Published in final edited form as:
Exp Brain Res. 2012 April ; 217(0): 397–411. doi:10.1007/s00221-011-2905-7.

Caenorhabditis elegans as a model organism to study APP function

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Abstract

The brains of Alzheimer's disease patients show an increased number of senile plaques compared with normal patients. The major component of the plaques is the β-amyloid peptide, a cleavage product of the amyloid precursor protein (APP). Although the processing of APP has been well-described, the physiological functions of APP and its cleavage products remain unclear. This article reviews the multifunctional roles of an APP orthologue, the *C. elegans* APL-1. Understanding the function of APL-1 may provide insights into the functions and signaling pathways of human APP. In addition, the physiological effects of introducing human β-amyloid peptide into *C. elegans* are also reviewed. The *C. elegans* system provides a powerful genetic model to identify genes regulating the molecular mechanisms underlying intracellular β-amyloid peptide accumulation.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized clinically by age-related cognitive dysfunction and pathologically by the accumulation of dense plaques and neurofibrillary tangles in the brains of AD patients (Kidd 1964; Krigman et al. 1965; Luse and Smith 1964; Terry et al. 1964). The major component of the plaques is the β-amyloid peptide (Aβ; (Glenner and Wong 1984; Masters et al. 1985), which is a cleavage product of the amyloid precursor protein (APP; Kang et al. 1987). Autosomal-dominant mutations in APP have been correlated with 10–15% of early-onset AD cases (Chartier-Harlin et al. 1991; Goate et al. 1991; Murrell et al. 1991; Bird 2010). The majority (30–70%) of the mutations correlated with early-onset AD, however, are in the presenilin genes, PS1 and PS2, which encode components of the γ- secretase complex that cleaves APP to produce Aβ (Kimberly et al. 2003; Takasugi et al. 2003; Bird 2010). These results suggest that Aβ plays a central role in the disease. The contribution of other APP cleavage products to the pathology of AD is unclear.

Function of the APP family

To ensure that therapeutic strategies do not interfere with essential functions of APP, the functions of APP have been addressed using mouse knockout (KO) models. Mice that carry a null allele of APP or that produce low levels of a truncated form of APP show several phenotypes, including a reduced body weight, reactive gliosis, agenesis of the corpus callosum, defects in spatial learning and exploratory behavior, and/or decreased locomotor activity and forelimb grip strength (Muller et al. 1994; Zheng et al. 1995). Overexpression of APP in mice also results in several phenotypes, which vary in severity, presumably because of varying levels of APP expression. These phenotypes include lethality, neophobia,

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impaired spatial alteration, reactive gliosis, and an increase in the number of synaptophysin and GAP-43 immunoreactive presynaptic terminals (Mucke et al. 1994; Hsiao et al. 1995). Analyzing the in vivo functions of APP in mammals, however, is complicated by the presence of two APP- related genes, APLP1 and APLP2 (Wasco et al. 1992, 1993a, b; Sprecher et al. 1993; Slunt et al. 1994). The APP-related proteins are similar to APP, but do not contain Aβ. APP and APP-related proteins share a cysteine-rich region (E1) and a region rich in acidic residues (E2) in their extracellular domains; the highest sequence similarity among these proteins, however, is within the small intracellular domain (AICD) (Kang et al. 1987; Yamada et al. 1987; Wasco et al. 1992, 1993b; Sprecher et al. 1993; Slunt et al. 1994; Paliga et al. 1997; Lenkkeri et al. 1998).

The functions of the APP-related genes have also been analyzed through KO mouse models. APLP1 KO mice have a reduced body weight (Heber et al. 2000), while APLP2 KOs show no obvious defects (von Koch et al. 1997). Interestingly, double KOs of APLP2 and APP or APLP2 and APLP1 result in postnatal lethality (von Koch et al. 1997; Heber et al. 2000). Moreover, triple KO mice in which all three APP family members are inactivated show postnatal lethality and type II lissencephaly; cortical areas are disorganized and show irregular protrusions (Herms et al. 2004). Collectively, these results suggest that APP family members have essential and overlapping functions during development, including proper brain development, and these functions do not require Aβ. The molecular pathways in which APP participates during development, however, remain unknown.

Several genes implicated in Alzheimer's disease (AD), such as APP and the presenilins, have ortho- logues in the nematode Caenorhabditis elegans (Daigle and Li 1993; Levitan et al. 1996; Li and Greenwald 1997; Westlund et al. 1999; Goutte et al. 2000; Francis et al. 2002); reviewed in (Ewald and Li 2010). Roughly 38% of the *C. elegans* genes have human orthologues (Shaye and Greenwald 2011); furthermore, an astonishing 40–50% of the identified human disease genes have *C. elegans* orthologues that show on average 50 and 70% amino acid identity and similarity, respectively (Ahringer 1997; Wheelan et al. 1999; Culetto and Sattelle 2000). Hence, *C. elegans* presents a powerful complementary genetic system to understand the function, processing, and regulation of APP and related proteins. In this review, we focus on the function of the *C. elegans* orthologue APL-1. In addition, we review using the power of *C. elegans* genetics to identify genes underlying the intracellular accumulation of the human β-amyloid peptide introduced by transgene expression.

Caenorhabditis elegans as a model system to examine neurodegenerative disorders

C. elegans is a multicellular eukaryotic organism with a short reproductive cycle of 3 days (Brenner 1974). From the fertilization of the egg to the adult hermaphrodite, each of the 959 somatic cells is characterized and delineated (Sulston and Horvitz 1977; Sulston et al. 1983). About one- third of all its somatic cells are neurons (302 neurons), which are connected by 5,000 chemical synapses, 2,000 neuromuscular junctions, and 600 electrical synapses (White et al. 1986). All of these synaptic connections have been analyzed by serial section electron micrograph reconstructions (White et al. 1986), establishing the first organism for which the entire neural wiring circuitry is known. Neurons use classical neurotransmitters, such as acetylcholine (Hosono et al. 1987), dopamine (Sulston et al. 1975), GABA (McIntire et al. 1993), glutamate (Lee et al. 1999), and serotonin (Horvitz et al. 1982), as well as numerous neuropeptides (Li and Kim 2008). Since *C. elegans* is transparent, neuronal structures and morphologies are easily traceable by expressing green fluorescent protein (GFP) in a neuron of choice, and the cellular localization of a protein can be visualized by tagging the protein with GFP (Chalfie et al. 1994). The function of individual neurons can be elucidated either by genetic alterations or by laser ablation of single cells (Chalfie et al. 1985; Bargmann and Horvitz 1991). Recently, optogenetic techniques have been adapted to *C. elegans*, such that the activity of neurons can be measured by monitoring calcium levels

(Kerr et al. 2000) and neurons can be activated by blue light stimulation of channelrhodopsin (Nagel et al. 2005). Despite *C. elegans* adults being only about 1 mm in size and its neuronal cell bodies only one to two μm (White et al. 1986), electrophysiological methods to patch-clamp neurons have been successfully described (Goodman et al. 1998), although it is unclear whether *C. elegans* produces action potentials (Lockery and Goodman 2009). Lastly, *C. elegans* is easy to manipulate genetically (Brenner 1974) and generating transgenic animals is relatively fast and straightforward (Mello et al. 1991; Mello and Fire 1995). In summary, *C. elegans* is an easy, tractable and well-established system to investigate developmental and neuron-specific functions.

