Clathrin-mediated endocytosis occurs at multiple independent import sites on the plasma membrane, but how these positions are selected and how different cargo is simultaneously recognized is obscure. FCHO1 and FCHO2 are early-arriving proteins at surface clathrin assemblies and are speculated to act as compulsory coat nucleators, preceding the core clathrin adaptor AP-2. Here, we show that the \( \mu \)-homology domain of FCHO1/2 represents an endocytic interaction hub. Translational silencing of \( fcho1 \) in zebrafish embryos causes strong dorsoventral patterning defects analogous to Bmp signal failure. The Fcho1 \( \mu \)-homology domain interacts with the Bmp receptor Alk8, uncovering an endocytic component that positively modulates Bmp signal transmission. Still, the \( fcho1 \) morphant phenotype is distinct from severe embryonic defects apparent when AP-2 is depleted. Our data thus challenge the primacy of FCHO1/2 in coat initiation.

Clathrin-mediated endocytosis is a major mechanism for the selective internalization of cell-surface components and extracellular macromolecules\(^1\). The import sites contain clathrin triskelia assembled into a polygonal lattice\(^1\). As the lattice curves by incorporating pentagonal facets and projects into the cell interior, select cargo is packaged into the clathrin-coated invagination. Preferential retention of cargo within the bud depends on cytosol-oriented sorting signals\(^4\). A heterotetrameric AP-2 adaptor complex and numerous clathrin-associated sorting proteins (CLASPs) identify structurally disparate sorting signals\(^2\); this recognition enables non-competitive grouping of dissimilar cargo into single clathrin-coated buds. The processes of coat assembly, cargo capture and budding take less than a minute, and eukaryotic cells have hundreds of spatially discrete clathrin-coated structures forming on the surface\(^3\). Precisely how buds initiate at defined locations is unclear\(^7\). Certainly, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)\( P_2 \)) is pivotal, because depleting this lipid triggers swift dissolution of surface coats\(^9\). Because AP-2 and numerous CLASPs and clathrin accessory proteins bind physically to PtdIns(4,5)\( P_2 \), current models invoke stochastic but simultaneous encounters of these molecules with PtdIns(4,5)\( P_2 \), themselves, cargo and clathrin to begin coat polymerization on a patch of membrane\(^6\). Recently, however, on the basis of two PtdIns(4,5)\( P_2 \)-binding proteins invariably preceding the arrival of AP-2 and clathrin at nascent bud sites, FCH domain only 1 (FCHO1) and FCHO2 were proposed to be functionally redundant founder proteins demarcating sites of future clathrin assembly\(^12\).

Here, we examine the endocytic activity of the modular FCHO1 and FCHO2 proteins to address the following questions. What molecular interactions distinguish the various protein domains? What role do Fcho1 and Fcho2 play in zebrafish embryonic development? Are Fcho1 and Fcho2 functionally interchangeable? And, if FCHO1/2 is obligatory for clathrin-coat nucleation, does the phenotype of Fcho1/2-compromised embryos parallel that of AP-2 morphants? We find Fcho1 operates during dorsoventral patterning of the embryo and associates with activin receptor-like kinase 8 (Alk8, also known as Acvr1l and Lost-a-fin), a type I BMP receptor involved in signalling ventral-cell fates\(^13\). Yet AP-2 depletion causes a much more penetrant, broadly severe and earlier developmental phenotype, indicating that AP-2 function is not dependent on Fcho1/2.

RESULTS

FCHO1 and FCHO2 are members of the muniscin subfamily of EFC (or F-BAR) domain proteins, evolutionarily conserved from unicellular eukaryotes to mammals\(^15,16\). These paralogues play a functionally redundant role beginning at the earliest assembly stages of clathrin-mediated endocytosis\(^2,12,15,17,18\). HeLa cells express transcripts for both FCHO1 and FCHO2, but endogenous FCHO1 protein is undetectable.

