77–80]. The proteolytic processing of APP have been studied in large detail (reviewed in [81]). In addition to ectodomain shedding, this proteolytic processing of APP results in the release of Aβ. As this is a continuous process, the brain faces constantly the challenging task of controlling the concentration of Aβ below the aggregation threshold. The Aβ region is a novel feature of the APP paralogue and is not present in APLP1&2 or in the APP-homologues in *C. elegans* (APL1) and *D. melanogaster* (APPL). Interestingly, all the proteases involved in APP processing, i.e., α-secretases, β-secretases and γ-secretases are conserved during evolution, together with the overall processing of the membrane bound APP paralogues resulting in the release of APP ectodomain and intracellular domain.

One *C. elegans* homologue of the catalytic subunit of γ-secretase or presenilin 1 (*PS1*) is called *sel12* [23]. Another *C. elegans* homologue of human presenilin is *hop1* which can compensate for loss of sel12 phenotypes such as egg-laying defect [82]. In flies, a 130KD fragment that misses the carboxyl terminus of full-length APPL was identified and proposed as a secreted form of APPL [25]. Kuzbanian is the α secretase-like protease of flies which was indentified because of its role in regulation of Notch signaling and neurogenesis [83]. More recently it was shown that Kuzbanian is also involved in the processing of flies' APPL [84]. Likewise, β-secretase activity has been described in flies and surprisingly dBACE can process APPL into a neurotoxic peptide which can deposit in the brain similar to human APP [84].

Similar to vertebrates, insect γ-secretase is a multiprotein complex whose activity depends on Presenilin. Takasugi et al. showed that *Drosophila presenilin* can restore the γ-secretase activity of *PS1/PS2* dko mouse fibroblast and increase Aβ production when expressed in N2A human cells [85]. In contrast to heterogeneous mammalian γ-secretase complexes [86,87], γ-secretase in Drosophila exists only in one composition because there is only one gene for each subunit of γ-secretase. In vivo reconstitution of Drosophila γ-secretase, showed that it can efficiently cleave Notch but has poor activity towards APP and APLP2 suggesting substrate specificity of the ancestral γ-secretase [88]. Therefore, although the cleavage activity of γ-secretase is conserved, it seems that the regulation of this activity has evolved in mammals by heterogeneity in composition of γ-secretase.

The biochemical similarities and conservation of the processing of APP and APP-like proteins supports the idea of functional redundancy. In contrast, the end products of APP, APLP1 and APLP2 processing differ in length and amino acid composition, and perhaps acquire different structures. Compared with the Aβ peptide with its variable length between 37 and 43 amino acids and its propensity to aggregate, sequential cleavage of APLP1 by β and γ secretases generates p28, an APLP1 β peptide-like fragment, with 25–28 amino acids which does not aggregate in the brain [89]. Interestingly, the analogy between the processing of APP and APLP1 has raised the possibility of using APLP1 derived p28 in the cerebrospinal fluid as a surrogate marker to detect altered activity of γ secretases in individuals with an increased risk of AD [89]. Processing of APLP2 appears even more elaborate with several c-terminus fragments that are variable in size [77]. Thus, APLP2 processing might release fragments that are different in length, sequence and structure compared to APP. Given these differences, it has been proposed that processed products from the different APP paralogues might regulate distinct signaling pathway, even though they experience similar biochemical processing [80].

6. Transcriptional divergence of APP and APLPs imply functional divergence

An important step in the evolution of gene duplicates is the modification of regulatory proximal elements leading to transcriptional divergence. In turn, transcriptional divergence is likely to result in diversification of the duplicates with regard to function. Such genetic differences in regulatory elements of genes expressed in the developing cortex has been proposed as a primary force driving the evolution of the brain [90,91].