C. elegans APL-1 has an essential function

Like *Drosophila* (Rosen et al. 1989), *C. elegans* contains only one APP family member, APL-1 (Daigle and Li 1993). APL-1 shows high amino acid similarity within the extracellular domain (46% for E1 and 49% for E2) and the intracellular domain (71%) to human APP; however, similar to *Drosophila* APPL and mammalian APLP1 and APLP2, *C. elegans* APL-1 does not contain an Aβ sequence (Daigle and Li 1993). Hence, the Aβ sequence may be a vertebrate-specific development. Loss of *apl-1* results in a completely penetrant early larval lethality (Hornsten et al. 2007; Wiese et al. 2010), showing that like the mammalian APP gene family, *apl-1* also has an essential function. By contrast, *Drosophila* mutants in which *appl* was inactivated were viable and had subtle behavioral deficits (Luo et al. 1992). Hence, *C. elegans* can be a useful model organism to reveal the essential function of APP in development.

APL-1 expressed in neurons is essential for survival in *C. elegans*

Like mammalian APP family members, but unlike *Drosophila, apl-1* is expressed in multiple cell types, including a subset of neurons, muscles, seam cells, hypodermal cells, and supporting cells (Hornsten et al. 2007; Niwa et al. 2008). By examining expression of APL-1 tagged at the C-terminus with GFP (APL-1::GFP), APL-1 appears to be continuously expressed starting from the 30-cell egg-stage through adulthood (Hornsten et al. 2007; Wiese et al. 2010). This broad expression pattern suggests that *apl-1* is involved in multiple functions and may explain the pleiotropic phenotypes of *apl-1* KOs. For instance, after hatching, *C. elegans* develops through four larval stages before arising as an adult; each larval stage transition is punctuated by a molt, whereupon the animals synthesize a new cuticle and undergo a stereotypic pattern of movements to emerge from the old cuticle, a process referred to as ecdysis (Singh and Sulston 1978). *apl-1* KOs are unable to fully shed their old cuticle, leading to the lethality seen during the first to second larval stage transition (Hornsten et al. 2007; Wiese et al. 2010). However, by pinching a hole in the old cuticle with a needle, second larval stage *apl-1* KO mutants can emerge and develop to the next larval stage, whereupon once again, the mutant is unable to molt properly and dies (C. Ewald and A. Silva, unpublished obs.). These data indicate that *apl-1* activity is essential during the molting ecdysis process not just during the first to second larval stage transition, but also during later larval transitions. In addition, the *apl-1* KO mutants have several other defects, including the formation of large vacuolar-like structures in hypodermal cells (Fig. 1b), the separation of organs from underlying basement membrane (Fig. 1d) and occasionally, the lack of posterior morphogenesis (Hornsten 2001; Hornsten et al. 2007). Germline transformation by introduction of full-length APL-1 or APL-1::GFP driven by its endogenous promoter completely rescued the *apl-1* phenotypes (Hornsten et al. 2007; Wiese et al. 2010).

To pinpoint the cells in which *apl-1* expression is critical for viability, APL-1 transgenes were constructed such that *apl-1* expression could be driven in specific tissues. Driving *apl-1* expression in hypodermal cells or muscle tissue did not rescue the lethality of *apl-1* KOs

(Hornsten et al. 2007). This result was somewhat surprising given that the cuticle is produced by hypodermal cells (Singh and Sulston 1978). By contrast, driving *apl-1* expression in neurons rescued the lethality of *apl-1* KO mutants, suggesting that APL-1 expression in neurons triggers the stereotypic pattern of movements to emerge from the old cuticle (Hornsten et al. 2007).

One noteworthy change in *apl-1* expression is during the last molt, when animals transition from the fourth larval stage into adulthood. This developmental transition is regulated by the *let-7* microRNA (miRNA), which regulates heterochronic transcription factors and the nuclear hormone receptor NHR-25 (Hayes et al. 2006; Hada et al. 2010). During this transition, seam cells, which act as stem cells to produce new hypodermal cells during molting, join the hypodermal syncytium (Sulston and White 1980). During the fourth larval stage, seam cells begin to express *apl-1* (Niwa et al. 2008), and this *apl-1* seam cell expression is regulated by the binding of NHR-25 to a 200-base pair (bp) enhancer element 6 kbp upstream of the *apl-1* ATG (Niwa et al. 2008; Niwa and Hada 2010; Hada et al. 2010). Loss of *let-7* miRNA results in extra hypodermal cell production, resulting in vulva bursting and lethality (Reinhart et al. 2000). RNAi of *apl-1*, which knocks down *apl-1* mRNA levels by 60%, rescues the lethal phenotype of *let-7* mutants (Niwa et al. 2008), suggesting that *let-7* miRNA downregulates *apl-1* to induce terminal differentiation of seam cells to join the hypodermal syncytium. Furthermore, double mutants of *let-7* miRNA family members *mir-48(n4097)* and *mir-84(n4037)* are unable to shed their old cuticle during the fourth larval stage to adult transition; this phenotype is also suppressed by *apl-1* knockdown (Niwa et al. 2008). These data suggest that several *let-7* miRNA family members directly or indirectly regulate *apl-1* expression during the larval to adult transition. *apl-1* expression is necessary in neurons for the molting process to proceed properly, but its expression must be downregulated in seam cells to allow seam cell terminal differentiation. In mammals, the role of APP in differentiation is more equivocal. For instance, sAPP, as a co-factor with epidermal growth factor, promotes cell proliferation of ventricular zone cells, but does not affect differentiation (Caille et al. 2004). By contrast, APP immunoreactivity was detected in embryonic mouse neural tube (Salbaum and Ruddle 1994), suggesting a role during neural differentiation. Moreover, downregulation of APP decreased neurite differentiation in vitro (Allinquant et al. 1995). In advanced stage melanomas, which express high levels of APP, APP knockdown by RNAi causes the terminal differentiation of metastatic melanoma cells (Botelho et al. 2010), again suggesting that APP expression is downregulated to allow cell differentiation. However, in certain instances, APP induces cell differentiation. Transfection of human APP into human embryonic stem cells induced rapid differentiation of stem cells into neural cells (Freude et al. 2011). Hence, mammalian APP can induce or inhibit cell differentiation depending on cellular context. Whether sAPL-1 also functions as a regulator for neuronal differentiation in *C. elegans* remains to be determined.

