Tol2 Gene Trap Integrations in the Zebrafish Amyloid Precursor Protein Genes appa and aplp2 Reveal Accumulation of Secreted APP at the Embryonic Veins

Hsin-Kai Liao,1,2† Ying Wang,1† Kristin E. Noack Watt,1 Qin Wen,1 Justin Breitbach,1 Chelsy K. Kemmet,1 Karl J. Clark,3 Stephen C. Ekker,3 Jeffrey J. Essner,1 and Maura McGrail1*

Background: The single spanning transmembrane amyloid precursor protein (APP) and its proteolytic product, amyloid-beta (Aβ) peptide, have been intensely studied due to their role in the pathogenesis of Alzheimer’s disease. However, the biological role of the secreted ectodomain of APP, which is also generated by proteolytic cleavage, is less well understood. Here, we report Tol2 red fluorescent protein (RFP) transposon gene trap integrations in the zebrafish amyloid precursor protein a (appa) and amyloid precursor-like protein 2 (aplp2) genes. The transposon integrations are predicted to disrupt the appa and aplp2 genes to primarily produce secreted ectodomains of the corresponding proteins that are fused to RFP.

Results: Our results indicate the Appa-RFP and Aplp2 fusion proteins are likely secreted from the central nervous system and accumulate in the embryonic veins independent of blood flow.

Conclusions: The zebrafish appa and aplp2 transposon insertion alleles will be useful for investigating the biological role of the secreted form of APP. Developmental Dynamics 241:415–425, 2012. © 2012 Wiley Periodicals, Inc.

Key words: Tol2 gene trap; endothelial cells; vein; vasculature; central nervous system

Key findings:
- Isolation of appa and aplp2 Tol2 gene trap alleles; appa and aplp2 expression in neuronal tissues during embryogenesis.
- Secretion of predicted appa-RFP and aplp2-RFP fusion proteins.
- Accumulation of appa-RFP and aplp2-RFP in living embryos at veinous vasculature.

Accepted 13 December 2011

INTRODUCTION

Alzheimer’s disease (AD) is the most prevalent form of human dementia, accounting for 60–70% cases worldwide. The neural pathology of AD includes senile plaques, neurofibrillary tangles, and loss of neurons. In addition, there is a significant vascular pathology in AD characterized by amyloid deposits in cerebral vessel walls (cerebral amyloid angiopathy), as well as structural abnormalities in the microvasculature (Revesz et al., 2003; Bailey et al., 2004; Storkebaum et al., 2011). The amyloid precursor protein (APP) is known to be the source of the hydrophobic peptide amyloid β (Aβ) that is a major component of amyloid deposits in the brains of AD patients (Kang et al., 1987; Newman et al., 2007; Kumar-Singh, 2008; Storkebaum et al., 2011). The amyloid precursor protein (APP) is known to be the source of the hydrophobic peptide amyloid β (Aβ) that is a major component of amyloid deposits in the brains of AD patients (Kang et al., 1987; Newman et al., 2007;
Philipson et al., 2010). Membrane bound APP is composed of a large extracellular amino-terminal domain, a single transmembrane domain, and a short cytoplasmic domain (reviewed in Gralle and Ferreira, 2007; Jacobsen and Iverfeldt, 2009). The processing of APP involves regulated intramembrane proteolysis, which can be divided into two major pathways. Approximately 90% of APP proteolytic processing is through the nonamyloidogenic pathway, in which cleavage of the extracellular domain by α-secretase releases a soluble form of APP (sAPPα) into the extracellular space. Subsequent cleavage by γ-secretase releases the sAPPβ into the cytoplasm. The remaining 10% of APP processing occurs by means of the amyloidogenic pathway in which the extracellular domain is cleaved at a different residue by β-secretase. This releases an alternative extracellular soluble form, sAPPβ. Cleavage of the remaining membrane bound protein by γ-secretase releases the hydrophobic Aβ peptide into the extracellular space. Although there are extensive studies on APP and Aβ peptides, the in vivo biological function and localization of secreted sAPP is not completely known, nor is the contribution of sAPP to the neural and vascular pathogenesis of AD.

Studies in Drosophila and mammalian cell culture systems have implicated sAPP in the regulation of neurite outgrowth (Small et al., 1994), neuronal survival (Araki et al., 1991) and neuroprotection (Goodman and Mattson, 1994). In addition, the sAPP peptide is sufficient to rescue motilting and morphogenesis defects from loss of APL-1 in Caenorhabditis elegans and is suggested to function in a cell-nonautonomous manner (Hornsten et al., 2007). In mice, a knock-in allele that produces sAPPα exclusively rescues the postnatal lethality in APP/APLP2 double mutants (Weyer et al., 2011), suggesting that much of the normal biological function of the APP gene family can be mediated through the soluble extracellular domains. It is also intriguing that patients with AD display reduced levels of sAPPα cleavage peptide (Lannfelt et al., 1995), raising the possibility that the sAPPα could contribute to the pathogenesis of AD. In humans and mice, the APP genes are predominantly expressed in neural tissues, and there is little evidence for expression in cell types other than neurons or ganglia (Goldgaber et al., 1987; Tanzi et al., 1987; Arai et al., 1991).

