

## AFAP1L1 is a novel adaptor protein of the AFAP family that interacts with cortactin and localizes to invadosomes

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### ARTICLE INFO

#### Article history:

Received 17 May 2010

Received in revised form

23 November 2010

Accepted 24 November 2010

#### Keywords:

AFAP1L1

AFAP1

XB130

Podosome

Cortactin

Dentate nucleus

Actin

### ABSTRACT

The actin-filament associated protein (AFAP) family of adaptor proteins consists of three members: AFAP1, AFAP1L1, and AFAP1L2/XB130 with AFAP1 being the best described as a cSrc binding partner and actin cross-linking protein. A homology search of AFAP1 recently identified AFAP1L1 which has a similar sequence, domain structure and cellular localization; however, based upon sequence variations, AFAP1L1 is hypothesized to have unique functions that are distinct from AFAP1. While AFAP1 has the ability to bind to the SH3 domain of the nonreceptor tyrosine kinase cSrc via an N-terminal SH3 binding motif, it was unable to bind cortactin. However, the SH3 binding motif of AFAP1L1 was more efficient at interacting with the SH3 domain of cortactin and not cSrc. AFAP1L1 was shown by fluorescence microscopy to decorate actin filaments and move to punctate actin structures and colocalize with cortactin, consistent with localization to invadosomes. Upon overexpression in A7r5 cells, AFAP1L1 had the ability to induce podosome formation and move to podosomes without stimulation. Immunohistochemical analysis of AFAP1L1 in human tissues shows differential expression when contrasted with AFAP1 with localization of AFAP1L1 to unique sites in muscle and the dentate nucleus of the brain where AFAP1 was not detectable. We hypothesize AFAP1L1 may play a similar role to AFAP1 in affecting changes in actin filaments and bridging interactions with binding partners, but we hypothesize that AFAP1L1 may forge unique protein interactions in which AFAP1 is less efficient, and these interactions may allow AFAP1L1 to affect invadosome formation.

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### Introduction

Adaptor proteins are non-enzymatic proteins that have the ability to link together different components of cellular signaling complexes through protein-binding motifs. Actin filament-associated protein of 110 kilodaltons (kDa) (AFAP-110/AFAP1) is an adaptor protein with multiple protein binding motifs that is known to function as both an actin binding protein and a cSrc activating protein (Flynn et al., 1993; Qian et al., 2000). The AFAP1 protein binding motifs include two juxtaposed poly-proline rich Src homology 3 (SH3) binding motifs of approximately 10 amino acids with essential prolines at the amino acid number seven and ten positions within the motif (Mayer, 2001), two Src homology 2 (SH2) bind-

ing motifs containing a phosphotyrosine residue amino terminal to 1 or 2 negatively charged amino acids followed by a hydrophobic amino acid (Songyang et al., 1993), two pleckstrin homology (PH) domains, a substrate domain (SD) rich in serine and threonine residues that is a substrate for serine/threonine kinases, a helical leucine zipper (Lzip) with a heptad repeat of leucine residues (Kouzarides and Ziff, 1988) for intra- and inter-molecular interactions within itself and other AFAP1 molecules and an actin binding domain (ABD) which is both necessary and sufficient for AFAP1 to interact with actin filaments (Baisden et al., 2001a; Qian et al., 1998, 2000, 2004). AFAP1 is known to bind actin filaments through its carboxy terminal actin filament-binding domain and will multimerize through its leucine zipper, thus enabling it to crosslink actin filaments (Qian et al., 2002). Activation by the serine/threonine kinase PKC $\alpha$  directs AFAP1 to colocalize with cSrc in the perinuclear region of the cell and activate cSrc by binding to the SH3 domain via its N-terminal SH3 binding motif (Gatesman et al., 2004; Walker et al., 2007). Subsequently, cSrc then has the ability to activate downstream cellular signals that affect cell adhesion, invasion and motility (Fincham et al., 1996; Frame and Brunton, 2002).

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Upon cSrc binding and activation, AFAP1 and cSrc move to podosomes, adhesion structures found on the ventral membrane of cells which contain an F-actin rich core (Gatesman et al., 2004; Linder and Kopp, 2005). Podosomes also secrete proteases which enable cells to degrade the extracellular matrix, cross tissue barriers and invade. As cSrc activation is known to switch cells from a normal to an invasive phenotype, the activation and movement of cSrc and AFAP1 may be important steps toward promoting cellular changes in adhesion and invasive potential. Also involved in this process is the ability of a cell to assemble, disassemble and remodel its actin cytoskeleton (Yilmaz and Christofori, 2009). As AFAP1 has been shown to affect dynamic changes in actin filament cross-linking (Qian et al., 2004), AFAP1 may also play a role in actin cytoskeleton remodeling in addition to cSrc activation.

AFAP1 represents a family of three proteins that also include actin filament associated protein 1 like 1 (AFAP1L1) and actin filament associated protein 1 like 2 (AFAP1L2/XB130) so named as a family by the Human Genome Project due to similarity in modular domain structure and amino acid sequence, most notably within their PH domains. There are 250 known PH domain-containing proteins in the human genome and while the amino acid sequences of these PH domains are not well conserved, they are predicted to have a similar structure to a  $\beta$ -barrel with four  $\beta$  strands on one side and three  $\beta$  strands on the other connected by three variable loops connecting  $\beta$ 1/ $\beta$ 2,  $\beta$ 3/ $\beta$ 4 and  $\beta$ 6/ $\beta$ 7 (DiNitto and Lambright, 2006). However, within the AFAP family, the amino acid sequence, as well as structure and placement within the protein, are well conserved between the PH domains, designating them as a related family of proteins. Overall, AFAP1L2/XB130 consists of 818 amino acids and is 35% identical (64% similar) to AFAP1. AFAP1L2/XB130 was also discovered as a cSrc binding protein and contains a shared functional domain structure to AFAP1 including one SH3 motif, three SH2 motifs, 2 PH domains, a coiled-coil region corresponding to the AFAP1 leucine zipper and a sequence similar to the AFAP1 actin binding domain (Xu et al., 2007). However, unlike AFAP1, AFAP1L2/XB130 did not appear to bind efficiently to actin filaments. AFAP1L2/XB130 does appear to have functions distinct from AFAP1 in that it acts as an intermediary between the RET/PTC kinase and PI-3kinase pathway in the thyroid (Lodyga et al., 2009).

In this report we characterize the third AFAP family member, AFAP1L1, which also contains a shared domain structure with AFAP1 and AFAP1L2/XB130. Cladistic analysis of the three AFAP family members (data not shown) indicated that AFAP1 and AFAP1L1 are more closely related to each other than to AFAP1L2/XB130, therefore we sought to compare and contrast AFAP1L1 to AFAP1 so as to characterize AFAP1L1 and determine whether it has functions that are shared or distinct relative to AFAP1.

## Materials and methods

### Cell culture and reagents

A7r5, rat aortic smooth muscle cells, were purchased from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-Glutamine (Gibco) and penicillin/streptomycin (Mediatech) at 37 °C with 5% CO<sub>2</sub>. MDA-MB-435, MDA-MB-231, B1A (Dorfleutner et al., 2007), and Cos-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech), 10% fetal bovine serum (FBS, Atlanta Biologicals), 2 mM L-Glutamine (Gibco) and penicillin/streptomycin (Mediatech) at 37 °C with 5% CO<sub>2</sub>. MCF-10A cells were grown in complete Mammary Epithelium Basal Medium supplemented with MEGM SingleQuots (Lonza) and MCF-7 cells were cultured in Mod-

ified Eagle's medium containing 10% FBS and 10  $\mu$ g/ml bovine insulin (Sigma).

Antibodies used for Western blotting and immunofluorescence are as follows: anti-AFAP1 (F1) (Flynn et al., 1993), anti-AFAP1 (BD Transduction Labs), anti-cortactin (Millipore), and  $\beta$ -actin (Sigma). Tetramethylrhodamine-isothiocyanate (TRITC)-conjugated phalloidin was from Sigma and Alexa Fluor conjugated phalloidin and secondary antibodies were from Invitrogen. The antibody against AFAP1L1, 1L1-CT, was generated by ProSci Incorporated. Rabbits were immunized with AFAP1L1 peptide corresponding to amino acids 714–727. 1L1-CT antibody was affinity-purified by affinity absorption against the AFAP1L1 peptide attached to an Ultralink Iodoacetyl column (Pierce) according to the manufacturer's protocol. Two additional antibodies against AFAP1L1, Ab1 and Ab2, targeting either the C terminus (amino acid 525–659) or the N terminus (amino acids 21–159) of 1L1, respectively, were from Sigma.

### Constructs

The *afap111* cDNA sequence was purchased in two vectors from OpenBioSource. The coding sequence for AFAP1L1 amino acids 1–340 was identified in a pCMV-SPORT6 vector. The coding sequence for AFAP1L1 amino acids 273–768 was identified in a pINCY vector. A BstYI restriction site, GATCC, in the overlap region was mutated to a BglIII restriction site, GATCT, to create a unique restriction site using the Stratagene QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol. AFAP1L1 N-terminal coding sequence was subcloned into pBlue-script II KS (Stratagene) using HindIII and an engineered EcoRI restriction site. AFAP1L1 C-terminal coding sequence was subcloned into pBluescript II KS using engineered HindIII and EcoRI restriction sites. Full length AFAP1L1 was created by restriction digest of pBluescript containing each AFAP1L1 coding sequence with the unique BglIII site in the overlap region and a unique ScaI site found in the vector followed by the fusion of the two halves of pBluescript. The AFAP1L1 full length sequence was confirmed by DNA sequencing.

Full length AFAP1L1 was subcloned into pEGFP (Clontech) using HindIII and EcoRI. Full length AFAP1L1 was subcloned from pEGFP into pcDNA3.1(+)hygro (Invitrogen) using HindIII and KpnI. GFP-AFAP1 was previously described by Qian et al. (2000).

### Transfection

For antibody characterization and GST pull down overexpression studies respectively, Cos-1 and 293T cells were transiently transfected with 5  $\mu$ g of either GFP-AFAP1 or GFP-AFAP1L1 using Lipofectamine and Plus reagent according to the manufacturer's protocol. For confocal overexpression studies, mouse embryo fibroblasts (MEF) were transfected with 5  $\mu$ g of either GFP-AFAP1L1 or untagged AFAP1L1 (in pcDNA3.1) using Lipofectamine and Plus reagent.

To determine endogenous AFAP1L1 localization to invadopodia, MDA-MB-435 cells were transfected with cSrc527F plasmid using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. A7r5 cells were transfected with GFP-AFAP1 or GFP-AFAP1L1 in increasing amounts from 0.1  $\mu$ g to 1.0  $\mu$ g using Lipofectamine and Plus reagent per well of a 6 well plate. Total DNA concentration for dose–response transfections was kept constant using an empty pcDNA3.1 vector to keep the total DNA transfected at 1.0  $\mu$ g to maintain equal transfection efficiency.

### Immunoblotting

Cos-1 cells transiently expressing GFP-AFAP1 or GFP-AFAP1L1 were lysed in 2 $\times$  SDS buffer (125 mM Tris–HCl pH 6.8, 20% glycerol,

4% SDS). Cell lines MCF-10A, MCF-7, MDA-MB-231, MDA-MB-435, B1A, and Cos-1 were lysed in 2× SDS buffer. Protein concentration was determined using a BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol. 50 µg of total lysate was resolved by 8% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) using semi-dry electroblotting. Proteins were detected by incubation with either anti-1L1-CT (ProSci) 1:250, anti-1L1-Ab1 (Sigma) 1:1000, anti-1L1-Ab2 (Sigma) 1:500, anti-AFAP1 (BD Transduction Labs) 1:1000, anti-AFAP1 (F1) 1:20,000, anti-GFP (Zymed) 1:1000 or anti-β-actin (Sigma) 1:10,000 in 5% powdered milk (TBS, 0.05% Tween-20) followed by incubation with 1:3000 dilution of donkey anti-mouse or donkey anti-rabbit horseradish peroxidase conjugated antibodies (GE Healthcare Bio-Sciences). Chemiluminescence was visualized with Pierce ECL Western Blotting Substrate.