Processing of APP and APL-1

In mammals, APP can be processed through two path- ways. In the first pathway, α-secretase cleaves APP to release a large extracellular fragment sAPPα. The small remaining C-terminal transmembrane fragment APP- CTFα (also known as C83) is subsequently cleaved by the γ-secretase complex to release the p3 fragment extracellularly and the AICD fragment intracellularly (Haass et al. 1993; reviewed in Nunan and Small 2000). Because the initial α-secretase cleavage is within the β-amyloid peptide sequence, this pathway does not generate the β-amyloid peptide. In the second pathway a β-secretase cleaves APP N-terminal to the α-secretase site to release the extracellular fragment sAPPβ. Subsequently, the remaining C-terminal transmembrane fragment APP- CTFβ (also known as C99) is cleaved by γ-secretase to release the β-amyloid peptide extracellularly and AICD intracellularly (Haass et al. 1992; reviewed in Nunan and Small 2000).

The functions of the *C. elegans* orthologues have provided significant insights into the role of the mammalian counterparts. Mutations in the presenilin genes were initially identified in familial Alzheimer disease patients (Levy-Lahad et al. 1995; Rogaev et al. 1995; Sherrington et al. 1995); however, the functional role of the presenilins was informed by the *C. elegans* SEL-12 presenilin homologue, which was identified as a suppressor of Notch signaling (Levitan and Greenwald 1995). The human presenilins not only show amino acid homology to the *C. elegans* SEL-12, but also functional conservation, since introduction of human presenilin can rescue *sel-12* deficiencies (Levitan et al. 1996). Moreover, PEN-2, a component of the γ-secretase complex, was first genetically identified in *C. elegans* in screens to enhance *sel-12* presenilin mutant phenotypes (Francis et al. 2002). The mammalian orthologue was similarly found in the γ- secretase complex (Chen et al. 2002). Lastly, the function of nicastrin/APH-1 was determined in *C. elegans* specifically to provide insights into its function in mammals (Yu et al. 2000).

APL-1 is similarly cleaved by secretases. However, like mammalian APLP1 and APLP2, *C. elegans APL-1* lacks sequence homology in the Aβ region (Daigle and Li 1993). Because only one sAPL-1 fragment was detected on Western blots (Hornsten et al. 2007), APL-1 is presumably processed by only one of the pathways. Bioinformatic searches of the sequenced *C. elegans* genome revealed no sequence homology to a β-secretase (C. Link, unpublished obs.). Furthermore, protein extracts from transgenic animals carrying human APP showed no detectable sAPPβ nor human Aβ peptide, suggesting that APL-1 is not cleaved by a β-secretase (Link 2006). These results suggest that APL-1 is first cleaved by an α-secretase to release sAPL-1 and subsequently cleaved by the γ-secretase complex (Hornsten et al. 2007).

The extracellular domain of APL-1 is essential for viability in *C. elegans*

In genetic screens to identify viable *apl-1* alleles, the *yn5* allele, which contains a deletion of the transmembrane domain, intracellular domain and large parts of the 3' UTR, was identified (Hornsten et al. 2007). *apl-1(yn5)* animals are fully viable, demonstrating that the extra-cellular domain is necessary and sufficient for viability. Furthermore, introducing a fragment containing only the extracellular domain of APL-1 corresponding to the *yn5* mutation (APL-1EXT) was sufficient to rescue the lethality of *apl-1* KOs. APL-1EXT is slightly larger than sAPL-1 because it encompasses the entire extracellular domain and does not appear to undergo further processing (Hornsten et al. 2007). Pan-neuronal expression of APL-1EXT was also sufficient to rescue the *apl-1* KO lethality (Hornsten et al. 2007), suggesting that neuronal release of sAPL-1 is sufficient for the molting ecdysis program to be activated. By contrast, introducing a fragment containing the APL-1 intracellular domain was not sufficient to rescue (Hornsten et al. 2007; Wiese et al. 2010). In mice, knockin of a fragment containing the extracellular domain of APP (sAPPα) was able to rescue the neuronal and behavioral deficits of $APP^{-/-}$ knockout mice (Ring et al. 2007). However, the lethality associated with $APP^{-/-}$;$APLP2^{-/-}$ double knockout mice was not rescued by knockin of the extracellular domain corresponding to sAPPβ (Li et al. 2010a) or by knockin of APP carrying the Swedish, Arctic, and London mutations, a humanized Aβ, and a mutated intracellular domain [APP(Swe-Artic-London)/hAβ/mutC] (Li et al. 2010b). Although the secreted sAPP levels from primary hippocampal neuronal culture of these two knockin mouse strains were similar to wild-type secreted sAPP levels, both knockin APP levels were (20–40%) less than endogenous APP levels in vivo (Li et al. 2010a, b), which could have a physiological effect on viability. Moreover, the sAPPβ knockin had a C-terminal FLAG tag that could affect functionality; similarly, the APP(Swe-Artic-London)/hAβ/mutC knockin had numerous mutations that could also affect the extracellular domain functionality. By contrast to the pan-neuronal APL-1 expression rescue of *apl-1* KOs, a conditional double KO of APP-APLP2 in neurons was not lethal (Li et al. 2010a). These data would argue that mammalian AICD is necessary for viability, suggesting a functional

difference between the orthologous proteins. Knockin of the intracellular domain of APP, untagged sAPPβ, or sAPPα would resolve whether these APP domains rescue the $APP^{-/-}$;$APLP2^{-/-}$ lethality.

To determine whether APL-1 is homologous to APP family members, the human and mouse orthologues were tested for rescue of the *apl-1* KO lethality. Introducing human APP, murine APP, human APLP1, human APLP2, or human APP, APLP1, and APLP2 together did not rescue the *apl-1* KO lethality (Hornsten 2001; Hornsten et al. 2007; Wiese et al. 2010); furthermore, swapping the extracellular domain of *C. elegans* APL-1 with human APP up to the putative α-secretase cleavage site also did not rescue the lethality (Hornsten 2001), suggesting that while APL-1 and APP share sequence similarities, slight changes in the structure affect protein interactions.