In the genome of *D. melanogaster* and *C. elegans*, there is only one APP-like gene. APl-1 of *C. elegans* is expressed in neurons as well as a few other cell-types [23]. Expression of APPL in *D. melanogaster* is restricted to the nervous system [92]. Similar to flies APPL, APLP1 shows a neuron specific expression in mammals whereas APP and APLP2 are expressed by various cell types. Until recently, APP was assumed to be expressed by all cell types in the brain [62]. However, using a specific antibody to stain APP in the adult brain, Guo et al showed that App is present predominantly in neurons in the adult brain [93]. At the transcript level, a complementary expression pattern has emerged from in situ analysis of App and Aplps transcripts. Lopez-Sanchez et al have demonstrated that Aplp1 expression is restricted to the post-mitotic cortical plate and Aplp2 transcripts show a specific distribution in proliferating ventricular/subventricular zone respectively. Interestingly, App is expressed both in ventricular zone and post-mitotic neurons of the cortical plate showing partial overlapping expression with Aplp1 and Aplp2 [54]. The same expression pattern is observed in publicly available atlases of the developing cortex including Genepaint and Eurexpress [94,95]. The region specific expression pattern of App, Aplp1 and Aplp2 suggests functional

specialization of each member during different stages of neuronal development.

The genetic factors that contribute to the transcriptional divergence of APP duplicates are not well defined. However, analysis of the proximal element of *APP* and *APP*-like genes in different species reveal a CAGA box within the *APP* 5′-UTR which is not present in *APP*-like genes [96]. This CAGA box might regulate expression of APP in response to inflammation linked signaling such as TGFβ [96]. More studies are needed to understand the contribution of the proximal element that control regulated expression of APP and APLPs. Even subtle changes in expression regulation of these genes may account for distinct biological functions.

7. The interaction networks of APP, APLP1 and APLP2 show specificity

Proteins are part of a dynamic network which show cell type and tissue specificity [97]. Evolutionary changes in specificity and strength of these interactions impact the function of the proteins and their networks [98]. Rewiring of the interaction network of paralogous proteins is a clear sign for their functional divergence [98].

Several binding partners have been proposed for APP with various functional implications. (For review see [16,38,99]). In general, the interactors of APP can be divided into those that bind at the extracellular side with a possible ligand mode of action and those that bind at the intracellular side with a possible signaling mode of action. F-spondin, Tag1, Reelin, Netrin, Lingo-1, Pancortins are some examples of extra-cellular binding proteins while Fe65, JIP, JNK, Mint1/X11, Dab1 are among intracellular binding proteins to APP [16,70,99]. If APP and APLPs share similar biological function, then they are expected to be part of similar protein networks. However, a study by Bai et al challenges this idea [100]. A significant proportion of identified APP interactors in their study were consistent with previously published data supporting the reliability of their analysis. Surprisingly therefore, comparing the interactors of the three paralogues showed only one interactor in common: Ras GAP-activating like Protein 1 that binds to both APLP1 and APLP2 [100]. Over-representation of ER chaperones in the APP interaction network might suggest sensitivity of APP folding, while APLP2 interacted distinctly with the Rho family of GTPases such as RhoA and RAC1 [100].

It is likely that the difference in the interaction networks of APP and APLPs reflects their differential subcellular localization and different tissue expression pattern. [101,102]. Indeed, using fluorescent tagged version of APP and APLPs in HEK293 cells, Kaden et al showed that APLP1-YFP is primarily localized to the cell surface. Most of the APP-YFP was found however in intracellular compartments such as the ER and endosomes and to a lesser extent the Golgi apparatus. APLP2 was equally distributed at the cell surface and intracellular compartments showing partial overlapping localization with both APP and APLP1 [102].