### Immunofluorescence

MDA-MB-435, MEF and A7r5 cells were grown on fibronectin-coated coverslips (50 µg/ml) overnight at 37 °C. For GFP-AFAP1L1 overexpression and overexpressed untagged AFAP1L1, MEF and A7r5 cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100 and incubated with or without anti-cortactin antibody (1:200). Actin filaments were stained with TRITC-phalloidin (1:600) or AlexaFluor conjugated phalloidin (1:100). For endogenous AFAP1L1 staining, MDA-MB-435 were fixed and permeabilized in 3.7% formaldehyde containing 0.1% Triton X-100, incubated with AlexaFluor conjugated phalloidin (1:100) in 3% BSA, and then stained with anti-AFAP1L1 antibodies Ab1 or Ab2 (Sigma, 2 µg/ml) and anti-cortactin antibodies. Fluorescence conjugated secondary antibodies (Invitrogen) were used at a 1:200 dilution. Coverslips were mounted on slides using Prolong Gold (Invitrogen) and imaged with a Zeiss LSM 150 microscope or Nikon eclipse Ti inverted epi-fluorescence microscope. Podosomes were confirmed by anti-cortactin localization along the basolateral membrane via confocal microscopy (0.5 µm scanning thickness). Images were further processed with Zeiss LSM Image Browser (Zeiss), CorelDRAW12, Nikon NIS element software (Nikon) and Adobe Photoshop.

### Immunoprecipitation

Cos-1 cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Igepal, 0.25% sodium deoxycholate, 10 mM β-glycerol, 1 mM sodium vanadate, 5 µg/µl aprotinin, 5 µg/µl leupeptin, 1 mM PMSF). 1 mg of Cos-1 lysate was used for each control IgG, anti-1L1-CT and AFAP1 (F1) pull down. Lysates were pre-cleared with agarose A beads for 1 h. The pre-cleared lysates were incubated on ice for 1 h with 2 µg IgG, 1L1-CT or F1 antibodies, and then 50% slurry agarose A beads were added and incubated for 1 h. Proteins were eluted from beads using 2× Laemmli sample buffer (LSB) with 200 mM dithiothreitol (DTT), separated by 8% SDS-PAGE and processed for Western blot analysis. 293T cells were transfected with human cortactin cDNA with either EGFP AFAP1 or AFAP1L1 construct using Polyfect (Qiagen) according to the manufacturer's specification. Forty-eight hours post transfection, cells were lysed in RIPA buffer and 2 mg of cell lysates were used for immunoprecipitation with either GFP antibodies (Polyclonal Av antibodies, Clontech) or cortactin antibodies (clone 4F11, Millipore) as described above.

### Immunohistochemical methods

Immunohistochemistry was performed on de-identified human brain, breast and colon tissue slices obtained from the West Virginia University Department of Pathology Tissue Bank. Tissues

were paraffin-embedded and cut into 5 µm-thick sections and mounted on positive-charge coated slides. Tissue sections were dried overnight in a 45 °C oven, deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval for 2 h in 1 mM citrate buffer (pH 6.00) in an 80 °C water bath. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and was followed by treatment with a serum-free protein blocker to block non-specific binding. Following each step of the immunoreaction except the protein blocker, sections were rinsed in Tris-buffered saline with Tween-20. Tissues were incubated for 2 h with either anti-AFAP1 antibody (F1) at a dilution of 1:50 or with anti-1L1-CT diluted 1:50 in 10% normal horse serum. Negative controls (i.e., preimmune serum or normal rabbit IgG) were incubated in 10% normal horse serum. Sections were incubated with biotinylated-linked secondary antibody, followed by treatment with streptavidin peroxidase and stained with diaminobenzidine substrate-chromogen solution. Counterstaining was performed with hematoxylin, followed by a water rinse and bluing solution. Tissues were then dehydrated and mounted with coverslips.

### Affinity precipitation with GST fusion proteins

293T cells transiently expressing GFP-AFAP1 or GFP-AFAP1L1 were lysed in modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Igepal, 0.25% sodium deoxycholate, 10 mM β-glycerol, 1 mM sodium vanadate, 5 µg/µl aprotinin, 5 µg/µl leupeptin and 1 µM PMSF). GFP-AFAP1 and GFP-AFAP1L1 lysates were pre-cleared using 50 µg of GST protein bound to Glutathione Sepharose 4B coated beads (GE Healthcare) and 1 mg of each pre-cleared lysate was incubated at 4 °C for 1 h with either 50 µg of control GST, 50 µg of GST-Src SH3 domain or 50 µg of GST-cortactin SH3 domain bound to GST beads. Proteins were eluted from beads by boiling for 5 min in 2× Laemmli sample buffer with 200 mM DTT and further processed with PAGE and Western blot analysis. Western blot results were examined with ImageJ densitometry analysis so as to provide a ratio between the amount of AFAP1 and AFAP1L1 pulled down with each GST or GST fusion protein.

## Results

### Identification of AFAP1L1

AFAP1L1 was discovered during an homology search of the human genome using sequences of the AFAP1 PH domains and was subsequently referred to as the third member of the AFAP family by the Human Genome Project. The *afap1l1* gene is located on chromosome 5q33.1 and consists of 19 exons that are predicted to encode 768 amino acids in the open reading frame (Fig. 1). All AFAP family members contain at least one predicted N-terminal SH3 binding motif, at least one predicted N-terminal SH2 binding motif, two PH domains separated by a region rich in serine and threonine residues and a C-terminal SH2 binding motif (Fig. 2A). One major difference is found in the C-terminus where AFAP1 and AFAP1L1 contain a leucine zipper and actin binding domain while AFAP1L2/XB130 contains a related coiled-coil in this respective region (Fig. 2B) (Baisden et al., 2001b; Xu et al., 2007).

There appear to be differences in the SH3 binding motif of AFAP1L1 compared to AFAP1. AFAP1 contains two juxtaposed SH3 binding motifs, PPQMPLPEIP and PPDGPPPLP, beginning at amino acids 65 and 76 respectively (Guappone and Flynn, 1997). AFAP1 will bind cSrc using the N-terminal P<sup>71</sup>EIP of the first SH3 binding motif (Guappone and Flynn, 1997), while AFAP1L2/XB130 has been reported to also bind cSrc through its SH3 binding motif, identified as PDLPPPKMIP (Xu et al., 2007). However, the AFAP1L1 coding sequence contains only one predicted SH3 binding motif in its amino terminus, DLPPPLPNKP, which is not consistent with a

**ATG** GAC CGA GGC CAG GTG CTG GAG CAG CTG CTC CCA GAG CTC ACC GGG CTG CTC AGC CTC CTG GAC CAC GAG  
**M** D R G Q V L E Q L L P E L T G L L S L L D H E  
TAC CTC AGC GAT ACC ACC CTG GAA AAG AAG ATG GCC GTG GCC TCC ATC CTG CAG AGC CTG CAG CCC CTT CCA  
Y L S D T T L E K M A V A S I L Q S L Q P L P  
GCA AAG GAG GTC TCC TAC CTG TAT GTG AAC ACA GCA GAC CTC CAC TCG GGG CCC AGC TTC GTG GAA TCC CTC  
A K E V S Y L Y V N T A D L H S G P S F V E S L  
TTT GAA GAA TTT GAC TGT GAC CTG AGT GAC CTT CGG GAC ATG CCA GAG GAT GGG GAG CCC AGC AAA GGA  
F E E F D C D L S D L R D M P E D D G E P S K G  
GCC AGC CCT GAG CTA GCC AAG AGC CCA CGC CTG AGA AAC GCG GCC GAC CTG CCT CCA CGC CTC CCC AAC AAG  
A S P E L A K S P R L R N A A D L P P P L P N K  
CCT CCC CCT GAG GAC TAC TAT GAA GAG GCC CTT CCT CTG GGA CCC GGC AAG TCG CCT GAG TAC ATC AGC TCC  
P P P E D Y Y E E A L P L G T G P G K S P E Y I S S  
CAC AAT GGC TGC AGC CCC TCA CAC TCG ATT GTG GAT GAG TAC TAT GAG GAC GCA GAC AGC AGC AGC TAC CCT GCA  
H N G C S P S H S I V D G Y Y E D A D S S Y P A  
ACC AGG GTG AAC GGC GAG CTT AAG AGC TCC TAT AAT GAC TCT AAG GAC GCA ATG AGC AGC TCC TAT GAG TCC TAC  
T R V N G E L K S S Y N D S D A M S S S Y E S Y  
GAT GAA GAG GAG GAG GAA GGG AAG AGC CCG CAG CCC CGA CAC CAG TGG CCC TCA GAG GAG GCC TCC ATG CAC  
D E E E E G K S P Q P R H Q W P S E E A S M H  
CTG GTG AGG GAA TGC AGG ATA TGT GCC TTC CTG CTG CGG AAA AAG CGT TTC GGG CAG TGG GCC AAG CAG CTG  
L V R E C R I C A F L L R K K R F G Q W A K Q L  
ACG GTC ATC AGG GAG GAC CTC CTG TAC AAA AGC TCC AAG GAT CGG CAG CCA CAT CTG AGG TTG GCA  
T V I R E D Q L L C Y K S S K D R Q P H L R L A  
CTG GAT ACC TGC AGC ATC ATC TAC GTG CCC AAG GAC AGC CGG CAC AAG AGG CAC GAG CTG CGT TTC ACC CAG  
L D T C S I I Y V P K D S R H K R H E L R F T Q  
GGG GCT ACC GAC GTC TTG GTG GCA CTG CAG AGC CGA GAG CAG GCC GAG GAG TGG CTG AAG GTC ATC CGA  
G A T E V L V L A L Q S R E Q A E E W L K V I R  
GAA GTG AGC AAG CCA GTT GGG GGG GCT GAG GGG GTG GAG GTC ACC AGA TCC CCA GTC CTC CTG TGC AAG TTG  
E V S K P V G A E G V E V P C R S P V L L C K L  
GAC CTG GAC AAG AGG CTG TCC CAA GAG AAG CAG ACC TCA GAT TCT GAC AGC GTG GGT GTG GGT GAC AAC TGT  
D L D K R L S Q E K Q T S D S D S V G V G D N C  
TCT ACC CTT GGC CGC CGG AGC TGT GAT CAC GCC AAA GGG AAG AGC AGC CTG GCA GAA CTG ATG AAG GGC  
S T L G R R E T C D H G K G K K S S L A E L K G  
TCA ATG AGC AGG GCT CGC GGC CGC AAG ATC ACC CGT ATC ATT GCG TTT TCC AAG AAG AAC ACA CTG GCC GAT  
S M S R A A G R K I T R I I G F S K K K T L A A  
GAC CTG CAG ACG TCC TCC ACC GAG GAG GAG GTT CCC TGC TGT GGC TAC CTG AAC GTG CTG GTG AAC CAG GGC  
D L Q T S S T E E V P C C G Y L N V L V N Q G  
TGG AAG GAA CGC TGG TGC CGC CTG AGC AAC TGC ACT CTG TAT TTC CAC AAG GAT CAC ATG GAC CTG CAG ACC  
W K E R W C R L K C N T L Y F H K D H M D L R T  
CAT GTG AAC GCC ATC GCC CTG CAA GGC TGT GAG GTG GCC CCG GGC TTT GGG CCC CGA CAC CCA TTT GCC TTT  
H V N A I A L Q G G E V A P G F G P R H P F A F  
AGG ATC CTG CGC AAC CGG CAG GAG GTG GCC ATC TTG GAG GCA AGC TGT TCA GAG GAC ATG GGT CGC TGG CTC  
R I L R N R Q E V A I L E A S C S E D M G R W L  
GGG CTG CTG GTG GTG GAG ATG GGC TCC AGA GTC ACT CCG GAG GCG CTG CAC TAT GAC TAC GTG GAT GTG GAG  
G L L L V E M G S R V T P E A L H Y D Y V D V E  
ACC TTA ACC AGC ATC GTC AGT GCT GGG CGC AAC TCC TTC CTA TAT GCA AGA TCC TGC CAG AAT CAG TGG CCT  
T L T S I V S A G R N S F L Y A R S C Q N Q W P  
GAG CCC CGA GTC TAT GAT GAT GTT CCT TAT GAA AAG ATG CAG GAC GAG GAG CCC GAG CGC CCC ACA GGG GCC  
E P R V Y D D V P Y E K M Q D E P E R P T G A  
CAG GTG AAG CGT CAC GCC TCC TCC AGT GAG AAG TCC CAT CGT GTG GAC CCG CAG CTC AAA GTC AAA CGC  
Q V K R H A S S C S E K S H R V D P Q V K V K R  
CAC GCC TCC AGT GCC AAT CAA TAC AAG TAT GGC AAG AAC CGA GCC GAG GAG GAT GCC CGG AGG TAC TTG GTA  
H A S S A N Q Y K Y G K N R A E D A R R Y L V  
GAA AAA GAG AAG CTG GAG AAA GAG AAA GAG ACG ATT CGG ACA GAG CTG ATA GCA CTG AGA CAG GAG AAG AGG  
E K E K L E K E K E T I R T E L I A L R Q E K R  
GAA CTG AAG GAA GCC ATT CGG AGC CCA GGA GCA AAA TTA AAG GCT GTG GAA GAA GCC GTG GCC ACC CTG  
E L K E A I R S S P G A K L K A L E E A V A T L  
GAA GCT CAG TGT CGG GCA AAG GAG GAG CGC CGG ATT GAC CTG GAG CTG AAG CTG GTG GCT GTG AAG GAG CGC  
E A Q C R A K E E R I D L E L V A V K E R  
TTG CAG CAG TCC CTG GCA GGA GGG CCA GCC CTG GGG CTC TCC GTG AGC AGC AAG CCC AAG AGT GGG GAA ACT  
L Q Q S L A G G P A L G L S V S S K P K S G E T  
GCA AAT AAA CCC CAG AAC AGC GTT CCA GAG CAA CCT CTC CCT CCT AAC TGT GTC AGC CTG GAG CTG AAG AGG  
A N K P Q N S V P E Q P L P V N C V S E L R K R  
AGC CCA TCC ATC GTA GCC TCC AAC CAA GGA AGG GTG CTA CAG AAA GCC AAG GAA TGG GAA ATG AAG AAG ACC  
S P S I V A S N Q G R V L Q K A K E W E M K K T  
**TAG**  
**STOP**

