*Vascular Biology, Atherosclerosis and Endothelium Biology*Caveolin-1 α and -1 β Perform Nonredundant Roles in Early Vertebrate Development

Ping-Ke Fang,* Keith R. Solomon,*[†]
 Liyan Zhuang,* Maosong Qi,[†] Mary McKee,[‡]
 Michael R. Freeman,* and Pamela C. Yelick[§]

From the Urological Diseases Research Center,* and the Department of Orthopaedic Surgery,[†] Children's Hospital Boston, Harvard Medical School; Massachusetts General Hospital;[‡] and the Department of Cytokine Biology,[§] The Forsyth Institute, and Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts

Caveolins are integral membrane proteins that localize predominantly to lipid rafts, where they oligomerize to form flask-shaped organelles termed caveolae and play important roles in membrane trafficking, signal transduction, and other cellular processes. To investigate potential roles for caveolin-1 (*cav-1*) in development, *cav-1 α* and *-1 β* cDNAs were functionally characterized in the zebrafish. *Cav-1 α* and *-1 β* mRNAs exhibited overlapping but distinct expression patterns throughout embryogenesis. Targeted depletion of either Cav-1 isoform, using antisense morpholino oligomers, resulted in a substantial loss of caveolae and dramatic neural, eye, and somite defects by 12 hours after fertilization, the time at which mRNA levels of both isoforms substantially increased in wild-type animals. Morphant phenotypes were rescued by injection of homotypic (*cav-1 α /cav-1 α*) but not heterotypic (*cav-1 α /cav-1 β*) zebrafish and human *cav-1* cRNAs, revealing nonredundant and evolutionarily conserved functions for the individual Cav-1 isoforms. Mutation of a known Cav-1 phosphorylation site unique to Cav-1 α (Y14 $\rightarrow$ F) resulted in a failure to rescue the *cav-1 α* morphant phenotype, verifying an essential role for Cav-1 α specifically and implicating this residue in early developmental functions. *Cav-1 α* and *-1 β* morphants also exhibited disruption in the actin cytoskeleton. These results support important and previously unanticipated roles for the Caveolin-1 isoforms in vertebrate organogenesis. (Am J Pathol 2006; 169:2209–2222; DOI: 10.2353/ajpath.2006.060562)

Lipid rafts are liquid-ordered membrane microdomains, enriched in cholesterol and glycosphingolipids, that have

been implicated in endocytosis, transcytosis, cholesterol transport, and signal transduction.^{1–8} Caveolae (“little caves”) are a form of lipid raft identifiable as ~50- to 100-nm plasma membrane invaginations visible in electron micrographs.⁹ Caveolae are predominantly found in the plasma membrane but also exist as detached vesicles and/or racemose structures that penetrate deep into the cell, forming transcytotic channels.^{10,11} Caveolae are abundant in endothelia, adipocytes, and muscle cells and have been implicated in diverse cellular processes, including polarized sorting of membrane proteins and regulation of signal transduction.^{12–14}

The invaginated morphology characteristic of caveolae is conferred by homo- or hetero-oligomerization of members of the caveolin family of structural proteins.^{15,16} Four caveolins have been identified in vertebrates: Cav-1 α and -1 β , which arise from differential splicing, and Cav-2 and -3, encoded by separate genes. Cav-1 α , -1 β , and -2 are present in nonmuscle cells, whereas myocytes express all four caveolins. Caveolins interact with and functionally regulate a number of signaling proteins via direct protein/protein interactions and, consequently, are believed to mediate many processes attributed to caveolar rafts.⁸ Despite extensive study, the *in vivo* roles of the caveolin proteins are not well understood. Surprisingly, *cav-1*-null mice, which lack most caveolae, and *cav-1/3*-null mice, which have been reported to lack all caveolae, are viable and fertile.^{17,18} However, *cav*-null mice do exhibit a variety of moderate pathologies, including hyperplasia of the genitourinary tract, cardiomyopathy and pulmonary hypertension, vascular abnormalities,

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The sequences of zebrafish *caveolin-1 α* and *caveolin-1 β* have been deposited in the GenBank database (accession numbers: AY124572 and AY124573).

Address reprint requests to Pamela C. Yelick, Ph.D., Director, Division of Craniofacial and Molecular Genetics, Tufts University, 136 Harrison Ave., M824, Boston, MA 02111; or Michael R. Freeman, Ph.D., Urological Diseases Research Center, Children's Hospital Boston, Enders Research Laboratories, Suite 1161, 300 Longwood Ave., Boston, MA 02115. E-mail: pamela.yelick@tufts.edu and michael.freeman@childrens.harvard.edu.

and reduced lifespan.^{7,19,20} Very recently, *cav-1*-null mouse mammary epithelium was found to exhibit defects in three-dimensional acinar architecture²¹ and, when deprived of exogenous extracellular matrix, to undergo spontaneous epithelial-mesenchymal transition and reorganization of the actin cytoskeleton.²¹ Targeted depletion of the muscle-specific Cav-3 was found to result in severe defects in muscle differentiation and function.²² The fact that *caveolin* genes exhibit significant evolutionary conservation suggests important biological functions for these proteins.^{23–27}

To investigate possible roles for the two Cav-1 isoforms in vertebrate development, zebrafish *cav-1α* and *-1β* cDNAs were isolated, the expression profiles of *cav-1* mRNAs were defined, and Cav-1 proteins were functionally characterized using a targeted protein depletion approach. Our studies revealed unique developmental functions for zebrafish Cav-1α and -1β isoforms, including roles in actin cytoskeletal organization, which were rescued by expression of human Cav-1α and -1β isoforms. We also demonstrate, for the first time in an *in vivo* context, that the Cav-1α Y14 phosphorylation site, which is a substrate of Src in other vertebrates, is required for Cav-1α function in early zebrafish development.