The application of Tol2 DNA transposons for gene trap, enhancer trap, and germline mutagenesis screens is well established in zebrafish (Balcuiñas and Ekker, 2005; Balcuiñas et al., 2006; Kawakami, 2007; Largaespada, 2009; Suster et al., 2009; Ivics and Izsvak, 2010). A major advantage of using gene trap transposons as a mutagen is that the integrated transposon acts as a molecular tag that facilitates gene cloning. Zebrafish are particularly well suited to gene trap insertional mutagenesis due to the optical clarity of the embryo and the amenability of the organism to largescale screens. Gene trap transposons are engineered to intercept splicing of the endogenous gene transcript and produce fluorescent proteins that act as reporters of the normal expression pattern of the mutated gene. This approach, using red fluorescent protein (RFP) or green fluorescent protein (GFP) trap Tol2 transposons, has identified many genes with tissue specific patterns of interest in the developing zebrafish embryo (Balcuiñas et al., 2004; Kawakami et al., 2004, 2010; Parinov et al., 2004; Choo et al., 2006; Kawakami, 2007; Sivasubbu et al., 2007; Asakawa and Kawakami, 2009). Recent modification of the Tol2 gene trap system has yielded a powerful tool for expression as well as functional annotation of the zebrafish genome (Clark et al., 2011). Live cell imaging of trapped fluorescent proteins in the zebrafish embryo can yield novel insights into the biological role of the gene product in a particular cell or developmental process.

In the present study, we carried out a germline mutagenesis screen using the Tol2 gene trap GBT-R15 (Petzold et al., 2009). The GBT-R15 transposon contains a splice acceptor, an RFP lacking the first initiating AUG codon, and a transcriptional terminator. Out of 227 founder adults, 37 produced progeny with tissue and cell-type specific patterns of GFP gene expression in the embryo (frequency of 16%). This frequency is comparable to previously published enhancer trap and gene trap reports in zebrafish (Balcuiñas and Ekker, 2005). Four of the 37 lines showed RFP expression in the embryonic vasculature, named V1–V4. To identify the Tol2 integration site in the vascular gene trap lines, we performed 5’-rapid amplification of cDNA ends (RACE) on total RNA isolated from 2 days post fertilization (dpf) RFP expressing embryos. The 5’-RACE product from line V2 contained the first 4 exons of the zebrafish amyloid beta (A4) precursor protein a (appa) gene fused to the 5’ end of RFP. The V3 line 5’-RACE product contained the first 12 exons of the amyloid beta (A4) precursor-like protein 2 (aplp2) gene fused to the 5’

RESULTS AND DISCUSSION
Isolation of appla and aplp2 Tol2 Gene Trap Alleles
To identify genes involved in early vascular development, we performed a germline mutagenesis screen using the Tol2 RFP gene trap GBT-R15 (Petzold et al., 2009). The GBT-R15 transposon contains a splice acceptor, an RFP lacking the first initiating AUG codon, and a transcriptional terminator. Out of 227 founder adults, 37 produced progeny with tissue and cell-type specific patterns of GFP gene expression in the embryo (frequency of 16%). This frequency is comparable to previously published enhancer trap and gene trap reports in zebrafish (Balcuiñas and Ekker, 2005). Four of the 37 lines showed RFP expression in the embryonic vasculature, named V1–V4. To identify the Tol2 integration site in the vascular gene trap lines, we performed 5’-rapid amplification of cDNA ends (RACE) on total RNA isolated from 2 days post fertilization (dpf) RFP expressing embryos. The 5’-RACE product from line V2 contained the first 4 exons of the zebrafish amyloid beta (A4) precursor protein a (appa) gene fused to the 5’ end of RFP. The V3 line 5’-RACE product contained the first 12 exons of the amyloid beta (A4) precursor-like protein 2 (aplp2) gene fused to the 5’
end of RFP. These results indicate that in gene trap line V2 the Tol2 transposon had integrated into intron 4 of the appa gene (Fig. 1A), while in line V3 the transposon had integrated into intron 12 of the aplp2 gene (Fig. 1A). Zebrafish appa (Ensembl/Zv9: ENSDARG00000059036) and aplp2 (Ensembl/Zv9: ENSDARG00000054864) are orthologues of human APP and APLP2, respectively, and are both members of the amyloid beta (A4) precursor protein family. The gene trap insertion alleles for the two lines isolated in this study are designated as appa<sup>appais22Gt</sup> and aplp2<sup>aplpis23Gt</sup>.