Fig. 1. AFAP1L1 sequence. AFAP1L1 coding sequence was divided into codons with corresponding amino acid sequence. Start and stop codons are indicated in bold type.

consensus cSrc SH3 binding motif but may have the ability to bind to other SH3 domains.

AFAP1 contains two SH2 binding motifs that interact with cSrc, YYEEA in the N-terminal portion of the protein and YDYI in the C-terminal portion, which are both sites for tyrosine phosphorylation (Guappone et al., 1998). AFAP1L1 shares 100% sequence identity with both AFAP1 and AFAP1L2/XB130 in its predicted N-terminal SH2 binding motif, YYEEA, and is highly similar in its C-terminal SH2 binding motif YDYV (Guappone et al., 1998; Xu et al., 2007). Thus, these may be potential sites for tyrosine phosphorylation.

The N-terminal and C-terminal PH domains of the AFAP family share sequence similarity and, thus, can be predicted to share

structural similarity. The amino acid sequence intervening between the PH domains of AFAP1 (substrate domain, SD, Fig. 2B) is rich in serine and threonine residues (Qian et al., 2002). Serine<sup>277</sup> in AFAP1 is a known target of phosphorylation by PKC $\alpha$  and plays a role in the ability of AFAP1 to regulate podosome formation and lifespan (Dorflautner et al., 2008). AFAP1L1 and AFAP1L2/XB130 also have multiple serine and threonine residues in the sequence flanked by their PH domains, although these sequences have not been confirmed to be phosphorylated by serine/threonine protein kinases.

The leucine zipper of AFAP1 is essential for intra-molecular regulation of AFAP1 and interaction with other AFAP1 molecules and