Materials and Methods

Zebrafish Husbandry

Wild-type and heterozygous *fli*-enhanced green fluorescent protein (EGFP) transgenic²⁸ zebrafish (*Danio rerio*) were maintained in The Forsyth Institute Zebrafish Facility. Embryos were generated by natural pairwise crosses, raised at 28.5°C, and staged as described previously.^{29,30}

Cloning of Zebrafish and Human *cav-1α* and *-1β* cDNAs

Full-length zebrafish *cav-1α* and *-1β* cDNAs were isolated using degenerate oligonucleotide primers, polymerase chain reaction, and rapid amplification of cDNA ends (DOP-PCR/RACE). In brief, degenerate *cav-1* primers were chosen from conserved amino acid sequences of human, bovine, mouse, rat, and chick *caveolin-1* genes. The *cav-1* forward primer was 5'-ATHGAYYTIGTIAAYM-GIGAYCC-3', and the *cav-1* reverse primer was 5'-AIG-GIAYIAIIRYCCADATRTG-3'. Total RNA was isolated from adult zebrafish using the TRIzol Reagent method (Invitrogen, Carlsbad, CA). Reverse transcription (RT) reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen) followed by PCR amplification. 5'- and 3'-RACE was performed using the GeneRacer kit (Invitrogen), and the resulting PCR products were cloned into the pCR4-TOPO TA cloning vector. Nucleotide sequence comparison with the previously identified zebrafish *cav-1* genomic clone confirmed the identity of full-length zebrafish *cav-1α* and *-1β* cDNAs, whose nucleotide sequences were submitted to GenBank (accession nos. AY124572 and AY124573).

Human *cav-1* cDNAs were generated as follows. Wild-type human *cav-1* was PCR amplified from a construct kindly provided by Dr. Michael Lu,³¹ adding *Bam*HI and *Eco*RI sites to 5' and 3' ends, respectively (sense primer, 5'-CAGGATCCATGTCTGGGGCAAATACGTG-3'; and antisense primer, 5'-CAGAATTCTTATTTCTTTCTG-CAAGTTGATGCG-3'), and cloned into pcDNA3. The human *cav-1α-Y14F* mutant cDNA was generated by introducing an A to T base change at position 41, using the Stratagene QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: sense primer, 5'-CGGAGGGACATCTCTTACCGTTC-CCATC-3'; and antisense primer, 5'-GATGGGAACGGT-GAAGAGATGTCCCTCCG-3'. Human *cav-1β* cDNA was cloned using the sense primer 5'-CAGGATCCATGGCA-GACGAGCTGAGCGAGAAG-3' and the above antisense primer used to clone human *cav-1α*, using a cDNA library from human prostate cancer cell (PC3) as template. The PCR product was cloned into pcDNA3 at *Bam*HI/*Eco*RI sites. The nucleotide sequence of generated human *cav-1* isoform expression constructs was confirmed by DNA sequence analyses.

Expression of Zebrafish Cav-1α and -1β in Mammalian Cell Lines

Full-length zebrafish *cav-1α* and *-1β* coding regions were cloned into pcDNA3.1/Myc-HisB (Invitrogen), generating the eukaryotic expression vectors pcDNA3.1-*zfcav-1α* and pcDNA3.1-*zfcav-1β*, respectively. LNCaP and human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD) were cultured and transfected using standard procedures. Protein extracts were generated from transfected cell lines and subjected to immunoblotting with the anti-human caveolin-1 polyclonal antibody (1:1000 dilution; Transduction Labs, San Diego, CA).

RT-PCR

Developmentally staged zebrafish were collected, frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated using TRIzol Reagent (Invitrogen), and 5 μg of each sample was used in RT reactions using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). One seventh of each RT reaction was used as template in 50-μl PCRs, which were performed per the manufacturer's specifications using the Platinum TaqDNA Polymerase High Fidelity (Invitrogen) and the following reaction conditions: denaturing at 95°C for 5 minutes; followed by 35 cycles at 95°C for 40 seconds, 60°C for 1 minute, and 72°C for 2 minutes; and final extension at 72°C for 7 minutes. Ten-microliter aliquots of each PCR reaction were resolved by agarose gel electrophoresis, stained with ethidium bromide, and digitally photographed. PCR primers were *β-actin* upstream, 5'-GGAGAAGATCTGGCATCACACCTTCTAC-3'; *β-actin* downstream, 5'-TGGTCTCGTGGATACCGCAAGATTCC-AT-3'; *cav-1α* specific upstream, 5'-AATATCCCCACGC-CTCCCACACTG-3'; *cav-1β* specific upstream, 5'-CTC-

CCACTCCACGCTCATGAACTTC-3'; and *cav-1 α* and -1 β common downstream, 5'-GCTGTCAGCAGCCTGT-AGCACC-3'.