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1. Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA.
2. Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218, USA.
3. Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, USA.
4. Correspondence should be addressed to L.M.T. (e-mail: traub@pitt.edu)

Received 16 November 2011; accepted 29 February 2012; published online 8 April 2012; DOI: 10.1038/ncb2473
Figure 1 Binding properties of FCHO1. (a) Schematic representation of FCHO1 with the location and relative binding properties of the various truncations tested. (b) Coomassie-stained gel and blot of supernatant (S) and pellet (P) fractions of a GST pulldown assay with brain cytosol and immobilized GST or the indicated GST-FCHO1 or GST-ARH fragments. Immunoblotted with anti-clathrin heavy chain and AP-1/2 subunit antibodies. Large adaptor subunits (arrowheads) indicated. (c) Pulldown assay with FCHO1-overexpressing HeLa-cell lysate and immobilized GST or the indicated GST-fusion proteins. Two independent anti-FCHO1 antibodies were used for detection. Non-specific bands (asterisks) are indicated. (d) Pulldown assay with brain cytosol and immobilized GST or the indicated GST-FCHO1 or GST-ARH fragments. Two independent anti-FCHO1 antibodies were used for detection. Large adaptor subunits (arrowheads) indicated. (e) Transferrin, GFP–FCHO1, or GFP–FCHO2 transfected with siRNA for the AP-2 subunit also expressing GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2) subunit. The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (f) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (g) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (h) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (i) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (j) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (k) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (l) GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). The lower panels show the intracellular localization of full-length GFP–FCHO1, compared with AP-2 (AP2A1+AP2A2). (m) Confocal microscopy image of a region of a HeLa cell transfected with siRNA for the AP-2 subunit also expressing GFP–FCHO1. Before fixation and staining with an anti-clathrin heavy chain monoclonal antibody (mAb), the silenced cells were incubated at 37°C with 25 µg ml⁻¹ Alexa 633-labelled transferrin. (n) Biochemical assessment of silencing with siRNA for AP-2 in HeLa-cell lysates by immunoblotting. (o-r) Representative confocal microscopy images of the intracellular localization of full-length (1–889), EFC-domain (1–275), ΔβHD (1–609) or μHD (609–889) GFP-tagged FCHO1, compared with AP-2 (α subunit). The lower panels of each part provide colour-separated and merged enlargements of boxed regions. Scale bar, 10 µm. Uncropped images of blots are shown in Supplementary Fig. S8.

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with antibodies. Transient expression of green fluorescent protein (GFP)-tagged FCHO1 reveals scattered bright puncta on the ventral surface, but a diffuse membrane-tethered population becomes evident with higher overexpression (Supplementary Fig. 5a). The heterogeneously sized fluorescent structures in cells expressing low-level GFP–FCHO1 co-localize with AP-2 but are spatially distinct from peripheral APPL1-positive endosomes or more centrally positioned EEA1-positive endosomes (Supplementary Fig. 5b–e; ref. 20). More abundant endogenous FCHO2 populates punctate structures similar to GFP–FCHO1 at steady state with considerable

![Figure 2](image_url)

**Figure 2** The μHD interaction hub. (a) Coomassie-stained gel and blot of supernatant (S) and pellet (P) fractions of a pulldown assay with full-length or ΔμHD (1–609) FCHO1-overexpressing HeLa lysates and immobilized GST or GST-eps15(595–896). Immunoblot with anti-FCHO1. Intact FCHO1 (orange arrowheads) and ΔμHD FCHO1 (black arrowheads) are indicated. (b) Pulldown assay using brain cytosol and either GST or 50 or 100 μg of the GST-μHD fusions indicated or the GST-αC appendage. Replicate immunoblots probed with the indicated antibodies with non-specific bands (asterisks) indicated. (c) Pulldown assay using HeLa lysate and either GST or 50 or 100 μg of the GST-μHD fusions indicated. Non-specific bands (asterisks) are indicated. All the GST-μHDs bind to eps15, eps15R, intersectin and Dab2, but only the FCHO1 μHD associates with Hrb, CALM and clathrin. Note that different eps15 splice isoforms are present in HeLa lysates when compared with brain (b). (d) Stained gel and replicate blots of first-stage assay pellets (red P) from incubations of brain cytosol with either GST or GST-FCHO2 μHD, compared with supernatant (S) and pellets (P) from subsequent second-stage pulldowns with the supernatant fractions resulting from the first-stage incubations. (e) Schematic representation of the eps15 C terminus with the relative positioning of the various truncations used indicated. (f) Pulldown assay using FCHO1-overexpressing HeLa lysate and the indicated GST–eps15 C-terminal fusions. Immunoblot with anti-FCHO1. (g) Stained gel and replicate blots from pulldown assays with GST or GST-FCHO1 μHD and HeLa lysates supplemented with 5 or 25 μM eps15(595–660) peptide as indicated. (h) Interaction diagram for FCHO1/2 and selected endocytic pioneer coat components. Presumptive contacts are indicated with dotted lines. Uncropped images of blots are shown in Supplementary Fig. S8.
(~75%) overlap, although not perfect coincidence, with AP-2 (Supplementary Fig. S1f–i). These findings confirm that FCHO1/2 operate at clathrin-coated structures at the cell surface.