To confirm the location of the Tol2 integration sites in lines appa<sup>appais22Gt</sup> and aplp2<sup>aplpis23Gt</sup>, genomic Southern blot analyses were performed with probes specific to the appa and aplp2 genes, and the Tol2 GBT:R15 transgene. Using a gene-specific probe a larger restriction fragment length polymorphism (RFLP) is detected in line appa<sup>appais22Gt</sup> and aplp2<sup>aplpis23Gt</sup> (Supp. Fig. S1A,B, which is available online), as expected based on the predicted location of the transposon integration site in each gene (Fig. 1A). Genomic Southern analysis on lines appa<sup>appais22Gt</sup> and aplp2<sup>aplpis23Gt</sup> using a probe complementary to the RFP cDNA detected a single RFLP of the expected size in each line (Supp. Fig. S2A,B). Together these results demonstrate that the RFP expression patterns observed in lines appa<sup>appais22Gt</sup> and aplp2<sup>aplpis23Gt</sup> are the result of a single Tol2 gene trap integration in the appa and aplp2 genes, respectively.

**Zebrafish App Family**

The human APP gene family encodes single-pass transmembrane proteins that are cleaved to produce extracellular and intracellular soluble peptides. The zebrafish appa and aplp2 genes encode proteins that have the same predicted structure, with a large extracellular domain containing multiple subdomains, a single transmembrane domain, and a short cytoplasmic tail (Musa et al., 2001; Fig. 1B). The conserved extracellular domain contains two heparin-binding domains (HBD), a growth factor like domain (GFLD), a metal-binding domain (Cu/Zn BD), an acidic domain (DE) and a Kunitz protease inhibitor domain (KPI; Fig. 1B). In addition, the locations of predicted cleavage sites recognized by α-, β-, and γ-secretases are conserved (Fig. 1B, Supp. Fig. S3). This indicates that the same pathways likely process the zebrafish proteins as human APP to create the soluble and secreted forms of APP. The location of the Tol2 gene trap integration in each gene suggests that the Appa-RFP and Aplp2-RFP gene trap proteins are secreted. The transposon had integrated into an intron upstream of the exons that code for the single-pass transmembrane domain (Fig. 1A,B; Supp. Fig. S3). The accumulation of the Appa-RFP and Aplp2-RFP gene trap proteins are secreted the transposon had integrated into an intron upstream of the exons that code for the single-pass transmembrane domain (Fig. 1A,B; Supp. Fig. S3). The accumulation of the Appa-RFP and Aplp2-RFP gene trap proteins are secreted. The transposon had integrated into an intron upstream of the exons that code for the single-pass transmembrane domain (Fig. 1A,B; Supp. Fig. S3). The accumulation of the Appa-RFP and Aplp2-RFP gene trap proteins are secreted. The transposon had integrated into an intron upstream of the exons that code for the single-pass transmembrane domain (Fig. 1A,B; Supp. Fig. S3). The accumulation of the Appa-RFP and Aplp2-RFP gene trap proteins are secreted.
Expression Patterns of Zebrafish appa and aplp2

The APP genes are widely expressed in human adult and fetal tissues (Goldgaber et al., 1987; Tanzi et al., 1987); however, tissues are composed of multiple cell types, and there is little evidence that the genes are expressed in cell types other than neurons. Although there is one report of human APP protein localization in endothelial cells of the gut (Cabal et al., 1995), there are no reports in the literature of APP mRNA expression in vascular cells. A previous study showed that appa gene expression is first detectable at the mid-gastrula stage in the zebrafish embryo (Musa et al., 2001). We used reverse transcriptase-polymerase chain reaction (RT-PCR) to examine the relative expression level of appa and aplp2 in the developing embryo and adult tissues (Fig. 2B). A low level of appa expression is detected in 1 to 12 hours post fertilization (hpf) embryos. This level increases substantially at 24 hpf, and remains high through 5 dpf. In contrast, the level of aplp2 is relatively low from day 1 through day 5. In adult tissues, both appa and aplp2 are widely expressed except in the muscle and liver. The level of expression of appa is higher in the adult brain compared with aplp2, while aplp2 is higher than appa in the ovary and intestine. These results suggest that zebrafish appa and aplp2 are widely expressed throughout development, similar to the human APP gene family.

To identify the cell types that express zebrafish appa and aplp2 during early development, we performed whole-mount in situ hybridization on wild-type embryos. Zebrafish appa has previously been shown to be expressed in the developing nervous system at 24 hpf (Musa et al., 2001). We also observed expression of appa, as well as aplp2, throughout the central nervous system at 36 hpf (Fig. 3). Control experiments using appa and aplp2 sense probes showed no detectable pattern above background (Supp. Fig. S4). Both appa and aplp2 show very similar patterns in the embryo and are expressed throughout the forebrain, midbrain and hindbrain (Fig. 3A,G) and along the length of the neural tube (Fig. 3C,F,K). Expression was also detected in cranial ganglia (Fig. 3B,H), the trigeminal ganglia (Fig. 3H,I) and anterodorsal and posterior lateral line ganglia (Fig. 3E,L). A cross-section through the trunk shows expression of both genes in the pronephric ducts and the neural tube (Fig. 3F,K). appa is expressed in the neuromasts of the lateral line (Fig. 3C,F), while a low level of aplp2 is detected in the floor plate (Fig. 3K, inset). Neither gene is expressed at detectable levels in the dorsal aorta, caudal vein, vessels in the head or intersegmental vessels of the trunk.