Lysate Preparation and Immunoblotting of Embryonic and Adult Zebrafish

Staged zebrafish embryos were collected, quick frozen in liquid nitrogen, and stored at -80°C . Frozen embryos were solubilized in immunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L NaF, and 1 mmol/L sodium orthovanadate) and COMPLETE protease inhibitor cocktail (Roche, Mannheim, Germany). After a 30-minute incubation on ice, insoluble material was removed by centrifugation at $18,000 \times g$ for 15 minutes at 4°C , and the supernatants were collected as whole cell lysates. Adult zebrafish lysates were prepared from anesthetized zebrafish quick frozen in liquid nitrogen and ground with mortar and pestle. Protein concentrations were determined using the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total cell lysate were solubilized in $3\times$ cell lysis buffer [6 mol/L urea (7.28 g/20 ml), 0.5 mol/L Tris, pH 6.8, 9 mmol/L ethylenediamine tetraacetic acid, pH 8.0, 3.6% β -mercaptoethanol, and 9% sodium dodecyl sulfate (SDS)], aliquoted, and stored at -20°C , boiled for 15 minutes, and fractionated by SDS-polyacrylamide gel electrophoresis using 20% gels. Ponceau S staining was used to confirm equal loading of protein extracts. Adult zebrafish lysates were diluted 1:20 relative to developmentally staged extracts, due to the very high Cav-1 expression in adults. Fractionated proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% milk, and immunoblotted with the anti-human Cav-1 polyclonal antibody (1:1000 dilution; Transduction Labs). Cav-1 proteins were visualized using horseradish peroxidase-conjugated secondary antibody and the Supersignal Chemiluminescence System (Pierce, Rockford, IL).

Whole-Mount in Situ Hybridization

Digoxigenin-labeled sense and antisense riboprobes (Roche Applied Science, Indianapolis, IN) were generated from unique 5'- and shared 3'-untranslated regions of *cav-1 α* and -1 β . Whole-mount *in situ* hybridization (WISH) was performed as previously described.^{32,33}

Targeted Depletion and Rescue of Cav-1 α and -1 β

Antisense morpholino oligomers (MOs) targeted to the ATG start sites of *cav-1 α* and -1 β mRNAs were designed and synthesized by Gene Tools, LLC (Eugene, OR). Antisense and 4-mismatch negative control MOs at 1, 2, and 4 mg/ml were injected at 1- to 3-picoliter volumes into single-cell staged embryos, which were then allowed to develop at 28.5°C with periodic examination using Leica

MZ12 (Wetzlar, Germany) and Zeiss M2Bio (Thornwood, NY) dissecting microscopes. The lowest MO concentration resulting in a consistent phenotype, 1 mg/ml, was used in all subsequent experiments. The specificity of each *cav-1* isoform morphant phenotype was determined using human *cav-1* (*hcav-1*) isoform cRNAs that do not contain the antisense MO binding site. Translation-competent *cav-1 α* and -1 β cRNAs were transcribed with mMESSAGE mMACHINE (Ambion, Austin, TX) and co-injected with antisense MOs, as detailed in Figure 6. We injected 100 pg per embryo of *cav-1 α* cRNA, 100 pg per embryo of *cav-1 α* Y14F cRNA, and 200 pg per embryo of *cav-1 β* cRNA.

Phalloidin Staining of Actin Microfilaments

At 12 hours after fertilization (hpf), embryos were fixed in 3.7% paraformaldehyde (PFA) in actin stabilizing buffer (ASB) (1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L EGTA, 10 mmol/L PIPES, pH 7.3, 5 mmol/L MgCl_2 , and 900 mmol/L KCl), washed extensively in ASB, blocked with 1% fetal bovine serum (FBS)/3% bovine serum albumin in ASB overnight, and labeled with 66 nmol/L Alexa 546 phalloidin (Molecular Probes, Eugene, OR) for 1 hour. At 24 hpf, embryos were fixed in sucrose/PFA for 3 hours and washed in PBS-Tween (PBT) and then in distilled water. Washed embryos were placed in prechilled -20°C acetone for 5 minutes and washed in PBT again. Embryos were then blocked with either PBT-2% dimethylsulfoxide (PBDT) and 10% goat serum or 5% goat serum and 5 mg/ml bovine serum albumin in PBDT for 2 hours and labeled with 165 nmol/L Alexa 546 phalloidin in PBDT and 1% goat serum for 2 hours and washed in PBDT. Labeled embryos were analyzed using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope.