The extracellular domain of APL-1 is required for proper synaptic function at the neuromuscular junction

The subset of neurons in which *apl-1* expression is detected includes neurons within the ventral nerve cord (Hornsten et al. 2007), which contains a large number of cholinergic excitatory motor neurons (Duerr et al. 2008). Upon stimulation, cholinergic neurons release acetylcholine, which is degraded by acetylcholinesterase in the synaptic cleft. Excessive acetylcholine in the synaptic cleft leads to constitutive activation of the acetylcholine receptor, resulting in constitutive muscle contraction that causes paralysis in *C. elegans* (Brenner 1974; Johnson et al. 1981). Similarly, pharmacological administration of aldicarb, an inhibitor of acetylcholinesterase, leads to *C. elegans* paralysis (Rand and Russell 1985; Nguyen et al. 1995). RNAi knockdown of *apl-1* resulted in faster paralysis of aldicarb-treated animals compared with control animals (Wiese et al. 2010). This hypersensitivity to aldicarb of RNAi *apl-1* worms was rescued by introducing full-length APL-1 or the extracellular domain of APL-1 (Wiese et al. 2010). These results suggest that lower levels of APL-1, and in particular the extracellular domain of APL-1, result in excessive acetylcholine levels, decreased acetylcholinesterase function, or enhanced acetylcholine receptor function. Whether the *apl-1* knockdown hypersensitivity is caused by morphological defects is unknown. In $APP^{-/-}$;$APLP2^{-/-}$ double KO mice, morphological defects of the neuromuscular junction displayed before the postnatal lethality were not rescued by knockin of sAPPβ (Li et al. 2010a), suggesting that sAPP might not function in the development of the neuromuscular junction in mice. However, sAPP in mammals might still function at the neuromuscular junction later in development or in adulthood, similar to sAPL-1 in *C. elegans*.

The extracellular domain of APL-1 binds heparin

In mammals, the extracellular fragment sAPPβ, but not sAPPα, is further cleaved in vitro by an unknown secretase to yield an E1 domain fragment and an E2 domain fragment (Nikolaev et al. 2009). In *C. elegans*, the presence of either E1 or E2 rescued the *apl-1* KO lethality (Hornsten et al. 2007), suggesting a redundant function of the two domains. Both domains have putative binding sites to heparin, which is abundantly found in the extracellular matrix between cells (Kramer 2005). Deleting the putative heparin-binding site of E1 together with the entire APL-1 E2 domain did not rescue the *apl-1* KO lethality (C. Ewald, unpublished obs.), suggesting an essential role for heparin binding. The APL-l E2 domain consists of six alpha helices (αA-αF; (Hoopes et al. 2010). Although only 34% of the amino acid sequence of APL-1 E2 is similar to human APP E2, the crystal structure of the APL-1 E2 domain almost perfectly overlays that of human APP E2, except that there is a 26 degree greater angle between the αC and αD helices of human APP E2 compared with *C. elegans* APL-1 (Hoopes et al. 2010). In *C. elegans*, the heparin binding to E2 was stronger at pH 5 than at neutral pH (Hoopes et al. 2010), suggesting that extracellular

changes in pH regulate heparin binding and presumably function. Human APP E2 was present as dimers in X-ray crystal structures, and heparin-binding sites were close to the dimer interface (Wang and Ha 2004), whereas *C. elegans* APL-1 E2 in solution was found to be monomeric (Hoopes et al. 2010). However, *C. elegans* protein extracts contained APL-1 dimers by SDS-PAGE analysis, suggesting that APL-1 can dimerize (Hornsten et al. 2007). One possible model is that when APL-1 is at the cell surface, it homodimerizes upon heparin binding to aid in cell adhesion.

LRPs are putative receptors for APL-1

If sAPL-1 is released to initiate the molting ecdysis program or to ensure proper cell adhesion, then sAPL-1 is likely to bind a receptor to activate a downstream signaling pathway. By analogy to mammalian APP, which binds LRP (Kounnas et al. 1995) and LRAD3 (Ranganathan et al. 2011), possible sAPL-1 receptors are the low-density lipoprotein receptor-related proteins LRP-1 and LRP-2. The *C. elegans* orthologue LRP-1 regulates cholesterol metabolism and molting (Yochem et al. 1999). Hence, sAPL-1 could bind LRP-1 or LRP-2 to activate a down- stream pathway. A second possible receptor would be a TNF-like receptor similar to DR6, a death domain containing receptor that binds and can be activated by mammalian sAPP (Nikolaev et al. 2009; Kuester et al. 2011). However, although a DR6-like orthologue has not been identified in *C. elegans*, a DR6 adaptor protein encoded by trf-1 TRAF has been found in *C. elegans* (Wormbase), suggesting that a functional equivalent to the DR6 receptor is present in *C. elegans*.

Intracellular domain of APL-1 has possible $G_o\alpha$ and FEH-1-binding sites

The intracellular domain of APL-1 shares 71% sequence similarity to human AICD (Daigle and Li 1993). This similarity includes a potential $G_o\alpha$-binding site (MRGFIEVDVYTPEERHVAG), suggesting that APP and related proteins can act as receptors. *C. elegans* that overexpress APL-1 have locomotory defects and sluggish movements (Hornsten et al. 2007). These locomotory defects were suppressed by constitutive activation of $G_o\alpha$ (G. O'Connor, unpublished obs.), suggesting a potential receptor function of APL-1. Such an APL-1 receptor function is independent of the role of APL-1 in activating the molting ecdysis program, as constructs that delete the $G_o\alpha$-binding site or the whole intracellular domain of APL-1 rescued the *apl-1* KO lethality (Hornsten et al. 2007). In mammals, the intracellular domain of APP, particularly the YENPTY motif, binds the adaptor protein Fe65 (Borg et al. 1996) to traffic the complex to the cell surface (Sabo et al. 2001). The APL-1 intracellular domain also contains a YENPTY motif and binds to FEH-1, the *C. elegans* orthologue of Fe65 (Zambrano et al. 2002). Loss of *feh-1* resulted in embryonic lethality or first larval stage arrest; RNAi knockdown of *feh-1* or *apl-1* led to hyperactive pharyngeal pumping compared with control worms, suggesting that *feh-1* and *apl-1* regulate pharyngeal pumping through a common pathway (Zambrano et al. 2002).