**A**

AFAP1	-----MEELIVELRLFLLELLDHEYLSTVREKKAVITNILLRIQSSKGFVDKHAQKQE	54
AFAP1L1	MDRGQVLEQLPELTGLLSLLDHEYLSDTTLEKKMAVASILQSLQPLFAKEVSYLYVNTA	60
AFAP1L2	MERYKALEQLLTELDDFLKILDQENLSSTALVKKSCLAELRLRYTKSSSDEE <b>ET</b> YLNKV	60
Consensus	E L EL L LD E L T KK L	
AFAP1	TANSL <b>---</b> ----- <b>APPOMPLPE</b> - <b>P</b> - <b>DPW</b> - <b>PDSGPP</b> -----	83
AFAP1L1	DLHSGPSFVSESLFEE <b>F</b> DCDLSDLRMPEDDGEPSKGASPELAKSPRLRN <b>AA</b> VL <b>PE</b> -----	116
AFAP1L2	TINKQQNAESQ <b>G</b> KAPEEQGLLP-----NGEPSQHSSAPQKSL <b>EDLPEEKVIL</b> ERKQLA	113
Consensus	P P L P	
AFAP1	<b>L</b> TS <b>SLP</b> <b>EGYEEA</b> V <b>L</b> SPGKAPEYITSN-----	112
AFAP1L1	<b>L</b> <b>PNR</b> <b>PP</b> <b>EDYEEA</b> L <b>P</b> LGPGKSPEYISS <b>H</b> NGCSPSHSIVDGYEDADSSYPATRVNGELK	176
AFAP1L2	IPKTES <b>P</b> <b>EGYEEA</b> <b>E</b> <b>P</b> YD <b>T</b> SL <b>NE</b> -----	136
Consensus	P PE YEEA P	
AFAP1	---YDSAMDSSSYESYDEEEEDGKGGKTRHQWPSEASMDLVK <b>DAKICAFLLRKKRFQGW</b>	169
AFAP1L1	SSYNDSDAMSSSYESYDEEEEGKSPQRHQPWSEASMLHVR <b>ECRICAFLLRKKRFQGW</b>	236
AFAP1L2	---DGEAVSSSYESYDEED-GSKGKSAPYQWSPPEAGIELMR <b>DARICAFLLRKKRFQGW</b>	191
Consensus	D A SSSYESYDEE K QWPS EA L ICAFL RKK GQW	
AFAP1	<b>TKLLCVIKDTKL</b> <b>CYKSSK</b> DQ <b>PQ</b> MEL <b>PLQGCN</b> ITYIPKDSK <b>KKK</b> HELK <b>IT</b> Q <b>QGT</b> DL <b>PLV</b>	229
AFAP1L1	<b>AKQ</b> L <b>TV</b> RED <b>Q</b> L <b>CYKSSK</b> DR <b>Q</b> PH <b>LRL</b> ALD <b>TCS</b> I <b>I</b> Y <b>VP</b> KDSR <b>HKR</b> HEL <b>RFT</b> Q <b>GATE</b> V <b>L</b>	296
AFAP1L2	<b>AKQ</b> L <b>CV</b> I <b>KD</b> N <b>RL</b> <b>CYKSSK</b> D <b>H</b> S <b>PQ</b> LD <b>VN</b> LL <b>GSSV</b> I <b>H</b> KE <b>Q</b> V <b>R</b> KK <b>EL</b> K <b>IT</b> <b>TP</b> M <b>NAD</b> V <b>I</b>	251
Consensus	K L VI L L C Y K S S K D P L L K K H L T V L	
AFAP1	<b>AVQ</b> S <b>K</b> EA <b>EQ</b> W <b>L</b> <b>VI</b> KEA <b>YS</b> <b>G</b> C <b>S</b> G <b>P</b> V <b>D</b> SE <b>C</b> PP <b>P</b> SS <b>P</b> V <b>H</b> KA <b>E</b> L <b>K</b> LS <b>S</b> ER <b>P</b> SS <b>D</b> GE <b>V</b>	289
AFAP1L1	<b>ALQ</b> S <b>R</b> EA <b>EE</b> W <b>L</b> <b>VI</b> RE <b>V</b> S <b>K</b> P <b>V</b> G <b>G</b> A <b>E</b> G <b>V</b> E <b>V</b> PR <b>S</b> <b>P</b> - <b>V</b> LL <b>CK</b> L <b>D</b> L <b>D</b> <b>K</b> RL <b>S</b> Q <b>E</b> K <b>T</b> S <b>D</b> S <b>D</b> S <b>V</b>	355
AFAP1L2	<b>GLQ</b> S <b>K</b> D <b>EA</b> EQ <b>W</b> L <b>VI</b> Q <b>E</b> V <b>S</b> G <b>L</b> P <b>S</b> E <b>G</b> A <b>S</b> E <b>G</b> N <b>Q</b> Y <b>T</b> P----- <b>DAQ</b> R <b>F</b> N <b>C</b> Q <b>P</b> D <b>IA</b> E <b>K</b> Y <b>L</b> S	303
Consensus	QS QAE WL VI E P	
AFAP1	<b>ENG</b> IT <b>T</b> C <b>NG</b> K <b>---</b> <b>E</b> <b>Q</b> V <b>K</b> R <b>K</b> S <b>S</b> K <b>S</b> E <b>A</b> R <b>G</b> T <b>V</b> S <b>K</b> V <b>T</b> G <b>K</b> K <b>I</b> T <b>K</b> I <b>I</b> S <b>L</b> G <b>K</b> K <b>---</b> P <b>S</b> T <b>D</b> E <b>Q</b> T <b>S</b> S <b>A</b>	344
AFAP1L1	<b>V</b> G <b>D</b> N <b>C</b> S <b>T</b> L <b>G</b> R <b>R</b> E <b>T</b> C <b>D</b> <b>H</b> G <b>K</b> K <b>S</b> S <b>L</b> A <b>E</b> L <b>K</b> G <b>S</b> M <b>S</b> R <b>A</b> A <b>G</b> R <b>K</b> I <b>T</b> R <b>I</b> I <b>G</b> F <b>S</b> K <b>K</b> K <b>T</b> L <b>A</b> D <b>L</b> Q <b>T</b> S <b>S</b> T	415
AFAP1L2	<b>A</b> S <b>E</b> Y <b>G</b> S <b>S</b> V <b>D</b> G <b>---</b> <b>H</b> P <b>E</b> V <b>P</b> E <b>T</b> K <b>V</b> K <b>K</b> K <b>C</b> S <b>---</b> <b>A</b> G <b>L</b> K <b>L</b> S <b>N</b> L <b>M</b> N <b>L</b> G <b>R</b> K <b>---</b> <b>S</b> T <b>S</b> L <b>E</b> R <b>---</b> <b>V</b>	350
Consensus	K S G K K K	
AFAP1	<b>E</b> D <b>V</b> P <b>T</b> <b>G</b> Y <b>L</b> N <b>V</b> L <b>S</b> N <b>S</b> R <b>R</b> W <b>R</b> W <b>R</b> V <b>K</b> D <b>N</b> K <b>L</b> I <b>F</b> H <b>K</b> D <b>R</b> T <b>D</b> L <b>K</b> T <b>H</b> I <b>V</b> S <b>I</b> P <b>L</b> R <b>G</b> C <b>E</b> V <b>I</b> P <b>L</b> D <b>S</b> K	404
AFAP1L1	<b>E</b> E <b>V</b> P <b>C</b> <b>G</b> Y <b>L</b> N <b>V</b> L <b>V</b> N <b>Q</b> W <b>K</b> R <b>W</b> C <b>R</b> L <b>K</b> C <b>N</b> T <b>L</b> Y <b>F</b> H <b>K</b> D <b>H</b> M <b>D</b> L <b>R</b> T <b>H</b> V <b>N</b> A <b>I</b> A <b>L</b> Q <b>G</b> C <b>E</b> V <b>A</b> P <b>G</b> P <b>G</b> P <b>R</b>	475
AFAP1L2	<b>E</b> R <b>S</b> L <b>E</b> T <b>S</b> Y <b>L</b> N <b>V</b> L <b>V</b> N <b>S</b> Q <b>N</b> K <b>S</b> R <b>W</b> C <b>S</b> V <b>R</b> D <b>N</b> H <b>L</b> H <b>F</b> Y <b>Q</b> D <b>R</b> N <b>R</b> S <b>K</b> V <b>A</b> Q <b>Q</b> P <b>L</b> S <b>I</b> V <b>G</b> C <b>E</b> V <b>D</b> P <b>S</b> P <b>D</b>	410
Consensus	E YLNV L N W RWC N L F D L GCEV P	
AFAP1	<b>H</b> P <b>L</b> T <b>F</b> R <b>L</b> L <b>R</b> N <b>G</b> Q <b>E</b> V <b>A</b> V <b>L</b> <b>A</b> S <b>S</b> E <b>D</b> M <b>G</b> R <b>W</b> I <b>G</b> I <b>L</b> L <b>A</b> E <b>T</b> G <b>S</b> S <b>T</b> D <b>PE</b> A <b>L</b> H <b>Y</b> D <b>Y</b> I <b>D</b> V <b>M</b> S <b>A</b> S <b>V</b> I <b>Q</b>	464
AFAP1L1	<b>H</b> P <b>F</b> A <b>F</b> R <b>I</b> L <b>R</b> N <b>R</b> Q <b>E</b> V <b>A</b> I <b>L</b> <b>A</b> S <b>S</b> E <b>D</b> M <b>G</b> R <b>L</b> G <b>L</b> L <b>L</b> V <b>E</b> M <b>G</b> S <b>R</b> V <b>T</b> <b>PE</b> A <b>L</b> H <b>Y</b> D <b>V</b> D <b>V</b> E <b>T</b> L <b>T</b> S <b>I</b> V <b>S</b>	535
AFAP1L2	<b>H</b> L <b>Y</b> S <b>F</b> R <b>I</b> L <b>H</b> K <b>E</b> E <b>L</b> A <b>K</b> L <b>A</b> K <b>S</b> E <b>E</b> M <b>G</b> H <b>L</b> G <b>L</b> L <b>L</b> S <b>E</b> S <b>G</b> S <b>K</b> T <b>PE</b> E <b>F</b> T <b>Y</b> D <b>Y</b> V <b>D</b> A <b>D</b> R <b>V</b> S <b>C</b> I <b>V</b> S	470
Consensus	H FR L E A LEA SE MG W G LL E GS PE YDY D	
AFAP1	TAK <b>Q</b> T <b>F</b> C <b>F</b> M <b>N</b> R <b>R</b> V <b>I</b> S <b>A</b> N <b>P</b> Y <b>L</b> G <b>G</b> T <b>S</b> N <b>G</b> -----Y <b>A</b> H <b>P</b> S <b>G</b> T <b>A</b> L <b>H</b> Y <b>D</b> D <b>V</b> P <b>C</b> I	507
AFAP1L1	A <b>G</b> R <b>N</b> S <b>F</b> L <b>Y</b> A <b>R</b> -----S <b>C</b> Q <b>N</b> Q-----W <b>P</b> E <b>P</b> R <b>---</b> V <b>Y</b> D <b>D</b> V <b>P</b> Y <b>E</b>	563
AFAP1L2	A <b>A</b> K <b>N</b> S <b>L</b> L <b>L</b> M <b>Q</b> R <b>K</b> F <b>S</b> E <b>P</b> N <b>T</b> Y <b>I</b> D <b>G</b> L <b>P</b> S <b>Q</b> D <b>R</b> Q <b>E</b> E <b>L</b> Y <b>D</b> D <b>V</b> L <b>S</b> E <b>L</b> T <b>A</b> A <b>V</b> E <b>P</b> T <b>E</b> E <b>A</b> T <b>P</b> V <b>A</b> D <b>D</b> P <b>N</b> E	530
Consensus	P D P	
AFAP1	NG <b>S</b> L-----	511
AFAP1L1	K <b>M</b> Q <b>D</b> -----	567
AFAP1L2	R <b>E</b> S <b>D</b> R <b>V</b> Y <b>L</b> D <b>L</b> T <b>P</b> V <b>K</b> S <b>F</b> L <b>H</b> G <b>P</b> S <b>S</b> A <b>Q</b> A <b>S</b> S <b>P</b> T <b>L</b> S <b>C</b> L <b>D</b> N <b>A</b> E <b>A</b> L <b>P</b> A <b>D</b> S <b>G</b> P <b>G</b> T <b>P</b> D <b>E</b> F <b>C</b> I <b>K</b> P	590
Consensus		
AFAP1	-----K <b>G</b> K <b>P</b> P <b>V</b> A <b>S</b> N <b>G</b> V <b>T</b> G <b>K</b> G <b>T</b> L <b>S</b> S <b>Q</b> P <b>K</b> K <b>A</b> D <b>P</b> A <b>V</b> V <b>K</b> R <b>T</b> G <b>S</b> -----N	549
AFAP1L1	-----E <b>E</b> P <b>R</b> P <b>T</b> G <b>A</b> Q <b>---</b> V <b>K</b> R <b>H</b> A <b>S</b> S <b>C</b> E <b>K</b> S <b>H</b> R <b>V</b> D <b>P</b> Q <b>V</b> K <b>R</b> H <b>A</b> S-----S	604
AFAP1L2	E <b>N</b> L <b>G</b> E <b>Q</b> L <b>E</b> S <b>L</b> E <b>P</b> E <b>D</b> P <b>S</b> L <b>R</b> I <b>T</b> V <b>K</b> I <b>Q</b> T <b>E</b> Q <b>R</b> I <b>S</b> F <b>P</b> S <b>C</b> P <b>D</b> A <b>V</b> V <b>A</b> T <b>P</b> P <b>G</b> A <b>S</b> P <b>P</b> V <b>K</b> D <b>R</b> L <b>R</b> V <b>T</b>	650
Consensus	S D S	
AFAP1	A <b>A</b> Q <b>Y</b> K <b>Y</b> G <b>K</b> N <b>R</b> V <b>E</b> A <b>D</b> <b>A</b> K <b>R</b> L <b>Q</b> T <b>K</b> E <b>E</b> E <b>L</b> L <b>K</b> R <b>K</b> E <b>A</b> L <b>R</b> N <b>R</b> L <b>A</b> Q <b>L</b> R <b>K</b> R <b>K</b> D <b>L</b> R <b>A</b> I <b>E</b> V <b>N</b> A <b>G</b> R <b>K</b> P <b>O</b> A	609
AFAP1L1	A <b>N</b> Q <b>Y</b> K <b>Y</b> G <b>K</b> N <b>R</b> A <b>E</b> E <b>D</b> A <b>R</b> R <b>V</b> L <b>E</b> K <b>E</b> K <b>L</b> E <b>K</b> E <b>K</b> E <b>T</b> I <b>R</b> T <b>E</b> L <b>I</b> A <b>L</b> R <b>Q</b> E <b>---</b> R <b>E</b> L <b>K</b> E <b>A</b> I <b>R</b> S <b>S</b> G <b>A</b> -----	663
AFAP1L2	<b>S</b> A <b>E</b> I <b>K</b> L <b>G</b> K <b>N</b> R <b>T</b> E <b>A</b> E <b>V</b> K <b>R</b> Y <b>T</b> E <b>E</b> K <b>R</b> L <b>E</b> K <b>K</b> E <b>E</b> I <b>R</b> G <b>H</b> L <b>A</b> Q <b>L</b> R <b>K</b> E <b>K</b> R <b>E</b> L <b>K</b> E <b>T</b> L <b>L</b> K <b>T</b> <b>D</b> K <b>E</b> V <b>L</b> A	710
Consensus	K GKN R E R EKE L K KE R L LR E L	
AFAP1	<b>L</b> L <b>E</b> <b>---</b> K <b>L</b> Q <b>L</b> E <b>E</b> E <b>C</b> R <b>Q</b> K <b>E</b> A <b>E</b> R <b>V</b> S <b>L</b> E <b>L</b> E <b>L</b> T <b>E</b> V <b>K</b> E <b>S</b> L <b>K</b> K <b>A</b> L <b>A</b> G <b>G</b> V <b>T</b> L <b>G</b> L <b>A</b> I <b>E</b> P <b>K</b> S <b>G</b> T <b>S</b> S <b>P</b> Q <b>S</b> P	669
AFAP1L1	<b>L</b> L <b>E</b> <b>---</b> A <b>V</b> A <b>T</b> L <b>E</b> A <b>Q</b> C <b>R</b> A <b>K</b> E <b>R</b> R <b>I</b> D <b>L</b> E <b>L</b> K <b>L</b> V <b>A</b> V <b>K</b> E <b>R</b> L <b>Q</b> S <b>L</b> A <b>G</b> G <b>P</b> A <b>L</b> G <b>L</b> S <b>V</b> S <b>S</b> K <b>---</b> <b>P</b> K <b>S</b> ---	717
AFAP1L2	<b>S</b> L <b>E</b> Q <b>L</b> K <b>E</b> I <b>D</b> E <b>C</b> R <b>G</b> E <b>S</b> R <b>R</b> V <b>D</b> L <b>S</b> I <b>M</b> E <b>V</b> K <b>D</b> N <b>L</b> K <b>K</b> A <b>E</b> A <b>---</b> G <b>P</b> V <b>T</b> L <b>G</b> T <b>T</b> V <b>D</b> T <b>T</b> -----	761
Consensus	LE CR E R LEL VK L AG LG	
AFAP1	V <b>F</b> R <b>H</b> R <b>T</b> L <b>E</b> N <b>S</b> P <b>I</b> S <b>S</b> C <b>D</b> T <b>S</b> D <b>T</b> E <b>G</b> P <b>V</b> P <b>V</b> N <b>S</b> A <b>A</b> V <b>L</b> K <b>K</b> S <b>Q</b> A <b>A</b> P <b>G</b> S <b>S</b> P <b>C</b> R <b>G</b> H <b>V</b> L <b>R</b> K <b>A</b> K <b>E</b> W <b>E</b> L <b>K</b> N <b>G</b>	729
AFAP1L1	--- <b>G</b> E <b>T</b> A <b>N</b> K <b>P</b> Q <b>N</b> S <b>V</b> P <b>---</b> <b>E</b> Q <b>P</b> L <b>P</b> V <b>N</b> C <b>V</b> S <b>E</b> L <b>R</b> R <b>K</b> S <b>P</b> S <b>I</b> V <b>A</b> S <b>---</b> N <b>Q</b> G <b>R</b> V <b>L</b> Q <b>K</b> A <b>K</b> E <b>W</b> E <b>M</b> K <b>K</b> T	768
AFAP1L2	--- <b>H</b> L <b>E</b> N <b>V</b> S <b>P</b> R <b>K</b> A <b>V</b> T <b>P</b> A <b>S</b> A <b>P</b> D <b>C</b> T <b>P</b> V <b>N</b> S <b>A</b> T <b>T</b> L <b>K</b> N <b>R</b> P <b>L</b> S <b>V</b> V <b>V</b> T <b>---</b> G <b>K</b> G <b>T</b> V <b>L</b> Q <b>K</b> A <b>K</b> E <b>W</b> E <b>K</b> G <b>A</b>	817
Consensus	PVN L G VL KAKEW K	
AFAP1	T 730	
AFAP1L1	-	
AFAP1L2	S 818	
Consensus		

**Fig. 2.** AFAP family members share both sequence and domain similarity. (A) AFAP1, AFAP1L1 and AFAP1L2 amino acid sequences were compared using ClustalW2 alignment (Larkin et al., 2007). Consensus sequence between all three family members is labeled as consensus. Intron/exon boundaries are marked by red letters. Predicted SH3 binding motifs are highlighted in green, predicted SH2 binding motifs in pink, predicted PH domains in light blue, predicted Substrate Domain (SD) in yellow, predicted leucine zipper (AFAP1, AFAP1L1) and coiled-coil (AFAP1L2) in red and predicted actin binding domain in dark blue. The AFAP1L1 peptide sequence used to create the 1L1-CT antibody is underlined. (B) Modular domain organization of AFAP family members was compared. SH3bm = SH3 binding motif, SH2bm = SH2 binding motif, PH1 = pleckstrin homology domain 1, PH2 = pleckstrin homology domain 2, SD = serine/threonine rich substrate domain, Lzip = leucine zipper, ABD = actin binding domain. Sequences that do not correlate with an identified type of modular domain or motif are labeled "A, B, C, D or E". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

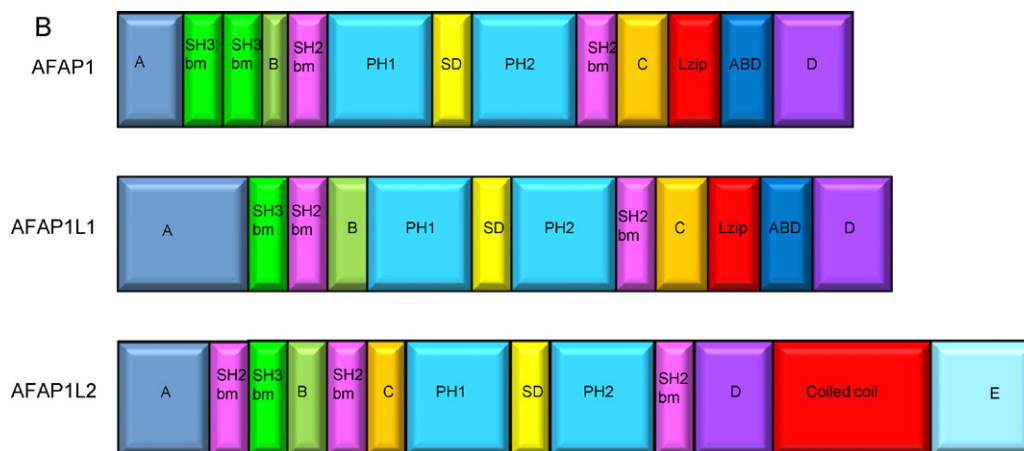


Fig. 2. (Continued)

is directly adjacent to the actin binding domain (Qian et al., 2004). AFAP1L1 contains a similar sequence that may have the ability to act as a leucine zipper or coiled-coil motif as well as a putative actin binding domain which is similar to the known actin filament binding domain found in AFAP1 (Qian et al., 2000). AFAP1L2/XB130 contains a coiled-coil motif in its C-terminus and a sequence that is similar to the AFAP1 actin binding domain, but AFAP1L2/XB130 has not been demonstrated to bind actin filaments (Xu et al., 2007). An amino acid sequence alignment of the three family members found that AFAP1 and AFAP1L1 overall share 44% identity and 71% similarity while AFAP1L1 and AFAP1L2/XB130 share 31% identity and 61% similarity and their sequences predict conserved functional domains within the proteins (Fig. 2B).