Transmission Electron Microscopy Analysis of Zebrafish Caveolae

Control and *cav-1 α* -1 β morphant 24-hpf embryos were dechorionated and fixed for 3 hours at room temperature and then overnight at 4°C in 2% PFA, 2.5% glutaraldehyde, and 0.1 mol/L sodium cacodylate, pH 7.4. Fixed embryos were treated with osmium collidine for 1 hour at room temperature, washed in 0.05 mol/L sodium maleate, pH 5.2, and dehydrated in successive incubations of 50, 70, 85, 95, and 100% ($2\times$) EtOH, for 15 minutes each. Dehydrated embryos were incubated in acetone for 3 minutes and infiltrated in a 1:1 araldite/acetone mixture overnight at 4°C , followed by overnight incubation in 100% araldite at 4°C . Embryos were then incubated in fresh 100% araldite for 2 hours at room temperature and in fresh araldite overnight at 68°C . Embedded embryos were thin sectioned for electron microscopy analysis. Transmission electron microscopy (TEM) was performed at the Massachusetts General Hospital East Core Facility (Charlestown, MA).

Figure 2. Developmental expression of *cav-1* isoform mRNAs and gene products. **A:** Developmental expression of *cav-1* isoform mRNAs. RT-PCR indicated that both *cav-1* mRNAs were weakly expressed as maternal mRNAs at 0.7 hpf, exhibited an increase in zygotic expression between 6 and 12 hpf, and were maintained at these levels throughout adulthood. NTC, no cDNA template control. **B:** Developmental expression of zebrafish Cav-1 protein isoforms. Protein extracts from developmentally staged and adult zebrafish were used to examine expression of endogenous Cav-1α and -1β isoforms by Western blot. Cav-1α was first detected as a faint band at 18 hpf and increased in intensity between 24 and 48 hpf. Cav-1β was first detectable as a faint band at 20 hpf and increased dramatically between 28 and 48 hpf. Both isoforms were highly expressed in the adult (adult protein extracts were diluted 1:20, relative to other extracts). Extracts from wt embryos injected with zebrafish *cav-1α* and -1β cRNAs served as positive controls. Ponceau S staining of the blot shows relative protein loading.

sequently expressed in heart, pharyngeal vasculature, notochord, somites, skin, and neuromast tissues. In contrast, only *cav-1α* mRNA was detectable in intestinal epithelium.

Zebrafish and Human Cav-1 Isoforms Exhibit Nonredundant and Conserved Functional Roles in Early Zebrafish Development

To identify putative developmental functions for individual Cav-1 isoforms, targeted protein depletion studies were performed using antisense MOs designed to hybridize to the unique translation start site of individual *cav-1* isoform mRNAs and block their translation.³⁷ Obvious defects in *cav-1* morphant embryos were first evident at 12 hpf (not shown) and were quite distinct at 24 hpf, when neural, eye, and somite defects were observed, and morphant embryos appeared shortened along the anteroposterior (AP) axis relative to control embryos (Figure 4, E and I). The specificity of targeted Cav-1 isoform protein depletions was confirmed by Western blot analysis of 28-hpf morphant embryos (Figure 4A, inset). At this time, Cav-1α-depleted embryos exhibited obvious defects in retinal pigmented

epithelium (RPE) differentiation, vacuolated neural ventricles, deformed notochord, and misshapen somites present in shortened tails composed of enlarged ventral tail tissue [Figure 4, E–H, versus wild type (wt) A–D]. Cav-1β-depleted embryos exhibited flattened heads, neural ventricle defects, abnormal RPE, and irregularly shaped somites in otherwise fairly well-patterned tail tissue (Figure 4, I–L). Analyses of thin-sectioned morphant embryo specimens confirmed abnormal cellular organization of eye, neural, RPE, and somite tissues (not shown). Molecular analyses revealed that 24-hpf *cav-1α* and -1β morphant embryos exhibited reduced or lack of expression of neural markers *pax2.1*,³⁸ *engr3*,³⁹ *fgf8*,⁴⁰ and *otx2*,⁴¹ examples of which are shown in Figure 5, and aberrant expression of the muscle differentiation marker *myoD*⁴² in somite tissue, consistent with the observed neural and somite tissue defects. These data illustrate the dramatic effects on the embryo of the selective knockdown of either Cav-1 isoform.

Targeted Cav-1 isoform protein depletion studies were highly reproducible. At 24 hpf, 99.5% (426 of 428) of Cav-1α-depleted embryos were phenotypic, compared with 2.3% (9 of 384) of *cav-1α* 4-mismatch control MO-injected embryos. Likewise, 100% (178 of 178) of Cav-1β-depleted embryos were phenotypic at 24 hpf, compared with 2.6% (5 of 192) of control MO-injected embryos. Specific depletion of only the targeted and not the alternate Cav-1 isoform was confirmed by Western blot analyses of 28-hpf morphant embryos (Figure 4A, inset). The specificity of our antisense MO-targeted protein depletion studies was confirmed by demonstrated rescue of morphant phenotypes with co-injected full-length cRNAs corresponding to human (Figure 6) and zebrafish (not shown) sequences. Criteria for rescue included proper neural fold formation, normal eye development including RPE differentiation, chevron-shaped somites, and proper vascular development. Human *cav-1α* and -1β cDNAs were isolated, sequenced, cloned into expression vectors, and used to generate translation-competent cRNAs. Human *cav-1α* and -1β cRNAs were tested for their ability to rescue isoform-specific and alternative isoform, morphant phenotypes as shown in Figure 6. These experiments demonstrated that injection of human *cav-1α* cRNA reproducibly rescued the zebrafish *cav-1α* morphant phenotype (98.6%, 275 of 279), and co-injection of human *cav-1β* cRNA reproducibly rescued the zebrafish *cav-1β* morphant phenotype (96.6%, 86 of 89). In contrast, zebrafish morphant phenotypes were not rescued by co-injection of alternative human *cav-1* isotype cRNAs. The rescue results were essentially identical using zebrafish instead of human *cav-1* cRNAs (not shown). These results suggest functionally nonredundant roles for individual Cav-1α and -1β isoforms in early zebrafish development. Importantly, they also demonstrate that human Cav-1 isoforms are capable of performing the functions of zebrafish Cav-1 isoforms within the early developmental time frame studied here.