Overexpression of APL-1 causes lethality that is rescued by decreased *sel-12* presenilin activity

Thus far, loss of APP function has not been correlated with AD. By contrast, duplication of the APP gene locus (Cabrejo et al. 2006; Rovelet-Lecrux et al. 2006; Sleegers et al. 2006) or Down syndrome, a trisomy of chromosome 21 on which APP is located (Glenner and Wong 1984), has been correlated with early-onset AD (Mann and Esiri 1989; Schupf et al. 1998; Korbel et al. 2009). These findings suggest that higher levels of APP may contribute to developing AD. Overexpression of APL-1 in *C. elegans* led to an incompletely penetrant early larval lethality, detachment of organs, and locomotive defects (Hornsten et al. 2007). This *apl-1* overexpression lethality was not suppressed by mutations in *ced-3*, which encodes a caspase required for apoptosis (Yuan et al. 1993), or *crt-1*, which encodes calreticulin, which is required for necrotic cell death (Xu et al. 2001) in *C. elegans*

(Hornsten et al. 2007); furthermore, APL-1::GFP generally did not co-localize with BEC-1, a component of the phosphatidylinositol class III kinase complex that regulates autophagy (Fig. 2b; (Melendez et al. 2003). These data suggest that the *apl-1* overexpression lethality is not due to ectopic activation of a cell death pathway. By contrast, reducing *sel-12* presenilin activity, thereby decreasing γ-secretase activity, partially suppressed the *apl-1* overexpression lethality (Hornsten et al. 2007). In a *sel-12* reduction-of-function mutant background, APL-1 was still cleaved (Hornsten et al. 2007), and the relative ratio of protein levels of sAPL-1 to full-length APL-1 was similar compared with animals with functional SEL-12. However, overall APL-1 protein levels were lower in *sel-12* presenilin mutants, suggesting that either APL-1 is being degraded more quickly or *sel-12* presenilin regulates APL-1 levels (Hornsten et al. 2007).

Missense mutations in *apl-1* destabilizes APL-1 and results in lethality

When screening for new alleles of *apl-1*, a missense mutation, yn32, which corresponds to a glutamic acid to lysine substitution at residue 371 (E371K), was identified (Hornsten et al. 2007). Germline transformation with an APL-1(E371K)::GFP transgene was unable to rescue the *apl-1* KO lethality; although low levels of GFP fluorescence were present, protein extracts showed no detectable APL-1 protein by Western blot analysis, suggesting that *yn32* is a null allele (Hoopes et al. 2010). APL-1(E371K) thermally unfolded faster than wild-type protein in stability assays in vitro, but compensatory mutations that restored wild-type properties in vitro did not rescue the *apl-1* KO lethality in vivo (Hoopes et al. 2010). The E371 residue is in the αD helix where it forms a salt bridge with lysine residue 439 and a hydrogen bond with tryptophan residue 435, both of which are located in the αF helix; hence, the E371K mutation disrupts the αD to αF helix interaction and destabilizes the protein (Hoopes et al. 2010).

Trafficking of APL-1

APL-1 tagged at the C-terminus with GFP (APL-1::GFP) was seen in neuronal processes (Fig. 2), indicating that APL-1::GFP is transported to the axon terminal (Hornsten et al. 2007). Anterograde trafficking of APL-1::GFP to the axon terminal is dependent on the kinesin motor UNC-104 (Wiese et al. 2010; Arimoto et al. 2011), whereas retrograde transport of APL-1::GFP back to the cell body is dependent on DL1-1 dynein, which is transported to the axonal terminals by UNC-116 kinesin 1 (see model in Fig. 3; Arimoto et al. 2011). Reduction in unc-108/rab2 GTPase activity, which functions in vesicular trafficking and is specifically localized to the Golgi (Sumakovic et al. 2009), resulted in retention of APL-1::GFP fluorescence in the cell body where APL-1::GFP was co-localized with UNC-108::RFP (Wiese et al. 2010). By contrast, loss of rab-5 GTPase activity, which mediates transport of vesicles from the plasma membrane to early endosomes, caused decreased APL-1::GFP levels, presumably because APL-1::GFP at the cell surface is degraded and subsequently internalized (Wiese et al. 2010). In mammals, vesicles containing APP are trafficked to the nerve terminals by fast anterograde transport with kinesin motors (Koo et al. 1990; Kaether et al. 2000; Kamal et al. 2000). APP at the cell surface is internalized through clathrin-mediated endocytosis (Nordstedt et al. 1993). The vesicles are shuttled to endosomes with the help of Rab5 GTPase (Ikin et al. 1996), where APP is cleaved by β- and γ-secretase (Vassar et al. 1999), and the vesicle shuttled back to the cell surface for exocytosis (Koo et al. 1996). Hence, APL-1 transport in *C. elegans* neurons may be similar to APP transport in mammalian neurons. Because *C. elegans* is transparent, APL-1 transport can be directly monitored in vivo (Arimoto et al. 2011). Therefore, *C. elegans* might be a new useful approach to study APL-1/APP transport in vivo.

Expression of human APP in *C. elegans*

To examine whether human APP can be expressed and processed in *C. elegans*, transgenic animals carrying a human APP transgene were generated (Link 2006). Whole animal lysates were probed with different antibodies against APP and Aβ; fragments corresponding to a- and γ- secretase cleavages, but not β-secretase cleavage of human APP, were detected (Link 2006). By contrast, human APP in *Drosophila* is cleaved by a β-secretase-like enzyme (Carmine-Simmen et al. 2009). These results indicate that *C. elegans* can produce human APP, but human APP is only processed by an α/γ-secretase pathway and, consequently, no Aβ is produced from APP introduced into *C. elegans.*

Expression of human β-amyloid peptide in *C. elegans* neurons causes learning defects

In familial AD cases, mutations of the APP gene have been linked to increased production of $A\beta_{1–42}$, which is highly fibrillogenic and abundantly found in brain plaques of AD patients (Haass and Selkoe 1993). In addition, a number of transgenic mice lines that overexpress Aβ neuronally or triple transgenic mice (APP; Presenilin; Tau) formed intracellular Aβ deposits, which have been correlated with several defects, including neurodegeneration and a severe decrease in lifespan (LaFerla et al. 1995, 1997; Chui et al. 1999; Li et al. 1999; Gouras et al. 2000; Kuo et al. 2001; Wirths et al. 2001). To investigate the in vivo effects of Aβ on *C. elegans* physiology, transgenes of human β-amyloid peptide ($A\beta_{1–42}$) were expressed with a signal sequence using different promoters in *C. elegans.* Pan-neuronal expression of human Aβ42 (P*snb-1*::h$A\beta_{1–42}$) caused several neuronal deficits, including a chemotaxis defect toward the volatile chemoattractant benzaldehyde (Wu et al. 2006), an impaired associative avoidance response toward another volatile chemoattractant diacetyl (Dosanjh et al. 2010), impairment in serotonin-mediated behaviors, and mildly decreased lifespan compared with control animals (Dosanjh et al. 2010). These behavioral deficits suggest that human $A\beta_{1–42}$ can affect synaptic function and has a detrimental effect on lifespan in *C. elegans*. In addition, the transgenic animals showed intraneuronal Aβ deposits, but no apparent neurodegeneration (Link 2005, 2006).