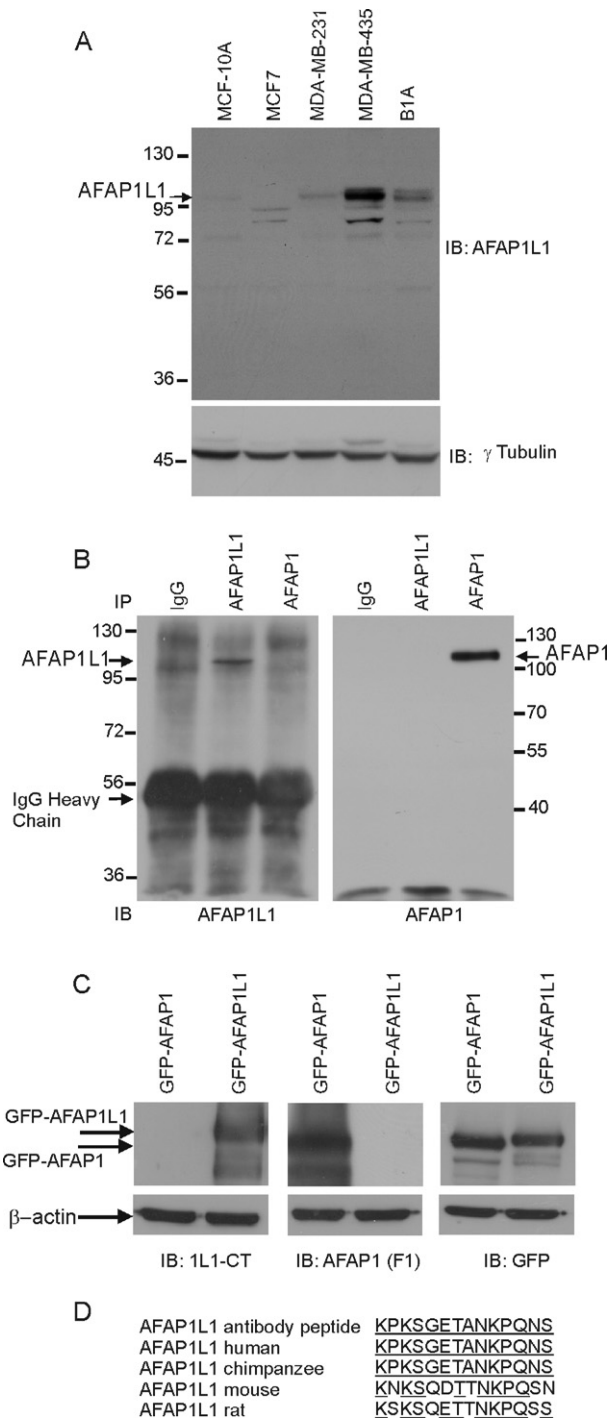
#### A novel antibody is specific for AFAP1L1

A rabbit polyclonal antibody was generated against the carboxy terminal amino acids 714–727 of human AFAP1L1. We refer to this antibody as 1L1-CT. We also utilized two commercially available AFAP1L1 antibodies, called Ab1 (epitope defined as amino acids 525–659) and Ab2 (epitope defined as amino acids 21–159). The ability of these antibodies to detect AFAP1L1 was studied using lysates of human breast lines MCF-10A, MCF-7, MDA-MB-231, B1A (a variant of MDA-MB-231 cells that had AFAP1 knocked down using shRNA, Dorfleutner et al., 2007), MDA-MB-435 as well as monkey kidney cell line Cos-1. All three AFAP1L1 antibodies detected a distinct protein band by Western blot analysis with a  $M_r$  of 115 kDa (Fig. 3A, result from Ab2 as a representative example) with MDA-MB-435 having the highest endogenous expression levels of AFAP1L1 among the tested cell lines. The  $M_r$  of AFAP1L1 was further confirmed by siRNA knockdown against AFAP1L1 (data not shown). The expression pattern of AFAP1L1 across breast cancer cell lines was further confirmed in messenger RNA levels through quantitative RT-PCR analysis (data not shown). To test the ability of the 1L1-CT antibody to immunoprecipitate endogenous AFAP1L1, Cos-1 cells were lysed and 1 mg of lysate (1  $\mu\text{g}/\mu\text{l}$ ) was incubated with either 5  $\mu\text{g}$  of 1L1-CT, 5  $\mu\text{g}$  of AFAP1 antibody F1 or 5  $\mu\text{g}$  of control rabbit IgG and the immunoprecipitate resolved by SDS-PAGE (Fig. 3B). Upon Western blot analysis with 1L1-CT, it was apparent that the 1L1-CT antibody specifically immunoprecipitated a protein with a  $M_r$  of 115 kDa, consistent with the predicted  $M_r$  for AFAP1L1 (Fig. 3B, left panel, middle lane); while the AFAP1 antibody F1 did not immunoprecipitate a protein of this  $M_r$  (Fig. 3B, left panel, right lane) that could be detected by the 1L1-CT antibody. To test the specificity of F1 antibody for endogenous AFAP1, Cos-1 cells were lysed and 1.5 mg (1  $\mu\text{g}/\mu\text{l}$ ) of lysate was incubated with

rabbit IgG, F1 or 1L1-CT. F1 antibody specifically immunoprecipitated the expected AFAP1 protein with an  $M_r$  of 110 kDa (Fig. 3B, right panel, right lane) and did not immunoprecipitate AFAP1L1 (Fig. 3B, right panel, middle lane), indicating that these two antibodies are specific for endogenous protein. To determine if 1L1-CT and F1 antibodies can specifically recognize overexpressed protein, plasmids encoding GFP-AFAP1L1 and GFP-AFAP1 were transfected into Cos-1 cells. Western blot analysis of these cells (Fig. 3C) showed that 1L1-CT antibody detected overexpressed GFP-AFAP1L1 but not overexpressed GFP-AFAP1 (Fig. 3C, left panel). AFAP1 antibody F1 detected GFP-AFAP1 but not GFP-AFAP1L1 (Fig. 3C, middle panel). These data indicate that the AFAP1L1 antibody 1L1-CT and AFAP1 antibody F1 have specificity for AFAP1L1 and AFAP1, respectively. These data also indicated that 1L1-CT antibody is specific to the AFAP1L1 of human and monkey species. 1L1-CT antibody did not recognize any distinct band in murine cell lysates (data not shown), because the epitope is not conserved in murine AFAP1L1 (Fig. 3D). Sigma antibodies Ab1 and Ab2 also failed to recognize AFAP1L1 in murine cell lysates. An alignment of the peptide sequence used to create 1L1-CT against the amino acid sequence of human, chimpanzee, mouse and rat AFAP1L1 was shown to illustrate the similarity of the 1L1-CT binding site across species (Fig. 3D).

#### Immunohistochemical analysis of AFAP1L1

The ability of the 1L1-CT antibody to specifically recognize endogenous AFAP1L1 in its native conformation by immunoprecipitation and to recognize AFAP1L1 by immunofluorescence (Supplemental Figure 1) demonstrated its usefulness for immunohistochemistry. To determine the tissue localization of AFAP1L1, immunohistochemistry was performed on human breast, colon and brain tissues using 1L1-CT antibody (Fig. 4). AFAP1 or AFAP1L1 tissue localizations were compared using sequential sections of tissue immunolabeled with F1 or 1L1-CT antibodies, respectively, in order to contrast expression patterns. In the breast, AFAP1 was found to strongly associate with the ductal cells and also the breast microvasculature (Fig. 4A panels a, c and e). AFAP1L1 was found in the contractile myoepithelial cell layer which surrounds the breast ducts and in the microvasculature (Fig. 4A panels b, d and f), similar to AFAP1. In the colon (Fig. 4B), AFAP1 was found in the epithelial mucous membrane as well as in the colonic crypts (Fig. 4B panels a and g) which aid in mucous production and production of new epithelial cells for the intestinal surface (Sherwood, 2003). AFAP1L1 was also found in the mucous membrane and colonic crypts (Fig. 4B panels b and h). Both AFAP1 and AFAP1L1 are found in the microvasculature (Fig. 4B panels a–d) while AFAP1 can also



**Fig. 3.** A novel antibody specifically recognizes AFAP1L1. (A) Cell lines were lysed in  $2\times$  SDS buffer, resolved by 8% SDS-PAGE and transferred to PVDF. The Sigma antibody Ab2 specifically recognized a protein band of 115 kDa. Bands identified as AFAP1L1 and recognized by the Ab2 antibody (top panel) are indicated by an arrow. Gamma tubulin was used as a loading control. Not shown, but in an adjacent lane, was a lysate prepared from 293T cells transfected with untagged AFAP1L1 which was used in identifying the band corresponding to AFAP1L1. (B) Endogenous AFAP1L1 and AFAP1 were specifically immunoprecipitated from Cos-1 cells using  $5\mu\text{g}$  of 1L1-CT and F1 polyclonal antibodies respectively and the resolved proteins detected with 1L1-CT (left panel) or AFAP1 monoclonal antibodies (BD transduction, right panel). Rabbit IgG antibody was used as a control. Note the differences in the molecular weight markers for each Western. (C) GFP-AFAP1L1 and GFP-AFAP1 were overexpressed in Cos-1 cells and lysed in  $2\times$  SDS buffer. Lysates were resolved by 8% SDS-PAGE, transferred to PVDF membrane and immunoblotted with either AFAP1L1 (1L1-CT) or AFAP1 (F1) antibody. GFP (top right Western) and  $\beta$ -actin (bottom Westerns) were used as loading controls. (D) The peptide sequence used to create antibody 1L1-CT was compared to analogous sequences in human, chimpanzee, mouse and rat to show similarity between antibody binding sites.