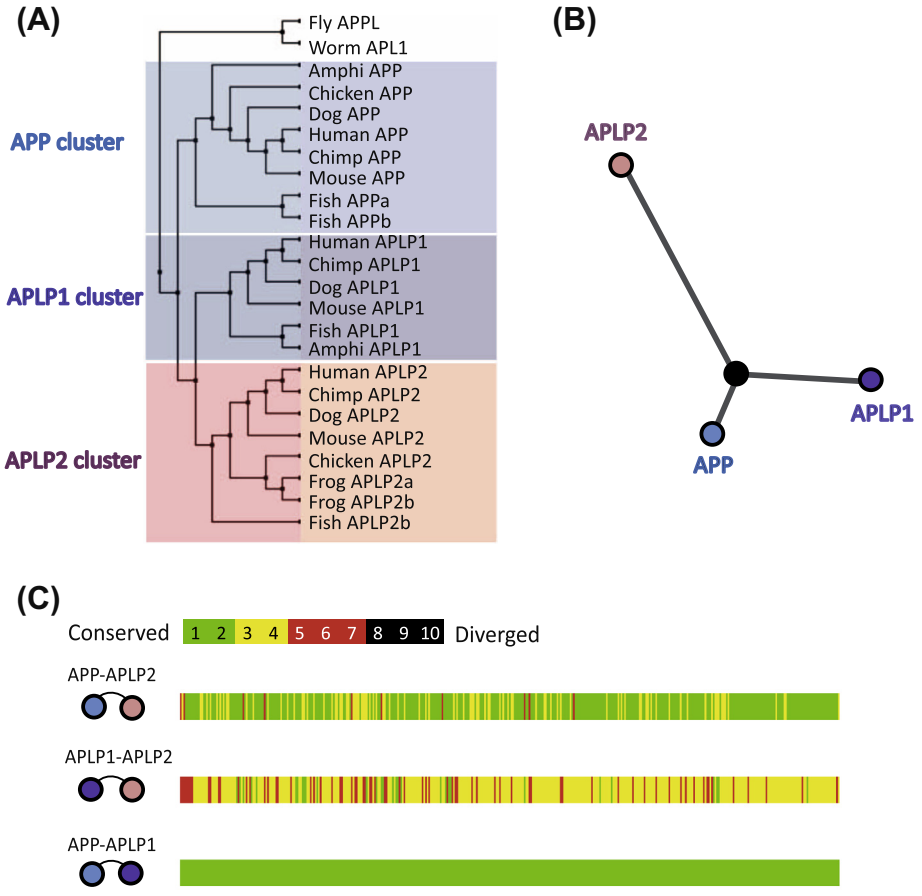

Fig. 2. (A) Phylogenetic tree of APP protein family with three clusters of APP homologues in different species highlighted in different colors. Notice that fly APPL and worm APL-1 cluster separately. (B) Functional distance analysis of phylogenetic tree of APP protein family shows that APLP2 is more distant from an inferred ancestral gene when compared to APP and APLP1. The diagram illustrates the degree of the divergence of each cluster based on (site-specific) shifted evolutionary rates after gene duplication or speciation. (C) The heatmap of (site-specific) shifted evolutionary rate from n-terminus (left) to c-terminus shows that APLP1 and APLP2 are the most divergent and APP and APLP1 are least divergent. The data is derived based on Gu Statistical method (Diverge2 software). Notice that many of the shifted sites are in the extracellular domain of APLP2.

8. Prediction of functional divergence of the APP protein family

Conservation of an amino acid residue in a sequence correlates with functional importance [103–105]. In brief, three possibilities exist for evolution of each residue of gene A and its duplicate B: 1) super conservation throughout the gene family (Type 0 evolution). A nice example of Type 0 evolution is the YENPTY motif at the carboxyl terminal of the APP family that is universally conserved (Fig. 3). Conserved in gene A and variable in the B duplicate (Type I evolution) 3) Conserved in both A and B but having different biochemical properties (Type II evolution) [104]. To test our hypothesis on functional divergence of APP and APLP, we used the statistical method developed by Gu in 1999 [105]. In this method, the altered functional constraint is estimated by calculating site-specific rate differences between duplicates.