Figure 3. Whole mount *in situ* hybridization analysis of zebrafish *cav-1α* and *-1β* mRNAs in the embryo. At 24 hpf, a probe recognizing both *cav-1* isoform mRNAs revealed ubiquitous *cav-1* expression throughout the embryo, with distinct notochord and epidermal expression domains (A–C). At 48 hpf, *cav-1* mRNAs were detected in skin (C) and in cranial and trunk neuromasts (E). At 90 hpf, *cav-1* transcripts were detected in pharyngeal vasculature, intestinal epithelium, and skin (G and J, arrows). A probe specific for *cav-1β* mRNAs shows expression of this isoform in pharyngeal vasculature, heart, and skin but not in intestinal epithelium (H). Sense controls were negative (D, F, and I). h, heart; ie, intestinal epithelium; nc, notochord; nm, neuromasts; pv, pharyngeal vasculature.

The Cav-1α Y14 Phosphorylation Site Is Required for Early Zebrafish Development

An interesting difference between Cav-1α and -1β is the presence of a Src kinase phosphorylation site at Y14 in the unique amino-terminal region of Cav-1α. The Cav-1α Y14 Src phosphorylation site has been implicated in Cav-1α function using mammalian cell culture models.⁴³ This site is conserved in the zebrafish Cav-1α isoform, suggesting that it performs a functional role. We took advantage of the zebrafish *cav-1* morphant phenotypes to determine whether a conservative but nonphosphorylatable human Cav-1α mutant (Y14F) was capable of rescuing the *cav1-α* morphant phenotype. We reasoned that if Y14 phosphorylation is not required for early developmental functions of Cav-1α, then co-injection of *cav-1α* Y14F cRNA would rescue the *cav-1α* morphant phenotype. We also examined whether the human Y14F Cav-1α isoform could rescue the *cav-1β* morphant phenotype, allowing for the possibility that the

Y14F site is the primary functional difference between the two Cav isoforms and that *cav-1α* Y14F might functionally mimic Cav-1β. If so, *cav-1α* Y14F cRNA would rescue the *cav-1β* morphant phenotype. Our results, summarized in Figure 6, demonstrated that *cav-1α* Y14F cRNAs could not rescue either the *cav-1α* or *cav-1β* morphant phenotype. Together, these results suggest that, although the Y14 phosphorylation site is required for Cav-1α function in early zebrafish development, this difference in the two proteins does not by itself define the functional difference between the Cav-1α and -1β isoforms.

Cav-1α and -1β Are Required for Caveolae Formation, Actin Cytoskeletal Organization, and Vascular Development

Transmission electron microscopy (TEM) was used to determine the effect of Cav-1 depletion on the formation

Figure 4. Distinct phenotypes are exhibited by *cav-1α* and *-1β* morphant embryos. **A–D:** Mismatch control MO-injected embryo; **E–H:** *Cav-1α* morphant embryo; **I–L:** *Cav-1β* morphant embryo. **A, inset:** Western blot of 28-hpf *Cav-1α* and *-1β* depleted and wt control embryos. **C, G, K** are dorsal views, and the remaining panels present lateral views, anterior to the left, dorsal up. *Cav-1α* morphant embryos exhibited curved bodies and kinked tails, lacked distinct mhb, and exhibited RPE defects (**E–G**) compared with control embryos (**A–C**). **F:** The heart was enlarged in *cav-1α* morphants, and neural tissues appeared vacuolated. **G:** Dorsal view shows disorganized RPE and abnormal neural folds. **H:** Lateral tail view reveals disorganized somites and shortened tails. **I:** *Cav-1β* morphant embryos exhibited slightly curved bodies. **J:** Lateral head view reveals heart edema (arrow), eye pigmentation defects, and reduced neural tissues. **K:** Dorsal head view shows pigmentation defects and aberrant neural folding. **L:** Lateral tail view reveals notochord and somite defects, which appeared less severe than those of *cav-1α* morphants. e, eye; hb, hindbrain; mhb, midbrain hindbrain; s, somite; y, yolk. Scale bars = 200 μm.