**Table 1**

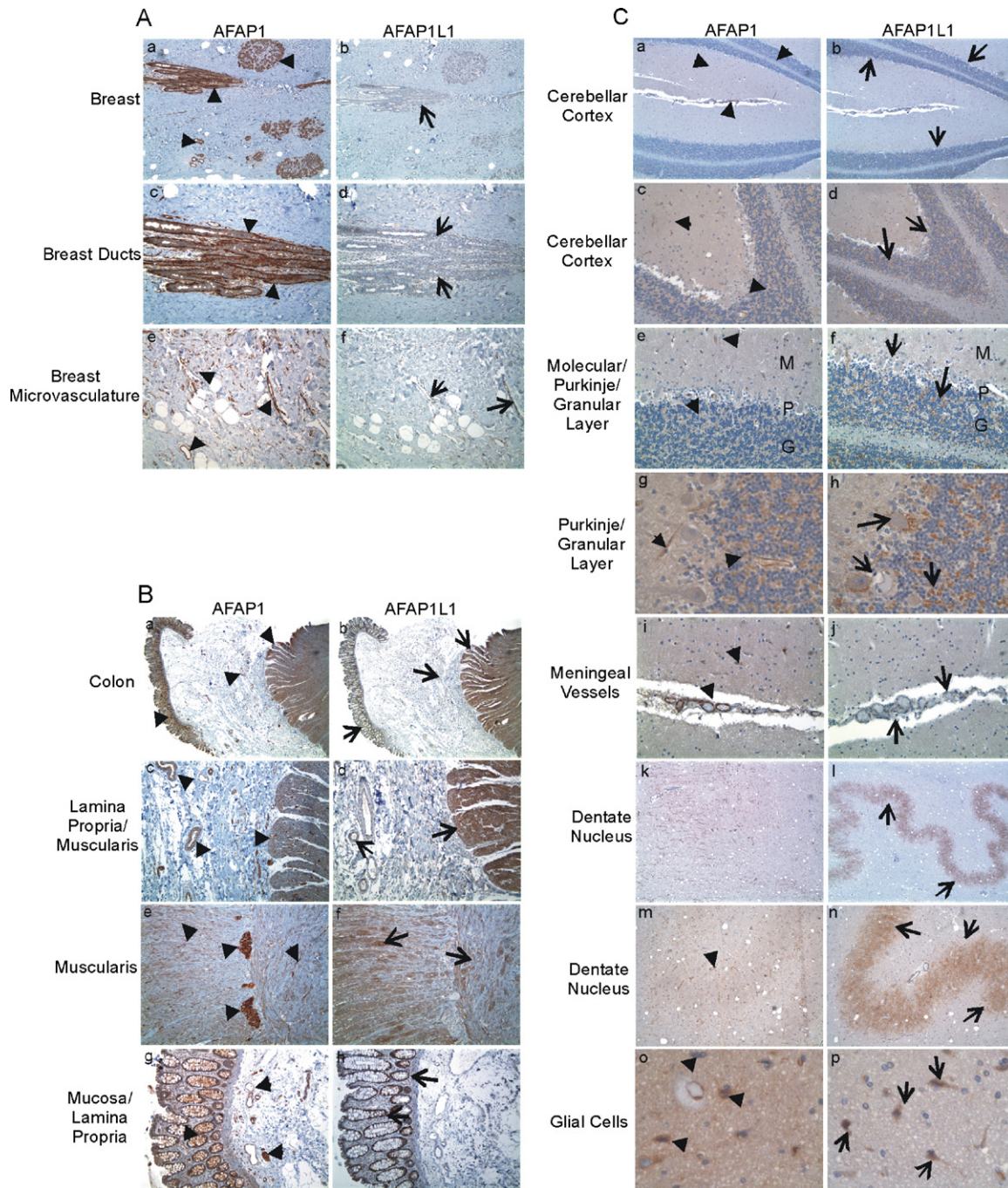
AFAP1 and AFAP1L1 immunohistochemical signal intensity in human tissue. The intensity of AFAP1 staining in human breast, colon and brain tissues was analyzed. Staining in breast ducts was scored as ++++ for highest level of intensity. Staining in the dentate nucleus was scored as – for no level of staining. Other tissues labeled for AFAP1 were compared using this scale. AFAP1L1 staining intensity was analyzed in a similar manner, designating AFAP1L1 staining in the dentate nucleus as ++++ and lamina propria as – for no level of staining. Other tissues labeled for AFAP1L1 were compared using this scale.

	AFAP1	AFAP1L1
Breast lobule	++++	+
Breast duct	++++	+
Breast microvasculature	+++	+
Colon muscularis	++	+++
Colon mucosa	+++	+++
Colon lamina propria	–	–
Cerebellar cortex	+	+
Cerebellar granule cell layer	++	++++
Cerebellar Purkinje layer	+	++++
Meningeal vessels	++++	+
Dentate nucleus	–	++++
Glial cells	++	+++

be found in nerves that pass through the lamina propria (Fig. 4B panel g). The lamina propria lies directly beneath the epithelial cell layer and consists of connective tissue, blood vessels, nerve fibers and lymphatic ducts (Widmaier et al., 2003). The muscularis, layers of smooth muscle which provide movement of the colon, expresses both AFAP1 and AFAP1L1 (Fig. 4B panels c–f) while AFAP1 is solely found in Auerbach's plexus (Fig. 4B panel e), the nerves that innervate the muscle of the gut (Sherwood, 2003). While AFAP1 and AFAP1L1 have overlapping localization in human breast and colon tissue, the human brain showed examples of differential expression patterns for these two proteins (Fig. 4C). The cerebellum is located at the base of the skull and is an important part of the motor system. Divided into three layers, the cerebellar cortex is composed of a granular cell layer consisting of small granule cells which receive input from mossy fibers and extend into the molecular layer, the outermost portion of the cerebellar cortex which houses stellate and basket cells. Purkinje cells are large neurons acting as the sole output of motor coordination from the cerebellar cortex and are found in a single layer between the molecular and granular layers (Squire et al., 2008). AFAP1 is found in the microvasculature of the brain, in the molecular layer and meningeal vessels and to a slight level around the granule cells of the granular layer (Fig. 4C panels a, c, e and g). AFAP1L1 is again found in low levels in the microvasculature of the brain but is localized around the Purkinje neurons and the granule cells of the granular layer (Fig. 4C panels b, d, f and h). This immunolabeling is not inside of either the Purkinje neurons or the granule cell bodies but instead extends away from the cell body. Although the source of this immunolabeling is unknown, mossy fibers and climbing fibers extend into the granule cell layer and Purkinje cell layer respectively, synapsing onto granule and Purkinje cells (Squire et al., 2008). This may provide the source of immunolabeling for AFAP1L1 away from the granule and Purkinje cell body. Outside of the cerebellar cortex, AFAP1 and AFAP1L1 are both found in glial cells (Fig. 4C panels o and p) but have a differential expression pattern in the dentate nucleus (Fig. 4C k–n), one of the four deep cerebellar nuclei responsible for voluntary movements of the extremities (Squire et al., 2008). Again, AFAP1L1 is highly expressed away from the cell bodies within the dentate nucleus, while AFAP1 was not detected in this structure. Relative intensities of immunohistochemical signal for AFAP1 and AFAP1L1 are presented in Table 1.

#### AFAP1L1 subcellular localization

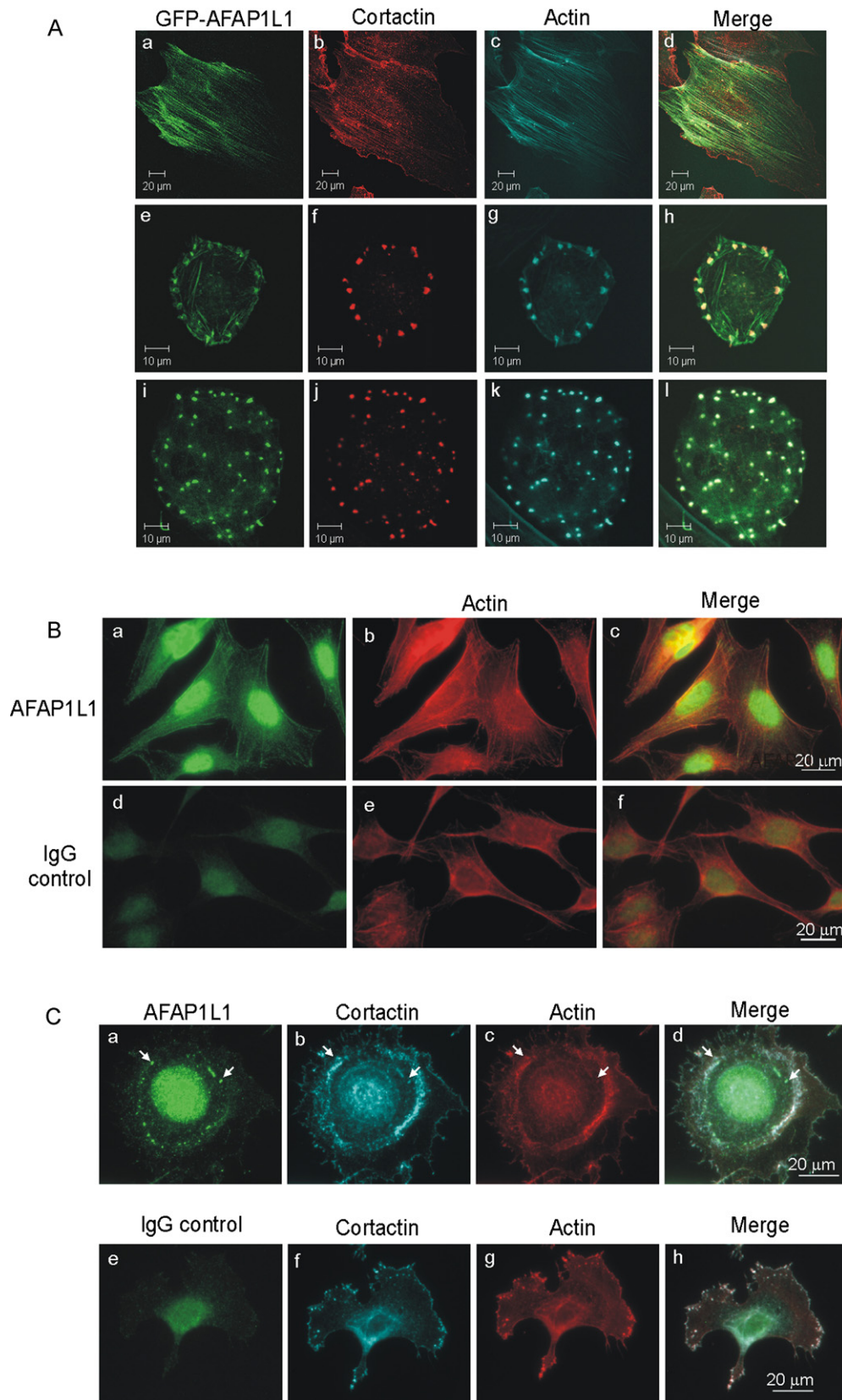
AFAP1 is known to bind actin filaments, move to actin rich structures called podosomes upon stimulation with the phorbol



**Fig. 4.** Immunohistochemical analysis of AFAP1L1 shows differential expression from AFAP1 in human tissue. Paraffin-embedded human breast (A), colon (B) and brain (C) tissues were analyzed for AFAP1 and AFAP1L1 localization using F1 and 1L1-CT antibodies respectively. Breast regions include breast ducts (A, panels a–d), breast lobules (A, panels a and b) and microvasculature (A, panels e and f). Colon regions include the mucosa (B, panels a, b, g, h), lamina propria (B, panels a–d, g and h) and muscularis (B, panels a–f). Brain regions include the cerebellar cortex (C, panels a–j), dentate nucleus (C, panels k–n) and glial cells (C, panels o and p). AFAP1L1 is designated by long thin arrows while AFAP1 is designated by arrowheads.

esterPDBu and also to be localized in podosome-like structures termed invadopodia in MDA-MB-231 cells, while AFAP1L2/XB130 is not known to bind actin filaments (Flynn et al., 1993; Gatesman et al., 2004; Xu et al., 2007). Thus, we sought to determine if AFAP1L1 had the potential to be localized with actin filaments and podosomes, similar to AFAP1 (Gatesman et al., 2004). For this analysis, we used the rat-derived A7r5 cell line, a well established cell model system for podosome formation (Hai et al., 2002). Overexpression of GFP-AFAP1L1 alone in A7r5 cells pro-

duced two distinct phenotypes (Fig. 5A). GFP-AFAP1L1 expressing cells either have GFP-AFAP1L1 decorating actin stress fibers while cortactin is found in the cytoplasm and around the cell periphery (Fig. 5A panels a–d) or both GFP-AFAP1L1 and cortactin colocalizing in punctate actin dots along the ventral membrane (Fig. 5A and Supplemental Figure 2). The adaptor protein cortactin was used as a marker for podosome formation. Podosome formation was observed either around the cell periphery (Fig. 5A panels e–h) or scattered across the ventral membrane (Fig. 5A



**Fig. 5.** Subcellular localization of GFP-AFAP1L1 shows association with actin and invadopodia. (A) A7r5 cells transiently expressing GFP-AFAP1L1 were plated onto fibronectin-coated coverslips and immunolabeled for cortactin (Millipore). Actin was visualized with TRITC-phalloidin (Sigma). Representative images of cells with well-formed stress fibers or podosome formation are shown. (B) MDA-MB-435 cells were plated on fibronectin coated coverslips and immunolabeled for endogenous AFAP1L1 (Sigma Ab2, panels a–c). Rabbit IgG was used as a control (panels d–f) and actin was visualized with AlexaFluor labeled phalloidin. Epifluorescence images of representative cells are shown. (C) MDA-MB-435 cells were transfected with Src 527F construct, plated onto fibronectin coated coverslips and immunolabeled for AFAP1L1 (Sigma Ab1, panels a–d). Rabbit IgG was used as control antisera for AFAP1L1 antibody (panels e–h). Cortactin was immunolabeled with monoclonal anti-cortactin antibodies (4F11, Millipore) and actin was visualized by AlexaFluor labeled phalloidin (panels c and g). Examples of AFAP1L1 co-localizing to invadopodia, actin and cortactin rich punctate structures are marked with white arrows.

panels i–l). Thus, AFAP1L1 decorates actin stress fibers and co-localizes with cortactin in podosomes. Overexpression of AFAP1L1 induced the formation of podosomes in a small percentage of cells.