Secreted Appa- and Aplp2-RFP Fusion Proteins Accumulate at the Zebrafish Embryonic Vasculature

We examined the localization of the Appa-RFP and Aplp2-RFP fusion proteins in the embryonic vasculature by whole-mount immunohistochemistry using a rabbit polyclonal antibody against human APP. Both proteins accumulate in the vasculature at later stages of development, with a strong signal in the yolk sac and developing yolk sac. The signal is particularly strong in the posterior region of the developing embryo, where the vasculature is most active. The signal is also detected in the developing heart and arterial vessels of the trunk. These results suggest that both Appa and Aplp2 are secreted by the vascular endothelial cells and accumulate in the extracellular matrix of the developing vasculature.
Fig. 3. Expression of appa and aplp2 in developing central and peripheral nervous system of 36 hpf zebrafish embryos. A: Lateral view, appa expression in the eye lens, optic tectum, midbrain tegmentum, hindbrain, and posterior lateral line ganglion. B: Dorsal view, appa expression in the cerebellar plate and ganglia. C: Lateral view, appa expression in the neural tube and lateral line neuromasts (arrowheads). Line labeled “F” refers to position of cross-section shown in panel F. D: Camera lucida drawing of 35 hours post fertilization (hpf) zebrafish embryo (Kimmel et al., 1995). Line labeled “E, J” refers to position of cross-sections in panels E and J. E: appa expression in the posterior lateral line ganglia and lateral regions of medulla oblongata (arrowheads). F: appa expression in the neural tube, neuromasts of the lateral line, and pronephric ducts. G: Lateral view of aplp2 expression in the epiphysis, olfactory placode, cerebellum, ganglia, and neural tube. Line labeled “K” refers to position of cross-section shown in panel K. H: Dorsal view, aplp2 expression in the epiphysis, trigeminal ganglia, and ganglia in the hindbrain. I: Lateral view, aplp2 expression in the trigeminal ganglia, anterodorsal and posterior lateral line ganglia. J: aplp2 expression in posterior lateral line ganglion and lateral regions of medulla oblongata (arrowheads). K: aplp2 expression in lateral regions of the neural tube, floor plate, and pronephric duct. Inset shows higher magnification view of boxed region of neural tube. Arrow points to expression of aplp2 in the floor plate region. ADLG, anterodorsal lateral line ganglia; Cb, cerebellum; CeP, cerebellar plate; EL, eye lens; Ep, epiphysis; FP, floor plate; Ga, ganglion; Hb, hindbrain; MO, medulla oblongata; MT, midbrain tegmentum; NL, neuromasts of lateral line; NT, neural tube; Op, olfactory placode; OT, optic tectum; PD, pronephric duct; PLLG, posterior lateral line ganglion; TG, trigeminal ganglia. Scale bars = 50 μm.
proteins by fluorescence and confocal microscopy in living 36 hpf embryos. Appa-RFP localization is primarily in the caudal vein but can be detected in some of the intersegmental vessels (Fig. 4A,B). A low level of expression can be detected throughout the central nervous system in the head and trunk (Fig. 4A). A similar pattern of RFP fluorescence was detected for the Appa-RFP protein in aplp2is23Gt embryos (Fig. 5A,B). Several veins are in close association with each region of the central nervous system and pronephric ducts where appa and aplp2 were detected by in situ hybridization. These vessels include the cerebral and cardinal veins, intersegmental vessels,
and the axial vein. It is possible that Appa-RFP fusion proteins are secreted by neurons and accumulate in the vessels located nearby. In situ hybridization with an RFP-specific probe on appaIs22Gt and aplp2Is23Gt 36 hpf embryos reveals a pattern similar to the endogenous appa and aplp2 genes. A high level of expression was present in the central nervous system and pronephric ducts, however, no signal was detected in the endothelial cells of the blood vessels (Supp. Fig. S5). These results are consistent with the prediction that the Appa-RFP and Aplp2-RFP fusion proteins are secreted from neuronal cells and cells of the pronephric ducts and subsequently accumulate at the embryonic vasculature.

To confirm that Appa-RFP and Aplp2-RFP localization is restricted to the venous vessels, we created double transgenic embryos using a line that expresses a GFP fusion protein in endothelial cells of both arteries and veins. The transgenic line Tg(flk1:moesin-egfp)Is1 (Wang et al., 2010) expresses a Moesin1-GFP fusion protein under the control of the flk1 promoter, which results in expression in the endothelial cells of the vasculature (Jin et al., 2005). The appaIs22Gt and aplp2Is23Gt lines were crossed to Tg(flk1:moesin-egfp)Is1 and living embryos imaged by confocal microscopy. Similar results were observed in appaIs22Gt; Tg(flk1:moesin-egfp)Is1/Is1 and aplp2Is23Gt; Tg(flk1:moesin-egfp)Is1/Is1 embryos (Fig. 4). Comparison of GFP and RFP localization in the trunk showed overlap only in the posterior caudal vein and the ventral region of the intersegmental vessels (Figs. 4C,D, 5C,D). In the head, GFP was detected throughout the veins and arteries, whereas RFP was primarily in the veins, including the common cardinal vein, midcerebral vein (MCeV), and the primordial hindbrain channel (PHBC; Figs. 4E,F, 5E,F). We confirmed the localization of RFP in the veins by immunolocalization of RFP in fixed 36 hpf transgenic embryos. A cross-section through the trunk shows RFP enriched at the posterior caudal vein but absent from the neural tube and posterior lateral line ganglia (Figs. 4H, 5H). Confocal microscopy of double transgenic living embryos suggested that Appa-RFP and Aplp2-RFP fusion proteins were localized to both the luminal and abluminal surface of the endothelial cells of the posterior caudal vein, as well as in intracellular regions possibly corresponding to endocytic vesicles (Figs. 4G,I, 5G,I).