Expression of human Aβ in *C. elegans* muscles causes paralysis and intracellular Aβ accumulation

In humans, intracellular accumulation of Aβ in muscles leads to myopathy (inclusion body myositis; (Askanas and Engel 2006). Similarly, transgene expression of human $A\beta_{1–42}$ in muscles led to the accumulation of intracellular Aβ and progressive paralysis during adulthood in *C. elegans* (Link 1995, 2001, 2006; Link et al. 2001). The paralysis phenotype was accompanied by oxidative stress and both were seen before Aβ fibril formation (Drake et al. 2003). Similarly, in transgenic mouse models, morphological and behavioral deficits preceded plaque formation (Jacobsen et al. 2006). Therefore, intracellular $A\beta_{1–42}$ accumulation could play a role in AD pathogenesis, and the *C. elegans* human $A\beta_{42}$ transgenic animals could be used to reveal the molecular mechanism of intracellular $A\beta_{1–42}$ accumulation.

Surprisingly, despite the presence of a signal peptide in the *C. elegans* human $A\beta_{1–42}$ model, only intracellular Aβ was detected (Link 1995, 2001; Link et al. 2001; Fonte et al. 2002). Furthermore, when the signal sequence is cleaved, two amino acids of $A\beta_{1–42}$ are included in the cleavage, so that actually human $A\beta_{3–42}$ is produced (McColl et al. 2009); we will refer to $A\beta_{1–42}$ and $A\beta_{3–42}$ produced in *C. elegans* collectively as $A\beta_{42}$. Despite missing two amino acids, a *C. elegans* Aβ transgenic model may nevertheless provide insights into the intracellular Aβ pathogenesis for AD and human myopathy.

Reduced insulin/IGF-1 signaling protects against intracellular Aβ pathogenesis

Reducing insulin/IGF-1 signaling prolongs lifespan in *C. elegans* (Kenyon et al. 1993), flies (Tatar et al. 2001), and mice (Bluher et al. 2003; Holzenberger et al. 2003). Furthermore, polymorphisms in genes involved in the insulin signaling pathway have been associated with increased lifespan in humans (Suh et al. 2008; Willcox et al. 2008; Flachsbart et al. 2009). In *C. elegans*, reducing the activity of the insulin/IGF-1 receptor DAF-2 increases the activity of FOXO transcription factor DAF-16 (Lin et al. 1997; Ogg et al. 1997) and bZip transcription factor SKN-1 (Tullet et al. 2008), both of which regulate transcription of distinct, but overlapping sets of genes involved in detoxification, protein folding, stress resistance and longevity (Honda and Honda 1999; Barsyte et al. 2001; McElwee et al. 2003; An and Blackwell 2003; Murphy et al. 2003; Oliveira et al. 2009; Park et al. 2009). Reducing DAF-2 signaling protected against the progressive paralysis and increased the lifespan of a *C. elegans* transgenic strain expressing human $A\beta_{42}$ in muscle (Cohen et al. 2006; Cohen et al. 2010). Interestingly, reduction in the insulin/IGF-1 signaling late in the worm lifespan was also sufficient to ameliorate the human $A\beta_{42}$-induced progressive paralysis (Cohen et al. 2010). Although overall mRNA or protein levels of human $A\beta_{42}$ were not altered (Cohen et al. 2006), those animals showed a shift from more soluble low molecular weight $A\beta_{42}$ aggregates to high molecular weight human $A\beta_{42}$ aggregates, which are presumed to be less toxic (Cohen et al. 2006, 2010). This active shift to high molecular weight $A\beta_{42}$ aggregated species, the protection against paralysis, and the increase in lifespan required DAF-16 activity (Cohen et al. 2006, 2010). Although insulin/IGF-1 signaling directly regulates the transcription factor SKN-1 (Tullet et al. 2008), the requirement of *skn-1* activity for the protection due to reduced *daf-2* activity has not been investigated. However, activation of *skn-1* by coffee extracts was required to delay progressive paralysis in the *C. elegans* transgenic strain expressing human $A\beta_{42}$ in muscle (Dostal et al. 2010). By contrast, reducing *skn-1* activity resulted in higher levels of human $A\beta_{42}$ protein of both low and high molecular aggregates and progressive paralysis (Dostal et al. 2010). SKN-1 might be involved in general disaggregation of proteins and their degradation, since SKN-1 also protects against aggregation of artificial GFP::degron protein (Dostal et al. 2010). Moreover, pharmacological suppression of human $A\beta_{42}$ aggregation and $A\beta_{42}$-induced paralysis by amyloid-binding compounds required *skn-1* but not *daf-16* activity (Alavez et al. 2011), suggesting a general importance for SKN-1 in $A\beta_{42}$ detoxification. Recently, polymorphisms in a *skn-1* orthologue Nrf2 (NFE2L2) gene and in human IGF-1 have been associated with early-onset AD (von Otter et al. 2010) and an increased risk for AD (Vargas et al. 2011), respectively. Some AD patients develop brain-specific diabetes that alters their insulin signaling (Steen et al. 2005). In mice, reducing IGF-1 signaling increased lifespan (Holzenberger et al. 2003), decreased APP-induced mortality rate (Freude et al. 2009), and protected against neuronal cell loss and behavioral deficits (Cohen et al. 2009). Furthermore, reducing IGF-1 signaling in transgenic AD mice resulted in a shift to high molecular weight $A\beta_{42}$ aggregates (Cohen et al. 2009), similar to what was seen in transgenic *C. elegans* $A\beta_{42}$ animals (Cohen et al. 2006, 2010). Taken together, these findings suggest that reducing insulin/IGF-1 signaling might be a preventive strategy against AD pathogenesis.

Molecular interactions of human $A\beta_{42}$ in *C. elegans* and AD patient brain tissue

Several approaches have been used in *C. elegans* to identify genes whose expression underlies intracellular human $A\beta_{42}$ accumulation. One approach is to identify physical binding partners to human $A\beta_{42}$. In transgenic animals that overexpress human $A\beta_{42}$ in muscles, $A\beta_{42}$ co-immunoprecipitated with six chaperone proteins: two proteins related to HSP70, which functions as an endoplasmic reticulum (ER) chaperone, a putative negative regulator of HSP70 R05F9.10, and three heat-shock proteins of the HSP-16 class with homology to αB-crystallin (Fonte et al. 2002). Interestingly, HSP-16/αB-crystallin also bound to $A\beta_{42}$ in muscle fibers of human inclusion body myositis patients (Banwell and