We aligned the APP and APLPs sequences from nine species (24 genes, Table 1) to generate the phylogenetic tree of the APP family. using Neighbor-Joining Tree making of Diverge 2 software developed by Gu [103]. After rerooting separate cluster of APPL and APL1, the tree revealed three distinct clusters for APP, APLP1, and APLP2 (Fig. 2A). Scanning the amino acid residues of the APP and APLP1 cluster did not reveal any sites that might significantly contribute to functional constraint alteration (Fig. 2C). The coefficient of type I functional divergence (θ) between the APP cluster and the APLP1 cluster is very low (0.10) suggesting functional conservation of APP and APLP1. Thus, structurally the two types of proteins are quite related and mainly transcriptional divergence has to be considered when evaluating specification of APLP1 function. Consistent with the data from the animal models, APLP2 shows significant divergence from both APP and APLP 1 with coefficients of type I functional divergence of 0.29 for APP-APLP2 and 0.53 for APLP1-APLP2 (Fig. 2B). Using Diverge 2 software, we identified several residues of APLP2 that contributed to its functional divergence from APP and APLP1 (Fig. 2B, C). Many of those residues are located in the extracellular domain of APLP2 (Fig. 2C) suggesting a specific role for this domain. Of note, the GTGFAVAE motif at the very N terminal APLP2 cluster, is not found in the APP and APLP1 cluster (Fig. 3). Experimental data will be valuable in understanding how those residues confer specific function. These findings are consistent with a model in which APLP2 is differentiated from APP and APLP1 by changing its extracellular fragment while the carboxyl terminus remains largely conserved between all three APP family members.

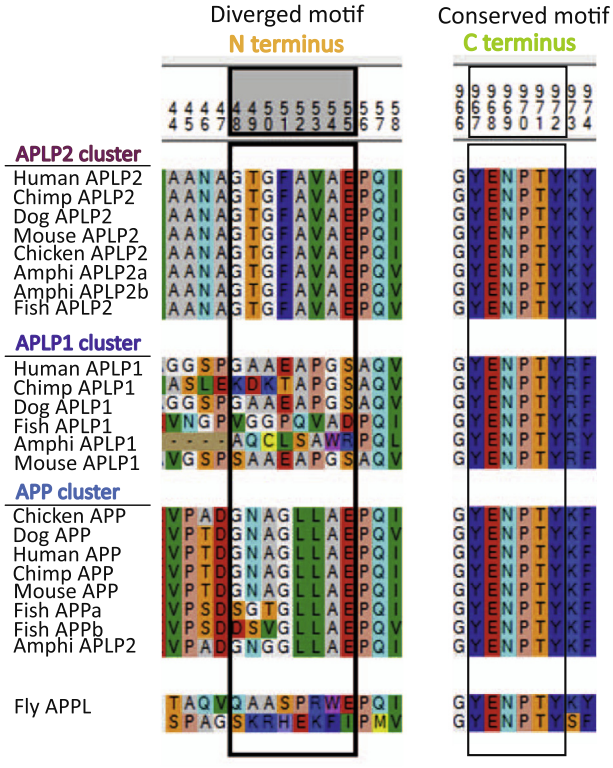

Fig. 3. An example of the specific APLP2 motif at the N terminus contributing to its divergence. Alignment of different cluster shows that GTGFAVAE motif at the N-Terminus is specific to APLP2 and opposite of this motif is super conserved YENPTY at the C-terminus.

9. Concluding remarks

The evolutionary tree of APP proteins reflects a complex trajectory which cannot be simplified into the "redundancy" or "divergence" model. Clearly, some functions of the family are preserved; especially the neuron specific functions at the synapse appear to fall under this category. However, also innovation has happened in this gene family leading to divergence of their expression and interaction networks. Further efforts to understand these specific features of each family member could address some of the following open questions.

1. What are the critical amino acid and motifs conferring specific features to each duplicate? Mutagenesis of the divergent motifs would be valuable in understanding the specific feature of each member [106].
2. How did cis-regulatory sequences of APP and APLPs diverge and how does this compare to the amino acid coding sequences?
3. Finally, the most challenging topic is to understand the specialized functions of each member which, by definition, should not be complemented by the other APP members.

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