Examination of Appa-RFP and Aplp2-RFP protein localization in embryos at earlier stages of development is consistent with the in situ hybridization data indicating expression is primarily restricted to neural tissues. Appa-RFP and Aplp2-RFP showed accumulation in locations in which blood vessels had not yet formed. At 24 hpf, Appa-RFP is detected at a low level throughout the neural tube and central nervous system (Supp. Fig. S6A–D). In the head Appa-RFP is enriched in an area around the lens in the eye (Supp. Fig. S6A,B, yellow arrows). Appa-RFP is also enriched at the somite boundaries in the trunk (Supp. Fig. S6C,D, yellow arrows), before the intersegmental vessels have completely formed. Later, at 28 hpf, Appa-RFP is detected in the region of the posterior caudal vein (Supp. Fig. 6G,H, white arrows). Similar results were observed with Aplp2-RFP protein localization (data not shown). Taken together, these results suggest that the Appa-RFP and Aplp2-RFP fusion proteins are not expressed in vascular cells, but instead are expressed in neurons, ganglia, and cells of the pronephros and are secreted. These results are consistent with studies of human β-APP that detected the presence of the proteins in neural tissues and only a few nonneural tissues, namely the pituitary and adrenal glands and cardiac muscle (Arai et al., 1991). The expression patterns in zebrafish indicate that following production in the neurons, the secreted Appa-RFP and Aplp2-RFP fusion proteins become enriched at the venous vessels in the embryo after 28 hpf.

**Appa-RFP and Aplp2-RFP Are Localized to Blood Vessels in the Absence of Blood Flow**

We next tested whether blood flow was required for the accumulation of the fusion proteins in the veins by crossing the gene trap alleles into a silent heart (tnnt2a) mutant background (Clark et al., 2011), which lacks a heartbeat and blood flow due to a mutation in the cardiac troponin T2a gene, tnnt2a (Sehnert et al., 2002). The appaIs22Gt/+, sihn0031Gt/+ or aplp2Is23Gt/+, sihn0031Gt/+ double heterozygous fish were further crossed with Tg(flk1:moesin1-egfp)Is1/Is1; sihn0031Gt/+ to generate homozygous appaIs22Gt/+, sihn0031Gt/+ or aplp2Is23Gt/+, sihn0031Gt/+ mutants in a Tg(flk1:moesin1-egfp)Is1/Is1 background. Despite the absence of a heartbeat or normal blood flow in sihn0031Gt/+ or aplp2Is23Gt/+ mutant embryos, Appa-RFP and Aplp2-RFP accumulated at the venous vessels, similar to control homozygous sihn0031Gt/+ siblings with a normal heart beat (Supp. Fig. S7). These results demonstrate that the accumulation of Appa-RFP and Aplp2-RFP at the veins occurs by a mechanism independent of blood flow.

We followed the expression of the Appa-RFP and Aplp2-RFP fusion proteins in older animals and found that RFP accumulated in the kidneys beginning at 5 dpf; however, RFP was still detected at the venous vessels even at 10 dpf (data not shown). Adult fish were dissected and the organs examined for RFP fluorescence on a standard epi-illumination microscope. RFP expression was detected in cells embedded in the membrane covering the brain (Supp. Fig. S8A,B,E–H). These cells are likely to be microglia, which are the resident macrophages of the central nervous system. RFP expressing cells were also present in the connective tissue in the ovary and the membrane covering the spleen (data not shown). RFP was not detected in muscle, liver, skin, intestine, or spleen. Consistent with the expression of Appa-RFP and Aplp2-RFP in older larvae, RFP was highly expressed throughout the kidney tubules (Supp. Fig. S8C–E,1).