of caveolae in *cav-1α/1β* double morphant embryos (Figure 7). At 24 hpf, numerous caveolae were clearly identifiable in notochord tissues of control MO-injected embryos, whereas few caveolae were evident in *cav-1α/1β* double morphant notochord tissue (Figure 7, A versus B, arrows). Caveolae present in notochord tissue sections obtained from similar AP positions of 24-hpf *cav-1α/1β* morphant and control embryos were quantified. *Cav-1α/1β* morphant notochord tissue contained only 16% of the caveolae present in control embryos (0.6 versus 3.8 caveolae/μm). Similarly, although caveolae were found in neural and somite tissues of 24-hpf wt and control MO-injected embryos, none were found in *cav-1α/1β* mor-

phant neural tissues (not shown). Interestingly, caveolae appear to be transient structures in the developing zebrafish notochord, with numerous caveolae present at 24 hpf and few to none detectable in 4-day postfertilization wt embryos (not shown). Together, these results demonstrate that targeted depletion of *Cav-1α* and *-1β* results in reduced numbers of caveolae. TEM analyses also revealed additional characteristics that distinguished wt and *cav-1α/1β* morphant tissues. Control 24-hpf embryos exhibited abundant, polarized somitic myofibrils, which were surrounded by sarcoplasmic reticulum (SR) transverse systems (Figure 7C). In contrast, 24-hpf *cav-1α/1β* morphant somites contained only disorganized

Figure 5. Molecular characterization of 24-hpf *cav-1α* and *-1β* morphant phenotypes. The neural markers *otx2* and *pax2.1* and the muscle marker *myoD* were used to characterize *cav-1α* and *-1β* morphant phenotypes. The expression of neural markers was reduced and *MyoD* expression revealed aberrant somite patterning in *Cav-1α* and *-1β* morphants.

cytoplasmic structures, in which myofibrils were not readily identifiable, and organized SR was not evident (Figure 7D).

Figure 6. Rescue of morphant phenotypes by co-injection of isoform specific human *cav-1* cRNAs. Human wild-type *cav-1* isoform cRNAs and Y14F *cav-1α* mutant cRNAs were tested for their ability to rescue zebrafish *cav-1* morphant embryo phenotypes. Representative rescue of *cav-1β* morphant phenotype by homotypic and not heterotypic human *cav-1β* cRNAs are shown (top), along with all of the experimental rescue results. Zebrafish *cav-1α* morphants were almost fully rescued by homotypic human *cav-1α* (98.6%) but not by *hcav-1α-Y14F* or heterotypic *hcav-1β* cRNAs. Likewise, zebrafish *cav-1β* morphants were rescued by homotypic human *cav-1β* cRNAs (96.6%) but not by heterotypic *hcav-1α* or *hcav-1α-Y14F* cRNAs. Embryos injected with *hcav-1α*, *hcav-1α-Y14F*, or *hcav-1β* cRNAs alone appeared normal.

We next examined actin cytoskeleton organization in *cav-1* morphant embryos because of published reports in cell culture models implicating caveolins and/or caveolae in the maintenance of actin cytoskeletal organization^{44,45} and because of the apparent disruption in the myofibrillar cytoskeleton seen by TEM in the *cav-1α-1β* morphants (Figure 7). Actin-specific phalloidin staining⁴⁶ of head and tail epidermis of 12-hpf *Cav-1α* and *-1β*-depleted embryos revealed highly irregular cell borders and punctate fluorescence patterns, indicative of disrupted actin microfilaments in these tissues (Figure 8). The actin microfilament networks in *Cav-1β*-depleted somites appeared less disrupted than those of *cav-1α* morphant embryos (Figure 8, 24-hpf tail panels), consistent with the relatively intact vasculature observed in *Cav-1β*-depleted embryos in comparison with *cav-1α* morphant embryos (Figure 9). At 24 hpf, somite defects were clearly evident in phalloidin-stained *cav-1* morphant embryos (Figure 8, bottom panels). *Cav-1α* morphants exhibited little to no organized somite tissues, whereas *cav-1β* morphant somites lacked a midline myoseptum and the classic chevron shapes were shortened between each intersegmental boundary and exhibited thinner and disorganized myofibrils relative to that of control embryos. We also observed aberrant pigment granule movements in retinal pigment epithelium (RPE) and melanocytes of *cav-1α* and *-1β* morphants (data not shown), suggestive of disrupted cytoskeletal organiza-

Figure 7. TEM analysis of caveolae in 24-hpf Cav-1 α -1 β -depleted embryos. Numerous caveolae were detected in notochord tissues of wt zebrafish (**A**, arrows), whereas very few were observed in Cav-1 α /1 β morphant notochord (**B**, arrow). **C:** Wt somite tissues exhibit organized myofibrils surrounded by distinct SR (arrows). **D:** Cav-1 α /1 β morphant somite tissues exhibited few myofibrils, and those were not attended by SR. Statistical analyses, using one-tailed ($P = 0.005$) and two-tailed ($P = 0.01$) Student's t -test, confirmed that the differences in the frequency of caveolae were significant. Cropped images were generated from original magnifications of 54,000 (**A**), 52,000 (**B**), 73,500 (**C**), and 73,500 (**D**).

tion and consistent with purported roles for actin microfilaments in intracellular pigment granule trafficking.^{47–49}

Caveolae are abundant in endothelial cells where they have been implicated in vascular function.⁵⁰ We took advantage of *fli*-EGFP transgenic zebrafish,²⁸ which express green fluorescent protein in vascular endothelial cells, to examine blood vessel formation in Cav-1-depleted embryos. Confocal imaging of 24-hpf control *fli*-EGFP embryos revealed well-patterned blood vessels in both head and tail tissues (Figure 9, A, A', B, and B'). In contrast, Cav-1 α -depleted embryos exhibited substantial vascular patterning defects in both head and tail tissues (Figure 9, C, C', D, and D'), whereas Cav-1 β morphant embryos exhibited extensively disrupted head but fairly well-patterned tail tissue vasculature (Figure 9, E, E', F, and F').