Engel 2000). A second approach is to take a more genomics approach and perform a microarray analysis to identify genes that are up- or downregulated in response to overexpression of human $A\beta_{42}$ in *C. elegans*. Such microarray assays revealed strong and specific up-regulation of two HSP-16/αB-crystallin chaperone transcripts, supporting the immunoprecipitation results, and two transcripts with homology to the mammalian TNFAIP1 (tumor necrosis factor a–induced protein) family in *C. elegans* (Link et al. 2003). Moreover, overexpression of HSP-16/αB-crystallin or knockdown of R05F9.10, the negative regulator of HSP70, partially suppressed the paralysis induced by $A\beta_{42}$ in transgenic animals that express human $A\beta_{42}$ in muscles (Fonte et al. 2008). In general, HSP chaperone proteins mediate trafficking of non-native or unfolded proteins for degradation in the lysosomes (Kettern et al. 2010). RNAi knock-down of lysosomal components or associated genes enhanced the paralysis rate and caused higher $A\beta_{42}$ levels of transgenic animals that overexpress human $A\beta_{42}$ in muscles (Florez-McClure et al. 2007). Hence, HSP-16/αB-crystallin and HSP70 proteins become upregulated by the excessive $A\beta_{42}$ levels and bind $A\beta_{42}$ directly for clearance via lysosomes. To determine whether these results have relevance for AD, αB-crystallin and TNFAIPI transcripts in AD brain tissue were examined and found to be upregulated as well (Link et al. 2003). Thus, a common mechanism for the detoxification or clearance of intracellular human $A\beta_{1\text{-}42}$ in neurons and muscle tissue is used by *C. elegans* and mammals.

Concluding remarks

A common theme in molecular processes has shown that once a pathway or mechanism has been established, it gets used over and over again in modified forms. The *C. elegans* APL-1 has multifunctional roles and acts non-cell autonomously. Either loss of APL-1 or overexpression of APL-1 results in lethality, showing the importance of maintaining APL-1 within homeostatic levels, particularly within neurons. Much remains to be understood about the mechanism of APL-1 function. Future genetic screens in *C. elegans* may reveal possible receptors to which sAPL-1 binds after its release. The greatest challenge in the field is to show the relevance of findings in *C. elegans* to mammalian biology or AD directly. Example of discoveries in *C. elegans*, such as the components of the γ-secretase complex, advanced the AD field tremendously. Furthermore, *C. elegans* has been a particularly useful model organism for outlining a pathway for clearance of intracellular Aβ deposits; mammals, including humans, use common elements in their clearance in both AD pathogenesis and human myopathy. Effects of intracellular Aβ accumulation are ameliorated by activation of general protein degradation pathways or longevity genes in *C. elegans* (Cohen et al. 2006; Florez-McClure et al. 2007; Fonte et al. 2008; Hassan et al. 2009). Drugs improving protein degradation might be a new strategy to fight Alzheimer's disease.

Acknowledgments

We wish to thank Alicia Melendez for her kind gift of the *C. elegans* strain carrying [RFP::BEC-1], Chris Link, Andrea Silva, and Greg O'Connor for permission to cite unpublished observations and members of our laboratory for many helpful discussions. This work was supported by grants from the Alzheimer's Association, National Science Foundation, and National Institutes of Health.

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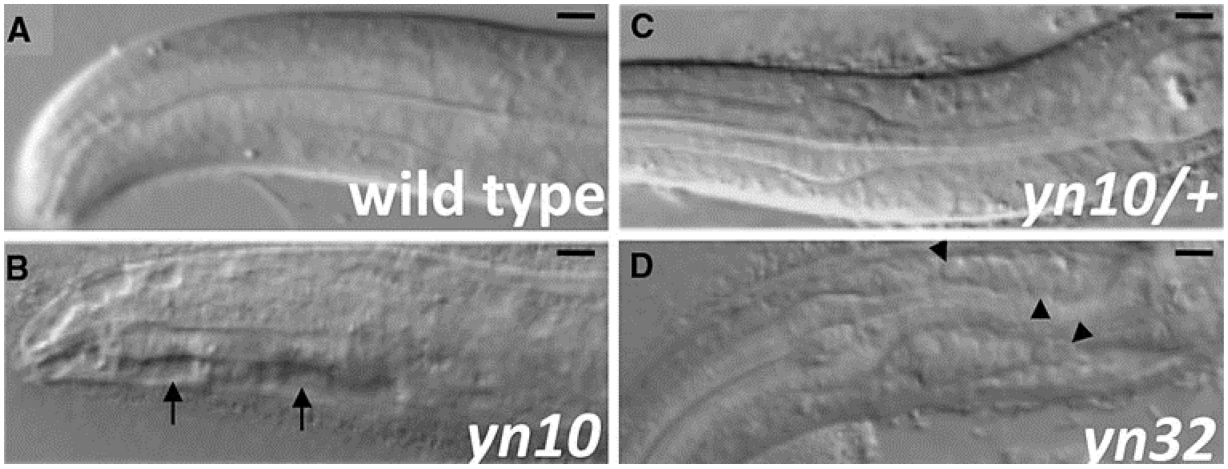

Fig. 1.

Loss of *apl-1* leads to lethality, detachment of cells, and formation of vacuolar-like structures. *apl-1* complete loss-of-function (KO) animals show a fully penetrant lethality due to a molting defect. KO animals also show other defects. a–d Show head regions of *C. elegans* at the first larval stage (L1; anterior left, ventral side down). *apl-1(yn10)* KO mutants (b) show vacuolar-like structures (arrow) in the hypodermis, which were not observed in wild-type animals (a). A heterozygous *apl-1(yn10)* animal (c) appears superficially wild type. By contrast, cells appeared detached (arrowheads) in an *apl-1(yn32)* KO mutant. Scale bar 10 μm