The localization of Appa-RFP and Aplp2-RFP is distinct from the mRNA expression patterns of appa and aplp2, suggesting that the fusion proteins are secreted by nonvascular cells and then accumulate at the veins. Because both fusion proteins contain nearly the entire N-terminal extracellular domain, similar to the soluble secreted form of sAPP, it is possible that the conserved subdomains (i.e., heparin binding/growth factor like, metal binding, acidic, or KIP domains) are responsible for the binding of the fusion proteins to the extracellular matrix at the veins.
Studies of the extracellular domain of APP indicate APP functions as an adhesion molecule/contact receptor, has the ability to self-dimerize, and binds to specific components of the extracellular matrix including collagen, heparin-sulfate proteoglycans, and F-spondin (Gralle and Ferreira, 2007; Jacobsen and Iverfeldt, 2009). Release of the intracellular domain of APP by the γ-secretase pathway is stimulated by binding of the extracellular domain to the GPI-linked ligand TAG1 (Ma et al., 2008), similar to the processing of other type-1 transmembrane proteins such as Notch. Fibulin-1, a member of the fibulin family of secreted glycoproteins that are components of basement membrane, has been shown to bind APP’s amino-terminal growth factor-like domain; this interaction contributes to the neurotrophic activities of APP (Ohsawa et al., 2001). In addition, Fibulin-1 and fibulin-5 have been shown to suppress angiogenesis in tumors derived from mice injected with HT1080 cancer cells (Xie et al., 2008). Fibulin-1 knock-out mice presented with a severe perinatal lethal phenotype with structural defects in endothelial cells of the microvasculature (Kostka et al., 2001). It is possible that sAPP might bind fibulin-1 in the vascular basement membrane in zebrafish, and this interaction could account for the localization of the Appa-RFP and Aplp2-RFP fusion proteins at the embryonic veins. Binding to other components of the extracellular matrix could also contribute to the localization of Appa-RFP and Aplp2-RFP in the zebrafish embryo.

Previous analyses of the zebrafish appb gene demonstrated that endogenous appb is expressed in the central nervous system (Musa et al., 2001), while an appb promoter-GFP transgene revealed GFP present throughout the nervous system and the embryonic vasculature (Lee and Cole, 2007). Our present study demonstrates that endogenous appa and aplp2 are expressed primarily in the central and peripheral nervous system, and in the pronephric ducts of the developing kidney, but not in the vasculature. Our results for appa are consistent with previous analyses of zebrafish appa and appb (Musa et al., 2001), and of human and mouse APP genes (Goldgaber et al., 1987; Tanzi et al., 1987; Arai et al., 1991). Studies on Presenilin-1 (PS1), one of three genes (APP, PS1, and Presenilin-2) known to be mutated in familial AD, suggest that signaling between neuronal and vascular tissues plays a role in the pathology of AD. Gama-Sosa et al. have shown that transgenic mice overexpressing a mutant form of human PS1 develop vascular pathologies (Gama Sosa et al., 2010). The PS1 transgene is specifically expressed in neural cells and is absent from vascular endothelial cells (Gama Sosa et al., 2010). Our similar findings on zebrafish appa and aplp2 expression suggest the appa<sup>is22Gt</sup> and aplp2<sup>is23Gt</sup> alleles could provide new insights into the role of secreted APP proteins in vascular biology and the mechanism linking the neural and vascular pathologies of Alzheimer’s disease.

In a recent study, the knockdown of appb was shown to cause defective convergent extension movements in zebrafish embryos, while knockdown of appa had no apparent effect on development (Joshi et al., 2009). The zebrafish appa<sup>appais22Gt</sup> and aplp2<sup>aplp2is23Gt</sup> insertion alleles isolated in the present study are homozygous viable, consistent with the previously published morpholino knockdown experiments. The lack of an obvious phenotype could be due to functional redundancy among APP family members in zebrafish. This could also be a result of low amounts of wild-type appa and aplp2 transcripts present in homozygous appa<sup>appais22Gt</sup> or aplp2<sup>aplp2is23Gt</sup> embryos, as detected by RT-PCR (Supp. Figs. S9C, S10C). This indicates that the gene trap alleles do not completely disrupt normal splicing of the gene and are not null alleles. Moreover, the production of soluble Appa-RFP and Aplp2-RFP fusion proteins may compensate for the loss of the full-length Appa and Aplp2 proteins, similar to studies in mice (Weyer et al., 2011). The ligand binding and adhesive activity of the extracellular domain of APP is important for its role in synaptogenesis, cell adhesion, and neurite outgrowth, but the exact biological function remains unclear (Jacobsen and Iverfeldt, 2009). The secreted Appa-RFP proteins expressed by the zebrafish appa<sup>appais22Gt</sup> and aplp2<sup>aplp2is23Gt</sup> gene trap alleles isolated in our study will be useful for investigating further the biological function of APP.

**EXPERIMENTAL PROCEDURES**

**Zebrafish Husbandry and Strains**

Wild-type and WIK strains of zebrafish were housed in an AHAB system (Aquatic Ecosystems, Inc., Apopka, FL) and maintained under a 14-hr light /10-hr dark cycle at 27°C. The WIK zebrafish strain was obtained from the Zebrafish International Research Center (http://zebrafish.org/zirc/home/guide.php). The silent hear<sup>p<sub>n</sub>oo001Gt</sup> allele contains a Tol2 gene trap integration in the cardiac tropinin T gene (Clark et al., 2011) and was obtained from Darius Balciunas, Temple University. The Tgf<sub>flk1</sub>:moesin-egfp<sup>j41</sup> line was described previously (Wang et al., 2010). Embryos were collected following fertilization and allowed to develop in a 28.5°C incubator under standard laboratory conditions. For in situ hybridization experiments, the embryos were placed in fish water (60.5 mg salts/l) containing 0.003% 1-phenyl-2-thiourea (PTU) to inhibit pigment formation in melanocytes. Staging of embryos was as published (Kimmel et al., 1995).