Discussion

This is the first report to demonstrate a requirement for both the Cav-1 α and - β isoforms during vertebrate development. We demonstrate here that targeted depletion

of either isoform results in embryonic lethality in the zebrafish and that the heterotypic isoform is incapable of substituting for the alternative form of the protein. These results are in marked contrast to Cav-1 knockout phenotypes observed in mice, where loss of both Cav-1 isoforms results in animals that are viable and fertile, live well into adulthood, and exhibit rather modest functional defects. The results in the mouse are surprising because, in the Cav-1 knockout animals, a prominent and presumably important class of subcellular organelles (caveolae) is completely absent in essential nonmuscle tissues, such as the vasculature. In the zebrafish, targeted depletion of either Cav-1 isoform resulted in morphological defects evident at 12 hpf and distinct neural, somitic, and vascular tissue defects evident by 24 hpf. The inability of one isoform to substitute for the other is an unanticipated observation because the proteins are very similar: Cav-1 β lacks the first 33 amino acids of Cav-1 α but is otherwise identical. In the present study, we demonstrate that early morphological defects observed in Cav-1 isoform-targeted morphant embryos coincided with a failure to form caveolae, a breakdown in actin cytoskeletal organization, and extensive disruption of vascular endothe-

Figure 8. Disrupted actin cytoskeletal organization in 12- and 24-hpf *cav-1α* and *-1β* morphants. Confocal images of phalloidin-stained actin cytoskeletal filaments in head and tail epidermis of 12- and 24-hpf *cav-1α* and *-1β* morphants and mismatch control-injected embryos. At 12 hpf, actin filaments of head and tail epidermis of *cav-1α* and *-1β* morphants appeared disorganized, particularly in *cav-1α* morphants, in which extensive depolymerized and disrupted cytoskeletal architecture was evident by punctate staining at cell-cell junctions. Cell size varied extensively in *cav-1α* and *-1β* morphants, consistent with cytokinesis defects. At 24 hpf, distinct defects were evident in actin cytoskeletal organization in somite myofibrils. *cav-1α* morphants exhibited little to no identifiable myofibrils, whereas myofibrils in *cav-1β* morphant somite tissue appeared dysmorphic and disorganized, lacked midline myoseptum and chevron shape, and were shortened along the AP axis.

lial tissue formation, consistent with published functions for caveolins (and caveolae) in each of these processes. Embryonic lethality of Cav-1 depletion in the zebrafish embryo, without regard to isoform specificity, was recently reported by another group, and our conclusion about the necessity for Cav-1 functions in early zebrafish development is consistent with theirs.⁵¹

Cav-1 α and -1 β isoforms, initially identified as alternative translation products from a single mRNA,³⁶ were subsequently demonstrated to form by differential RNA splicing.²⁵ Collectively, our studies indicate that the two Cav-1 isoforms perform nonoverlapping roles during zebrafish development. We arrived at this conclusion from several independent observations. 1) Defects arising from targeted depletion of one Cav-1 isoform were not rescued by the heterotypic isoform but were rescued by the homotypic isoform. The ability of the human *cav-1*

isoform cRNAs to rescue defects arising from depletion of the zebrafish proteins suggests strong functional conservation across a long evolutionary timeline. 2) Targeted depletion of Cav-1 α resulted in severe neural and tail tissue defects, whereas targeted depletion of Cav-1 β more selectively affected neural tissues. Isoform-characteristic patterning defects of *cav-1α* and *-1β* morphant tissues were also clearly evident 3) with phalloidin-staining of the actin cytoskeleton and 4) in the vasculature of *flil*-EGFP, *cav-1* morphant zebrafish. 5) WISH analyses revealed that only *cav-1α* mRNA was detected in intestinal epithelium, consistent with a recent report of Cav-1/Annexin 2 heterocomplex formation in intestinal sterol transport.⁵¹ 6) Mutation of the Y14 Src phosphorylation site to the nonphosphorylatable residue phenylalanine disrupted the ability of human *cav-1α* cRNA to rescue the zebrafish *cav-1α* morphant defect. This mutation also did

Figure 9. Cav-1 α and -1 β are required for proper patterning of the vascular endothelium. Lateral views of control (A, A', B, and B'), *cav-1 α* morphant (C, C', D, and D'), and *cav-1 β* morphant (E, E', F, and F') transgenic *ll-EGFP* embryos. Anterior is to the left, and dorsal is up. Control embryos exhibited green fluorescent protein expression in well-patterned head and tail vascular endothelial cells. In contrast, Cav-1 α -depleted head and tail vascular endothelium appeared mispatterned and did not form patent vessels (C, C', D, and D'). Head vasculature of Cav-1 β -depleted embryos appeared disorganized, whereas tail vasculature was less affected (E, E', F, and F').