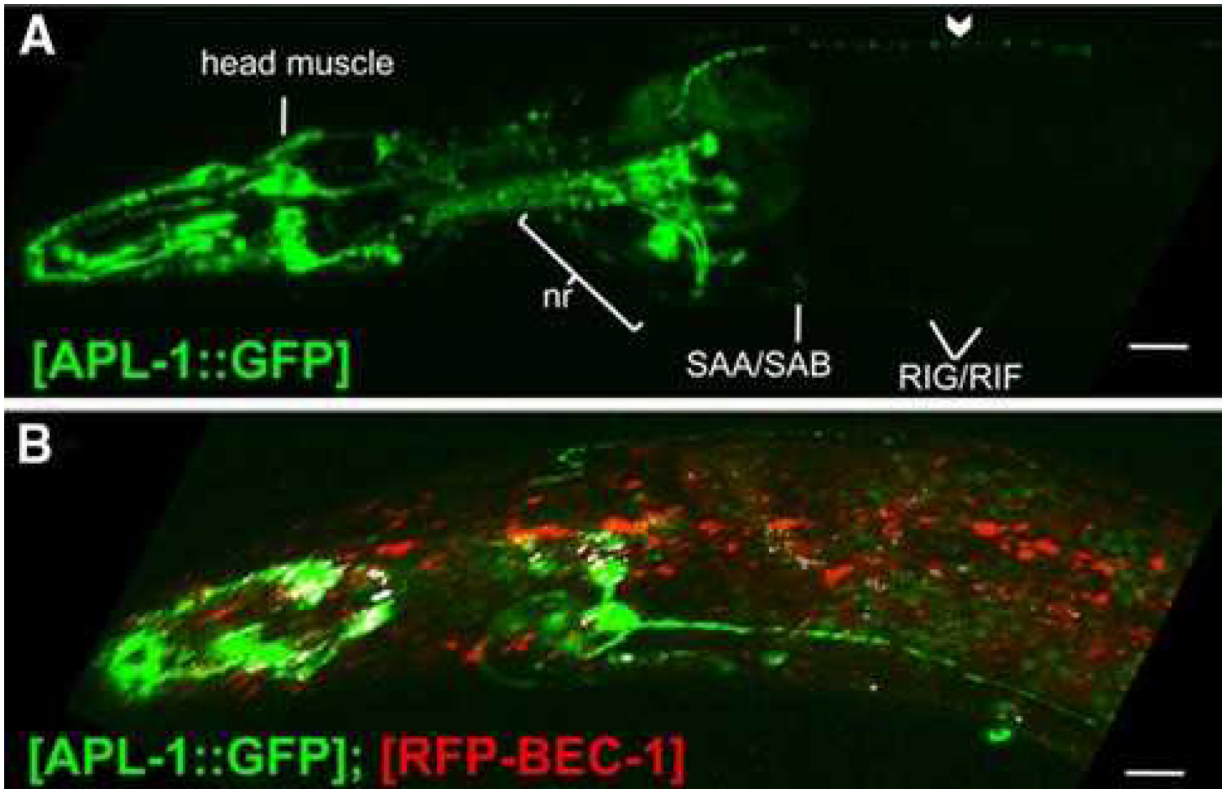

Fig. 2.

APL-1 is expressed in multiple cell types and is not degraded via autophagy. a) In wild-type animals, APL-1::GFP is expressed in neurons (SAA/SAB and RIG/RIF can be seen with faint expression) and processes in the nerve ring (nr). APL-1::GFP showed punctate expression in axonal processes (chevron) and head muscles in the anterior region of wild-type worms. b) APL-1::GFP (green) shows little co-localization with RFP::BEC-1 (red). Overlapping areas are shown in white of the confocal z-stack. Scale bars 10 μm

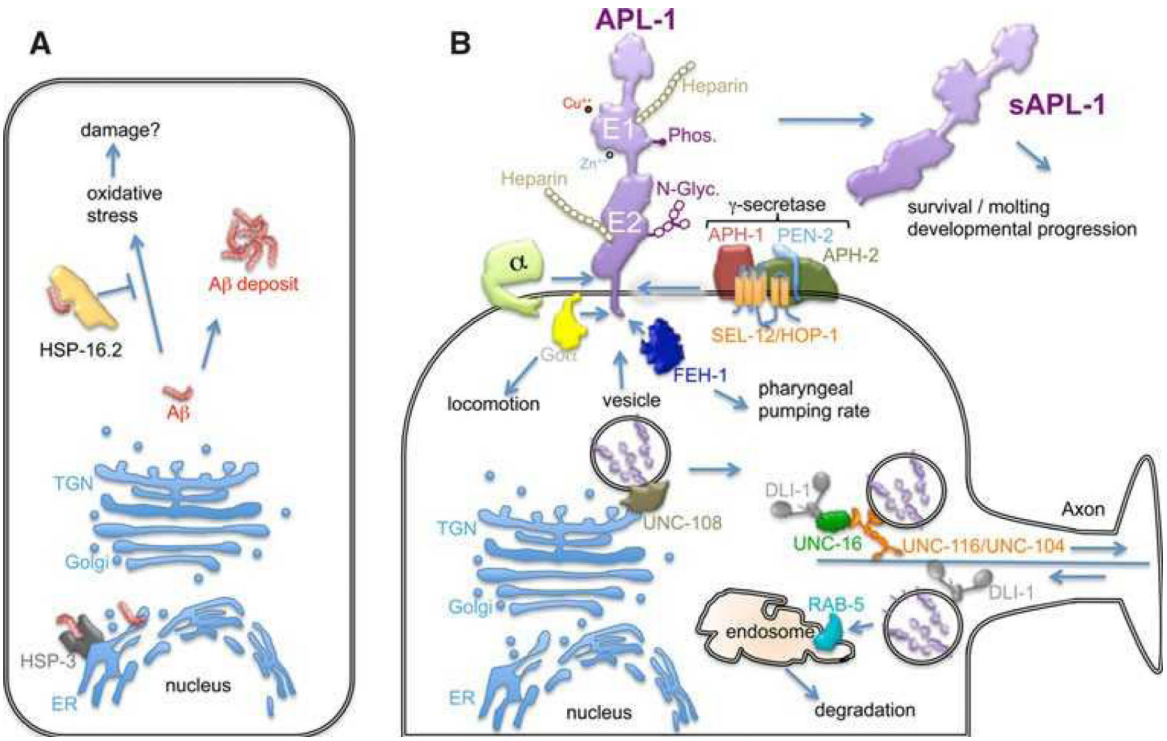

Fig. 3.

Model for Aβ and APL-1 trafficking and function. a) Aβ in the endoplasmic reticulum (ER) is bound by the chaperone HSP-3, an orthologue of HSP70, to mediate Aβ folding or degradation. Soluble Aβ in the cytoplasm can lead to oxidative stress and presumably cellular damage or can form intracellular deposits. HSP-16.2, an orthologue of αB-crystallin, binds Aβ in the cytoplasm to target it for degradation. b) By passing through the ER and Golgi, APL-1 is N-glycosylated before budding off in vesicles from the trans-Golgi-network (TGN) with the help of UNC-108/GTPase. The UNC-104/ UNC-116 kinesin motors transport the APL-1 vesicles along micro- tubules in the anterograde direction, while the DL1-1 dynein motor transports the vesicle retrogradely; UNC-16 functions as a cargo adaptor for anterograde transport. On the cell membrane, APL-1 could potentially bind heparin, copper (Cu^{++}), and zinc (Zn^{++}) and be phosphorylated. APL-1 affects multiple functions: (1) locomotion via intracellular $G_o\alpha$; (2) pharyngeal pumping rate via binding of FEH-1 to the YENPTY site on APL-1; and (3) developmental progression, survival and molting by α/γ-secretase cleavage and release of the extracellular domain of APL-1 (sAPL-1). The remaining intracellular fragment and full-length APL-1 are endocytosed and transported via DLI-1/dynein along microtubules to the early endosome containing RAB-5 GTPase for degradation