**Embryo Injections for the Germline Insertional Mutagenesis Screen**

Capped Tol2 transposase mRNA was synthesized in vitro from 1 μg of linearized pT3TS-Tol2 plasmid template (Balciunas et al., 2006) using the mMESSAGE mMACHINE High Yield Capped RNA transcription kit (Ambion, Cat. No. AM1348) and purified using the RNasey MinElute Cleanup Kit (Qiagen). The GBT-R15 Tol2 RFP gene trap plasmid (Petzold et al., 2009) was propagated in bacteria and purified using the QiAprep spin column kit (Qiagen). The mRNA and plasmid DNA were diluted in water, and 50 pg of GBT-R15 plasmid DNA and 300 pg of Tol2 mRNA were coinjected into 1-cell embryos. 227 injected embryos were raised to adulthood. Adults were intercrossed or outcrossed with WIK, and the F1 progeny were
screened for RFP expression from the 
Tol2 gene trap at 24 and 48 hpf. F1 
embryos for each Tol2 integration line 
were raised to adulthood, and subse-
quently generated were maintained by 
out-crossing with WIK.

**Computational Analysis**

Protein sequence alignment was 
performed using Clustal W (Swiss Insti-
tute of Bioinformatics). Phylogenetic 
tree construction was carried out using 
MEGA4: Molecular Evolution-
ary Genetics Analysis (MEGA) soft-
ware (Tamura et al., 2007).

**In Situ Hybridization and 
Confocal Imaging**

Whole-mount in situ hybridization on 
zebrafish embryos and larvae was 
performed using digoxigenin anti-
sense RNA probes as described previ-
ously (Larson et al., 2004). appa and 
aplp2 cDNAs were amplified by PCR 
using the following primers: appa, 
forward 5'-TGTGGAGTCCGTGCTGCT 
GTCT-3'; appa, reverse 5'-CGTGAAGA 
CGATGATGGTGG-3'; aplp2, forward 5'-CCTCTGGTGCATCAAGAAGT-3'; 
and aplp2, reverse 5'-CCGACTGTCG 
TCTTCTCAG-3'. The PCR products 
were cloned into the pCRII TOPO 
vector. A 541-bp fragment of the 
RFP was amplified from the 
GTBTR15 plasmid using primers rfp-L: 5'- 
CGAGGAGCGTATCAAGGAGT-3' and 
rfp-R: 5'-CTTGGCCATGTAGGTGGTC 
TCTTCCTCAG-3', and cloned into the pCR4 TOPO 
vector. The cloned appa, aplp2, and 
RFP cDNAs were used as template for 
synthesizing digoxigenin-labeled anti-
sense and sense RNA probes. Digoxige-
nin-labeled RNA probes were synthe-
sized by in vitro transcription in the 
presence of dig-labeled UTP (Roche) 
and purified as described (McGrail et 
al., 2010). In situ labeled embryos 
were analyzed on a Zeiss Axioscope 
using DIC optics and photographed 
with a Nikon Coolpix camera. Living 
embryos were mounted in 1% low-
melting agarose and imaged on a Zeiss 
LSM 700 confocal microscope. Confocal 
z-series were analyzed with Zeiss Axio-
Vision software. The identification of 
blood vessels in the embryonic vascula-
ture was determined according to 
zebrafish anatomy described at http:// 
zfish.nichd.nih.gov/zfatlas/FinalDesign1/

**Immunohistochemistry**

Zebrafish embryos were selected for 
RFP expression and were fixed over-
night in 4% paraformaldehyde in phos-
phate buffered saline (PBS) at 4°C. 
Embryos were dechorionated, dehy-
drated through an ethanol series and 
stored at −20°C. Embryos were trans-
ferred to acetone at −20°C for 15 min 
and washed in H2O with 0.1% Triton 
X-100 for 5 min. Following two rinses in PBS/0.1% Tri-
ton X-100 (PBSx), embryos were 
blocked for one hour at room temper-
ature in 2% sheep serum, 1% bovine se-
rum albumin (BSA), 1% dimethyl sul-
foxide (DMSO), 0.1% Triton X-100 in 
PBS (blocking solution). DaRed rabbit 
polyclonal antibody (Clontech) was 
diluted 1:400 in blocking solution and 
absorbed to embryos overnight at 4°C. 
Embryos were washed at room temper-
ature eight times over two hours in 1% 
BSA, 1% DMSO, 0.1% TX-100 in PBS 
and placed in blocking solution for 30 
min. An anti-rabbit horseradish perox-
idase (HRP) antibody (Zymed) was 
edited for 30–35 cycles using 
Taq DNA polymerase (Promega). PCR 
products were cloned and sequenced at the DNA Fa-
cloning and sequenced at the DNA Fa-