not convert Cav-1 α into a Cav-1 β mimic. These results suggest that this tyrosine residue is essential for Cav-1 α function and therefore is an important molecular difference between the two proteins. 7) Finally, the genomic organization (including intron/exon boundaries) specifying the zebrafish and mammalian *cav-1* isoforms are conserved, implying strong evolutionarily constraints on the expression of the Cav-1 splice variants. We submitted the zebrafish *cav-1 α* and -1 β cDNA sequence data to GenBank in 2003 (accession nos. AY124572 and AY124573). After GenBank released this information to the public, the existence of two zebrafish *cav-1* isoform mRNAs was reported by another group.²²

To date, very little published evidence exists supporting discrete functions for individual Cav-1 isoform proteins. One recent study, reporting Cav-1 isoform-specific interactions with bone morphogenetic protein (BMP) receptors Rla and RII, suggests that Cav-1 β inhibits BMP signaling, whereas Cav-1 α does not.⁵² Another study used immunofluorescence and Western blotting to show that, although both Cav-1 isoforms were initially co-distributed in cardiac tissue at birth, at later times, Cav-1 α exhibited restricted expression in endothelial tissues.⁵³

At the present time, it is not clear whether developmental defects observed in Cav-1-depleted embryos are a primary or secondary effect of cytoskeletal disruption. Actin microfilament defects preceded and mirrored later

tissue specific defects, suggesting a functional link between these observations. This study is the first *in vivo* demonstration that down-regulation of Cav-1 isoforms results in disruption of the actin cytoskeleton, consistent with mammalian cell culture studies demonstrating that actin microfilament defects arise from loss of Cav-1.⁵⁴ Izumi et al⁵⁵ first demonstrated that caveolae present in endothelial membranes could be decorated with phalloidin, which binds to actin at the junction between subunits.^{56–58} More recently, filamin, an F-actin cross-linking protein and Cav-1 ligand, was shown to directly link Cav-1 to the actin cytoskeleton, and Cav-1-positive membranes were demonstrated to redistribute to filamin-decorated stress fibers in response to the low-molecular weight GTPase RhoB, known to promote cytoskeletal reorganization *in vitro*.⁴⁴ That study also used a quantitative yeast two hybrid approach to demonstrate that Cav-1 β , and not Cav-1 α , might be the primary ligand for filamin. Further confirmation of this result would provide additional evidence for discrete biochemical functions for the two Cav-1 isoforms. Additional evidence for an actin-caveolin interaction includes the demonstration that Cav-1 resides in structures at the plasma membrane that appear to be assembled into the cortical actin cytoskeleton^{59,60} and that disruption of filamentous actin by cytochalasin D treatment results in enhanced mobility and clustering of cell surface caveolae.⁵⁹ Finally, numerous

reports have shown that a variety of proteins known to communicate with or participate in the formation of cytoskeletal structures localize to lipid rafts and/or caveolae.^{8,61–69}

The biological function(s) of caveolae is incompletely understood, despite a substantial body of literature linking these specialized organelles to multiple signal transduction processes and membrane trafficking. Consistent with observations from the Cav mouse knockout studies, we have shown that simultaneous targeted depletion of Cav-1 α and -1 β in zebrafish resulted in dramatically reduced numbers of caveolae, in accord with the view that caveolins are integral membrane proteins required for the distinctive caveolar architecture.^{7,44} The reason for the disparity in the requirement for Cav-1 during development between mouse and zebrafish is unknown. It is possible that Cav-1 serves specialized functions in zebrafish that can be compensated for by redundant system(s) in the mouse. Another possibility is that the difference may arise from features characteristic of placental versus nonplacental embryonic development. Further elucidation of the molecular mechanisms of mammalian and zebrafish Cav-1 signaling pathways will clarify this issue.

Conclusions

In this study, we present a functional characterization of Cav-1 α and -1 β , key structural proteins of caveolar lipid rafts, in the developing zebrafish. Our primary conclusions are that 1) expression of both Cav-1 isoforms is required for embryonic development in the zebrafish, because targeted depletion of either Cav-1 isoform results in tissue patterning defects evident as early as 12 hpf; 2) morphological characterization of Cav-1 morphants, tissue distribution analysis, and rescue experiments indicate that Cav-1 α and -1 β exhibit distinct and nonredundant roles in early zebrafish development; 3) human Cav-1 α and -1 β rescued the early developmental functions of zebrafish Cav-1 isoforms; 4) the Cav-1 α Y14 Src phosphorylation site is required for the developmental function(s) of Cav-1 α ; and 5) targeted depletion of either Cav-1 α or -1 β results in disrupted actin cytoskeletal organization, which likely contributes to later vascular and developmental defects. Together, these findings suggest important and previously unrecognized developmental roles for Cav-1 and, by implication, for caveolar lipid rafts in vertebrate organogenesis.

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