Next, we utilized MDA-MB-435 cells to determine the subcellular localization of endogenous AFAP1L1 based on the high level of expression of AFAP1L1 in this cell line. Immunolabeling of MDA-MB-435 with Sigma Ab2 (Fig. 5B, panels a–c) and Ab1 (data not shown) showed a population of AFAP1L1 decorating actin stress filaments while the rest of AFAP1L1 was detected diffusely across the cytoplasm. A similar pattern of expression was observed for the 1L1-CT antibody in MEF cells overexpressing untagged AFAP1L1 (Supplemental Figure 1).

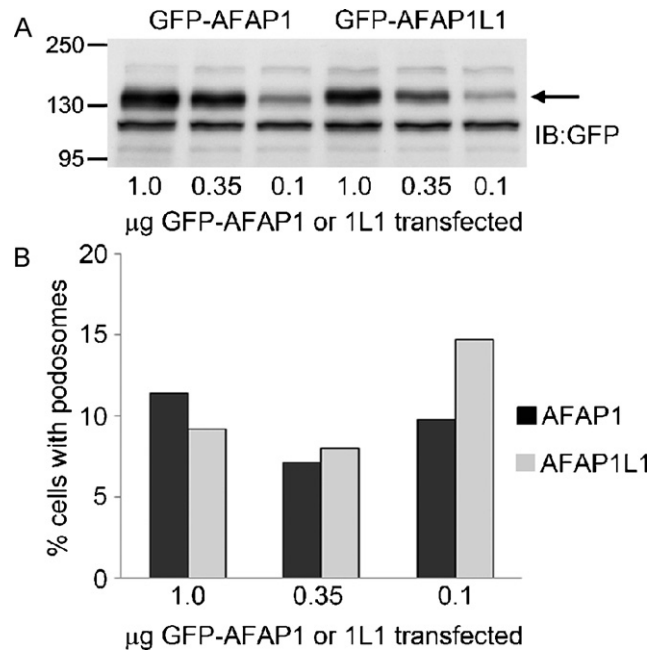
To determine whether endogenous AFAP1L1 could co-localize to invadopodia, we transiently transfected a constitutively active Src construct (Src527F) into MDA-MB-435 to induce invadopodia, podosome-like structures found in cancer cells. The overexpression of Src527F induced punctate actin- and cortactin-rich structures in these cells (Fig. 5C, b and c, f and g) accompanied by the loss of stress filaments, which were consistent with the appearance of typical invadopodia. The immunolabeling of endogenous AFAP1L1 (Fig. 5C, a) showed discernable association with invadopodia, co-localizing with cortactin and actin (Fig. 5C, a–d, marked with a white arrow as an example of colocalization). These data indicate that AFAP1L1, like AFAP1, is associated with actin stress filaments and localizes to invadosomes (the collective term for podosomes and invadopodia) at both overexpressed and endogenous levels (also see Supplemental Figure 3) (Linder, 2009; Saltel et al., 2011).

#### Quantification of podosome formation in A7r5 expressing AFAP1 or AFAP1L1

In a previous study of AFAP1, expression of GFP-AFAP1 was sufficient to induce podosomes in a portion of the A7r5 cells in which it was expressed (Dorflautner et al., 2008). Therefore, we sought to compare the potency of the GFP-AFAP1 and GFP-AFAP1L1 constructs to induce podosomes using a dose–response analysis. A7r5 cells were transfected with equal amounts of total plasmid cDNA containing the indicated amount of GFP-AFAP1 or GFP-AFAP1L1 plasmid (Fig. 6). Transfection of equal amounts of plasmid (combination of empty vector and GFP-AFAP) ensured that transfection efficiencies were identical for each dose of plasmid. Forty eight hours after transfection, the cells expressing either GFP-AFAP1 or GFP-AFAP1L1 were assessed for podosome formation. Reduction of plasmid DNA resulted in a corresponding reduction of GFP-AFAP1 and GFP-AFAP1L1 expression (Fig. 6A). The reduction of expression was also reflected in a reduction in the percent of cells exhibiting podosomes (Fig. 6B). When 1  $\mu$ g and 0.35  $\mu$ g of plasmid DNA was used, the percentage of GFP-AFAP1 expressing cells exhibiting podosomes dropped from 11.4% to 7.1% while in GFP-AFAP1L1 expressers the change was more modest, 9.2% to 8.0%. At 0.1  $\mu$ g of DNA the number of podosome cells appeared to increase. This increase resulted from the variability in expression levels among cells and the dimness of the low expressers which made these cells difficult to detect by immunofluorescence. Overall, this experiment demonstrated that under identical conditions of transfection, expression, processing and immunofluorescence analysis, expression of GFP-AFAP1 and GFP-AFAP1L1 induce podosome formation similarly in A7r5 cells.

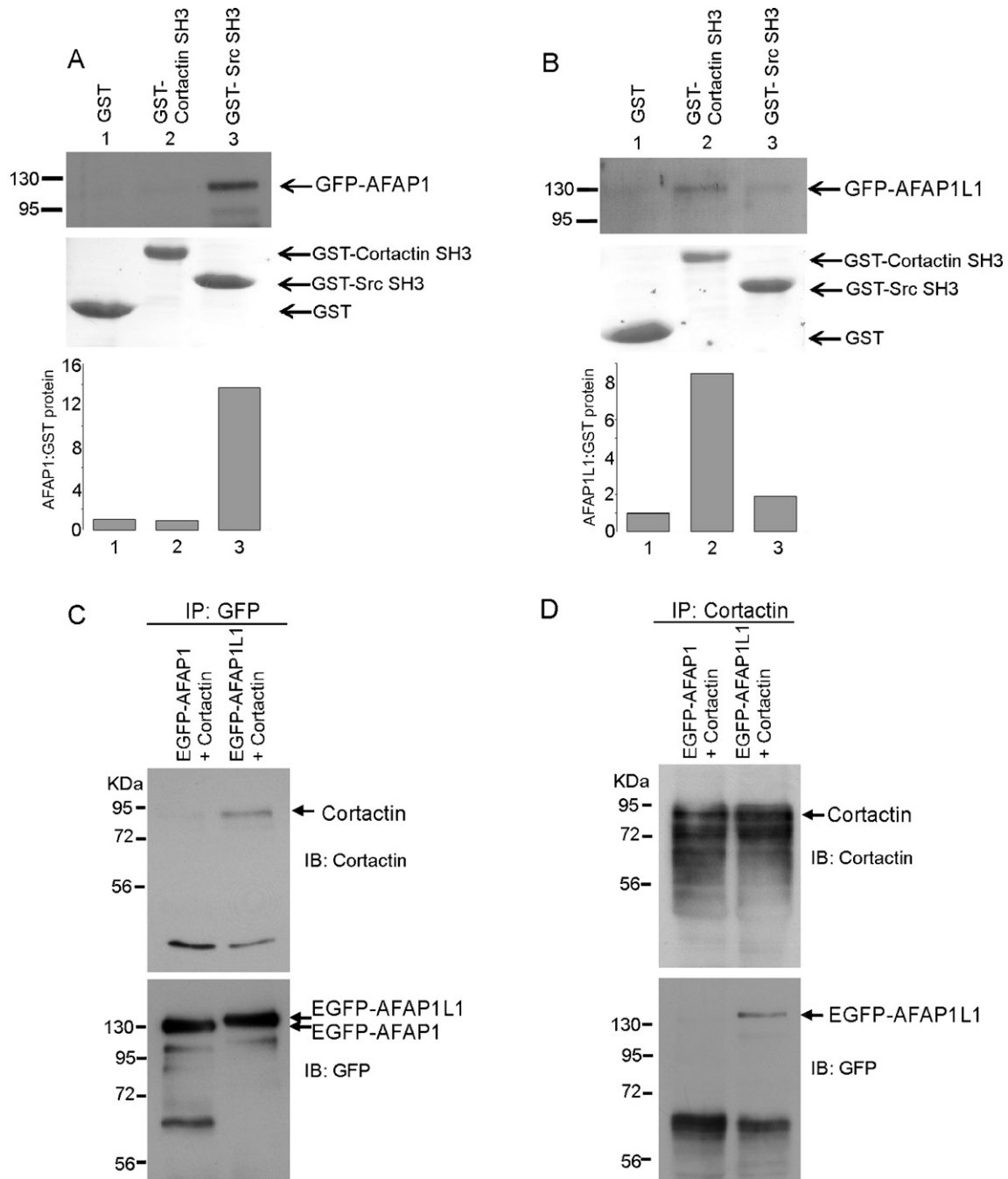
#### Interaction of AFAP1L1 and cortactin

To determine if AFAP1L1 might have a differential ability to interact with proteins relative to AFAP1, we screened SH3 domains from various proteins for the binding to AFAP1L1. A panomics array screening of SH3 domains showed that the SH3 domain



**Fig. 6.** Podosome formation in A7r5 transfected with GFP-AFAP1 or GFP-AFAP1L1 plasmids. A7r5 cells were transfected with the indicated amounts of plasmids encoding either GFP-AFAP1 or GFP-AFAP1L1 (in combination with pcDNA3.1) to bring the total amount of plasmid DNA to 1  $\mu$ g for each transfection. Twenty four hours post-transfection, the cells were transferred to fibronectin-coated coverslips. Forty-eight hours after transfection, cells were processed for immunofluorescence analysis or used to prepare whole cell SDS lysates. (A) Equal amounts of SDS lysates were resolved by 8% SDS-PAGE and then transferred to PVDF membranes and subsequently probed with an antiserum that recognizes GFP. (B) Cells expressing GFP-AFAP1 or GFP-AFAP1L1 were assessed for podosome formation and the percentage of cells exhibiting podosomes for each transfection was calculated. 150–300 cells were counted for each transfection. Panels A and B represent one experiment out of two independently performed experiments.

of cortactin and the SH3 binding motif of AFAP1L1 had a strong potential to interact (data not shown). To confirm these screening results, GST fusion proteins containing the cortactin SH3 domain and the Src SH3 domain were incubated with 1 mg of cell lysate containing either overexpressed GFP-AFAP1L1 or GFP-AFAP1. As expected from the known binding of AFAP1 to Src, AFAP1 showed a strong interaction with the Src SH3 domain with a 14-fold increase in the ratio of AFAP1 pulled down by GST-Src SH3 as compared to GST control while precipitation by the cortactin SH3 domain was undetectable (Fig. 7A). However, AFAP1L1 was more efficiently precipitated by GST-SH3-cortactin than GST-SH3-Src with an approximately 4.5-fold increase in the ratio of AFAP1L1 pulled down by GST-cortactin-SH3 compared to GST-SH3-Src (Fig. 7B). These data indicate that the SH3 binding motifs of AFAP1 and AFAP1L1 have the ability to interact with different affinities toward different SH3 binding partners. To further examine the potential binding of AFAP1L1 with cortactin, we transiently overexpressed either GFP-AFAP1 or GFP-AFAP1L1 with cortactin in 293T cells. GFP-AFAP1L1 co-immunoprecipitated with cortactin (Fig. 7C, upper panel) more efficiently than did GFP-AFAP1 (the amounts of immunoprecipitated GFP proteins were relatively equal Fig. 7C, lower panel). Conversely, cortactin specifically co-immunoprecipitated GFP-AFAP1L1, but not GFP-AFAP1 (Fig. 7D, upper panel). These data suggest that cortactin can form a complex with AFAP1L1, but is either unable to form a complex with AFAP1 or its ability to bind in a complex with AFAP1 is below detection limits. This interaction is hypothesized to occur through SH3 interactions based on our affinity precipitations with the SH3 domains and suggests a unique function for AFAP1L1 that is distinct from AFAP1.