**Genotyping and RT-PCR Analysis**

Genotyping was based on PCR ampli-
fication of the junction fragments 
between the flanking regions of the 
Tol2 transposon integration site and 
the Tol2 transposon. The locations of 
primers are shown in Figure S9A and 
Figure S10A. Genomic DNA was 
exttracted from 3 to 4 dpf embryos 
using the DNeasy Blood and Tissue 
DNA Extraction kit (Qiagen). The 
primers used to amplify from appa gene 
were V2 jf-L: 5'-CCATCATCATTCTTCC 
TCGAGCA-3' and V2 jf-R: 5'-TCCAGG 
CGCAGATGCTGTTGTA-3'. For aplp2 gene, 
primers were V3 jf-L: 5'-CAACA 
CTTCCAGTTTTCCCGCTA-3' and V3 
jR: 5'-CACAAAATTTAACGTTGG 
GCAA-3'. The PCR primer used to 
amplify out from the Tol2 transposon 
was Tol2-L: 5'-CGGCTGCTGCTGAG 
GCGTT-3'. The PCR products were gel 
purified and sequenced to confirm the 
amplified products.

RT-PCR analysis of total RNA was 
carried out from staged embryos, lar-
vae, and adult organs as described 
(McGrail et al., 2010). The sequences 
of primers used for PCR were as fol-
ows: appa, forward 5'-GTGGAGGC 
CATGGAGAA-3' in exon 4; appa, 
reverse 5'-CGATGATGGTCGCGTG 
ATG-3' in exon 9; aplp2, forward 5'- 
CGCATCGTCGCGAGAAAC-3' in 
exon 2; aplp2, reverse 5'-ACACTCC 
AGAGATGCGCTGA-3' in exon 16. Con-
trol primer sequences for ribosomal 
protein S6 kinase b (rps6kb1) were 
forward 5'-CATGGGCGCGTGTTGTT 
CAT-3' and reverse 5'-AGCGGGCCGC 
CGTGCTGAAA-3'.

**Genomic Southern Blot 
Analyses**

Genomic DNA was extracted from 
adult fish and nonradioactive South-
ern blot analysis performed as
previously described (McGrail et al., 2011). The sequences of primers used to amplify digoxigenin-labeled PCR probes were as follows. A 297-bp probe for the *appa* gene was amplified from genomic DNA using primers *appa*-L: 5'-TCTGACTGAGGCTTGA TGA-3' and *appa*-R: 5'-GGGTATTTCT ATAGCCTGCTTCT-3'. A 292-bp probe complementary to the *aplp2* gene was amplified from genomic DNA using primers *aplp2*-L: 5'-GGGGCTGCTG-3' and *aplp2*-R: 5'-CTTGATGTCGAGG-3'. A 541-bp probe complementary to the *R15* cDNA was amplified from the *GBT-R15* plasmid using primers *rflp*-L: 5'- CGAGGACGTACTCAAGGGT-3' and *rflp*-R: 5'-CTTGGCCATGTAAGG-3'. Images of blots were captured on a ChemiDoc XRS imaging system.

**ACKNOWLEDGMENTS**

The authors thank Dr. Darius Balcianas for zebrafish harboring the *sil*<sup>m00316</sup> allele. The WIK strain of zebrafish used in this study was obtained from the Zebrafish International Resource Center. This work was supported by Iowa State University startup funds (J.J.E.), the Center for Integrated Animal Genomics and the Roy J. Carver Charitable Trust (J.J.E. and M.M.), and the National Institutes of Health (S.C.E. and K.J.C.).

**Author Contributions**

H.K.L. and M.M. wrote the manuscript; H.K.L., Y.W., J.J.E., M.M., K.J.C., S.C.E. designed the screen; K.J.C., S.C.E. designed the reporter; H.K.L., Y.W., J.J.E., M.M., and J.B., and C.K.K. cloned RACE products; J.B., and C.K.K. cloned RACE-PCR cloning strategy; Y.W., and M.M. designed and performed genotyping, immunofluorescence, and RT-PCR analyses; H.K.L. performed Southern analyses; H.K.L. and M.M. designed the screening strategy; Y.W., and M.M. performed screening; M.M. designed and provided plasmids; Y.W. and M.M. designed and performed experiments; K.J.C. and S.C.E. designed the screen and experiments; H.K.L., Y.W., J.J.E., M.M., and J.B., and C.K.K. cloned RACE products. Y.W. and M.M. designed and provided plasmids; Y.W. and M.M. designed and performed experiments; K.J.C. and S.C.E. designed the screen and experiments; H.K.L., Y.W., J.J.E., M.M., and J.B., and C.K.K. cloned RACE products.

**REFERENCES**


Lannert L, Basun H, Wahlund LO, Rowe BA, Wagner SL. 1995. Decreased alpha-secretase-cleaved amyloid precursor protein as a diagnostic marker for...