**Fig. 7.** AFAP1L1 interacts with the cortactin SH3 domain. (A) 1 mg of lysate from 293T cells transiently expressing GFP-AFAP1 was incubated with 50  $\mu$ g of GST, the GST-Src-SH3 domain or the GST-cortactin-SH3 domain bound fusion protein to glutathione Sepharose 4B beads and probed for GFP through Western blot analysis (upper panel). The lower panel represents a GelCode Blue Stain (ThermoScientific) of GST or GST fusion protein. A graph representing the ratio of AFAP1 pulled down by each GST fusion protein compared to GST control using scanning densitometry is shown. (B) 1 mg of lysate from 293T cells transiently expressing GFP-AFAP1L1 was incubated with 50  $\mu$ g of GST, the GST-Src-SH3 domain or the GST-cortactin-SH3 domain bound to glutathione Sepharose 4B beads and probed for GFP through Western blot analysis (upper panel). The lower panel represents a GelCode Blue Stain (ThermoScientific) of GST or GST fusion protein. A graph representing the ratio of AFAP1L1 pulled down by each GST fusion protein compared to GST control using scanning densitometry is shown. (C) 293T transiently expressing cortactin with either GFP-AFAP1 or GFP-AFAP1L1 were immunoprecipitated with anti-GFP antibody, probed for cortactin through Western blot analysis (upper panel) and then re-probed for GFP tagged proteins (lower panel). (D) 293T transiently expressing cortactin with either GFP-AFAP1 or GFP-AFAP1L1 were immunoprecipitated with anti-cortactin antibody, probed for GFP tagged proteins through Western blot analysis (lower panel) and then re-probed for cortactin (upper panel).

## Discussion

### AFAP family amino acid sequence

AFAP1 is a well-studied cSrc binding partner and actin filament cross-linking protein that is the prototype member of what is pre-

dicted to be a family of three proteins which also includes AFAP1L1 and AFAP1L2/XB130. There are no published reports on AFAP1L1 and the goal of this study was to analyze some of the similarities and differences between AFAP1 and AFAP1L1 in order to understand what functions AFAP1L1 may have relative to AFAP1. The AFAP family consists of three family members that share similarity in protein

binding motifs including both PH domains. Analysis of the overall amino acid structure of previously described AFAP1 and newly presented AFAP1L1 show 44% identity and 71% similarity between the two proteins. AFAP1 contains two juxtaposed SH3 binding motifs in its N-terminus, PPQMPLPEIP and PPDSGPPPLP, of which the first is necessary for efficient cSrc binding. Mutation of a necessary proline, Pro<sup>71</sup>, to an alanine, Ala<sup>71</sup>, in the AFAP1 SH3 binding motif prevented cSrc binding (Guappone and Flynn, 1997). While AFAP1L1 only contains one putative SH3 binding motif, DLPPPLPNKP, this sequence is not consistent with the conserved consensus cSrc SH3 binding motif. Rather, the AFAP1L1 SH3 binding motif more closely resembles that of a cortactin SH3 domain binding site, which preferentially binds a +PPΨPXKPKWL motif where + is a basic residue, Ψ is an aliphatic residue, and X is any amino acid (Sparks et al., 1996).

In addition to the SH3 binding motif, cSrc also has the ability to phosphorylate and bind two SH2 binding motifs in AFAP1. In AFAP1, an N-terminal YEEA sequence adjacent to the SH3 binding motifs and a C-terminal YDYI sequence have been shown to be phosphorylated by cSrc (Guappone et al., 1998). AFAP1L1 contains the conserved YEEA sequence in its N-terminus adjacent to the SH3 binding motif and a similar YDYV sequence in its C-terminus. Although we did not analyze AFAP1L1 as a possible target for phosphorylation by tyrosine kinases, these similar SH2 binding motifs may be potential targets for tyrosine phosphorylation. A ScanSite motif scan comparison between AFAP1 and AFAP1L1 predicts these sites for tyrosine phosphorylation and also predicts similar sites for serine/threonine phosphorylation as well (data not shown). The amino acid sequences between the PH1 and PH2 domains of AFAP1 and AFAP1L1 are rich in serine and threonine residues that are predicted to be sites of serine and threonine phosphorylation. In particular, AFAP1 is known to be phosphorylated on serine 277 by PKC $\alpha$  and this phosphorylation is important for podosome turnover (Dorfleutner et al., 2008). Although we have not validated AFAP1L1 as a PKC substrate, AFAP1L1 also contains a similar PKC $\alpha$  phosphorylation site.

The conservation of domain structure, overall sequence similarity and high similarity of sequence between the two PH domains indicates that AFAP1, AFAP1L1 and AFAP1L2/XB130 are members of a family of proteins, referred to here and in genome databases as the AFAP family. The amino terminal PH1 domain of AFAP1 has been known to function in intra-molecular regulation of AFAP1 (Qian et al., 2004). In addition, the PH1 domain is a binding partner for PKC $\alpha$  and phospholipids (Cunnick and Flynn, manuscript in preparation) (Qian et al., 2002).

The actin binding domain within AFAP1 is necessary and sufficient for the binding of AFAP1 to actin filaments (Qian et al., 2000). Comparison of the AFAP1 actin binding domain sequence to the corresponding sequence in AFAP1L1 does not indicate a high level of similarity although our data indicate that AFAP1L1 also has the ability to associate with actin filaments. We predict that the sequences within the C-terminus of AFAP1L1 that are similar to the known actin filament binding domain in AFAP1 may promote actin filament association, however, as the binding affinity of AFAP1 for f-actin is relatively low, we predict that the putative actin binding domain of AFAP1L1 may have a lower affinity for f-actin. Studies are underway to determine the mechanism of actin filament binding and if the leucine zipper/coiled-coil motif adjacent to the putative actin filament binding domain can regulate self-association and actin filament cross-linking, similar to AFAP1 (Qian et al., 2004).

#### *AFAP1L1 Western blot analysis*

AFAP1L1 can be found in human breast and breast cancer cell lines, as well as the Cos-1 monkey cell line. The predicted molec-

ular weight of AFAP1L1 is 85 kDa, but it is detected as a single protein band by Western blot analysis with a  $M_r$  of 115 kDa. Similarly, AFAP1 has a molecular weight of 82 kDa but is detected on Western blot as a 110 kDa protein. For AFAP1, it was hypothesized that the difference in molecular weight and  $M_r$  might be due to overall charge and the shape of the protein, which may also apply to AFAP1L1. The AFAP1L1 specific antibodies, 1L1-CT, Ab1 and Ab2 antibodies (latter two from Sigma), have the ability to specifically detect AFAP1L1 but not AFAP1 by Western blot. In our hands, all three antibodies could detect AFAP1L1 by immunoprecipitation, immunofluorescence and Western blot; however, the 1L1-CT antibody was determined to be less efficient in detecting denatured AFAP1L1 by Western blot, indicating that this antibody may preferentially recognize a conformational epitope and be less efficient in detecting denatured AFAP1L1, relative to Ab1 and Ab2, which appear to detect denatured AFAP1L1 efficiently. Conversely, the anti-AFAP1 antibody F1 is able to specifically immunoprecipitate AFAP1 but does not immunoprecipitate AFAP1L1.

#### *AFAP1L1 tissue expression pattern*

AFAP1 and AFAP1L1 have similar distribution in breast and colon tissue with the exception that AFAP1 can be found in the nerves that innervate the gut while AFAP1L1 was not detected there. While immunolabeling of the brain shows similarities between AFAP1 and AFAP1L1 in the granule cell layer and glial cells, differences occur in the brain where AFAP1L1 is found in the Purkinje cell layer and the dentate nucleus, while AFAP1 was not detected here. The dentate nucleus is known to be involved in motor coordination; alterations in the neuronal composition of signaling to and from the dentate have been implicated in diseases with motor dysfunction such as Alzheimer's disease, autism spectrum disorders and Pick's disease (Braak et al., 1999; Fukutani et al., 1999; Lotspeich and Ciaranello, 1993). Consistent immunolabeling of AFAP1L1 between the granule cell layer, Purkinje layer and dentate nucleus indicates that the AFAP1L1 immunohistochemical signal is not in the cell bodies of the granule cells, Purkinje neurons or cells of the dentate nucleus, but is instead found extending away from the cell body. Thus, we hypothesize that AFAP1L1 may be playing a role in either the axon terminals of afferent neurons or the dendritic spines of efferent neurons in these areas, both of which have a dynamic actin cytoskeleton and are rich in proteins involved in cytoskeletal remodeling, such as cortactin (Hering and Sheng, 2003). As we see the most marked difference between AFAP1 and AFAP1L1 expression in the dentate nucleus, we speculate that AFAP1L1 may play a role in the association of proteins involving signaling to or from the dentate nucleus. Notably, podosomes have recently been shown to be involved in maturation of the neuromuscular junction and a similar mechanism may be involved in neuron–neuron interactions (Proszynski et al., 2009).

#### *AFAP1L1 subcellular localization and podosome formation*

GFP-AFAP1L1 and endogenous AFAP1L1 colocalized with actin filaments and invadosomes similar to the subcellular localization pattern of AFAP1. Overexpression of GFP-AFAP1L1 induced podosome formation in the absence of extracellular signals (e.g., phorbol ester). A direct comparison of the ability of GFP-AFAP1 and GFP-AFAP1L1 to induce podosomes demonstrated that both proteins had similar capacities to induce these structures. Thus, like AFAP1, AFAP1L1 may have the ability to interact with proteins involved in podosome formation such as f-actin, cSrc or possibly cortactin. A panomics array of various SH3 domains was scanned for the ability to bind the AFAP1L1 SH3 binding motif, and these data indicated that the AFAP1L1 SH3 binding motif and the cor-

tactin SH3 domain showed the strongest interaction among those SH3 domains surveyed (data not shown). GST fusion proteins containing either the cortactin SH3 domain or the Src SH3 domain show that AFAP1 interacted with GST-Src SH3 much better than with GST-cortactin SH3. However, with AFAP1L1 the reverse was true. AFAP1L1 interacted with GST-cortactin-SH3 better than GST-Src SH3. These data indicate that the SH3 binding motifs of AFAP1 and AFAP1L1 likely have different affinities for different SH3 binding partners. The co-immunoprecipitation of cortactin with full length AFAP1L1 and not with AFAP1 further supports the possibility of differential characteristics of these two proteins and also suggests the interaction of AFAP1L1 with cortactin as a possible mechanism of AFAP1L1 localization to invadosomes. Investigations are underway to determine if the SH3 binding motif of AFAP1L1 mediates the binding of AFAP1L1 to cortactin and to determine the functional relevance of this interaction.

## Conclusion

The goal of this study was to characterize the AFAP family member AFAP1L1 and to determine if it had functions common and distinct to AFAP1. Our data indicate that AFAP1L1 has strong similarity and conservation of domain structure with AFAP1. Also similar to AFAP1, it has an ability to associate with actin filaments, can be found in actin-rich structures such as invadosomes, and is capable of independently inducing podosomes upon overexpression. AFAP1L1 shares expression patterns with AFAP1 in several tissues. However, AFAP1L1 did display some unique properties. For example, unlike AFAP1, AFAP1L1 was found in the dentate nucleus and its expression appeared to occur along neuronal processes. Interestingly, it is in these processes that podosomes are hypothesized to be associated with synaptic connections (Proszynski et al., 2009), and these same connections in the dentate nucleus contain cortactin binding proteins (Hering and Sheng, 2003). As podosomes play a role in enabling cells to traverse and cross tissue barriers, we hypothesize that AFAP1L1 may play a unique role in the innervation of the dentate nucleus. Although it may have a weak affinity, AFAP1L1 is not predicted to be a strong binding partner for cSrc through SH3 interactions; however, it does interact with cortactin. As a potential binding partner for actin filaments and cortactin, AFAP1L1 may associate with podosomes via interactions with these proteins and regulate podosome formation in cells, including neurons within the dentate nucleus.

## Acknowledgements

We thank the lab of Scott Weed for the GST cortactin SH3 construct and the full length cortactin construct. We thank the lab of Steven M. Frisch for MCF-10A cells. We also thank Albert Berrebi for advice and consultation on evaluation of the dentate nucleus. This work was supported by the NIH (R01CA60731). The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejcb.2010.11.016.

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