The FCHO endocytic hub

FCHO1 and FCHO2 are modular proteins (Supplementary Fig. S1j). The amino-terminal EFC domain is connected to a ~280-residue carboxy-terminal μ-homology domain (μHD) by linkers of differing length in the paralogues. The first 270 residues of human FCHO1 and FCHO2, encoding a helical EFC-domain monomer, are 58% identical. Accordingly, dose-dependent association of purified FCHO1 EFC domain with PtdIns(4,5)P₂ containing liposomes parallels that of FCHO2 (Supplementary Fig. S1k; refs 12, 21). Binding to liposomes lacking PtdIns(4,5)P₂ is ~10-fold less efficient. The membrane-binding and curvature-sensing/inducing properties of the antiparallel EFC-domain dimer in FCHO1 and FCHO2 are thus similar.
Protein-disorder predictions indicate that the EFC- and μHD-separating residues (300–600) of FCHO1 comprise a low-complexity, largely unstructured segment. Intrinsic disorder is a hallmark of numerous endocytic factors that bind AP-2 using short, tandemly arrayed peptide motifs in the flexible region of the membrane. In the absence of an assigned structure, the Fcho1 protein is expected to be unstructured in solution and to adopt a variety of conformations that depend on local interactions with other proteins and on the local environment.

Glutathione S-transferase (GST)-fusion proteins encompassing the short, tandemly arrayed peptide motifs in the flexible region of the membrane bound AP-2 using short, tandemly arrayed peptide motifs in the flexible region of the membrane. In the absence of an assigned structure, the Fcho1 protein is expected to be unstructured in solution and to adopt a variety of conformations that depend on local interactions with other proteins and on the local environment.

Fcho1 participates in early embryonic development. (a–f) Representative images of the morphant phenotypic range on injection with 2 ng control or 2 ng fcho1 morpholino oligonucleotide (MO). Dorsalized phenotypes are classified as normal (pale blue, a), weak (C1, white, b), moderate (C2, yellow, c), strong (C3, orange, d) and severe (C4, brown, e). The most severe C5-like morphants (brown, f) are rare and are grouped with the C4 class. Arrows: ventral fin defects. Scale bar, 250 μm.

Figure 4 Quantification of dose-dependent fcho1 morphant phenotypes at 24 hpf, colour-coded as in a–f. (h,i) Typical vegetal pole view of 10 ng control or 10 ng fcho1 early-stage morphants at 10 hpf. A, anterior; P, posterior. Arrow: expanded A–P axis. Scale bar, 125 μm. (j–m) Representative mRNA expression patterns of both krox20 (brackets) and gata1 (arrows) in control (j,k) or 10 ng fcho1 morpholino oligonucleotide-injected (l,m) five-somite-stage embryos. A, anterior; P, posterior. (j,l) Group dorsal survey view; (k,m) individual lateral view. Scale bar, 225 μm (j,l) or 125 μm (k,m).

(n) Schematic representation of the general location of opposing morphogen gradients that dictate dorsoventral patterning at shield stage. An, animal pole; Vg, vegetal pole; D, dorsal; V, ventral. (o–r) Representative mRNA expression patterns of indicated genes in 10 ng control (o,q) or fcho1 (p,r) shield-stage morphants. Relative expression zones (arrowhead in animal and bracket in lateral views) are shown. Scale bar, 250 μm. (s,t) Dorsal view of gross morphology of developing notochord and somite regions of live 10 ng control (s) and fcho1 (t) morphants at the six-somite stage. Scale bar, 50 μm.

(u–x) Representative phenotypes of 2 ng fcho1 morphants co-injected with indicated human (Hs) FCHO1 mRNA. Scale bar, 250 μm. Colour coded as in y, (y) Quantification of morphant phenotypes at 24 hpf. WT, wild type.
the central portion of FCHO1 engage AP-2 in pulldown assays (Fig. 1a,b). The apparent affinity of this association with AP-2 is weaker than that of the C-terminal segment of ARH (autosomal recessive hypercholesterolaemia), which engages AP-2 with a $K_D$ of $\sim1\mu M$ (refs 23,24). The main AP-2 interaction determinant(s) in FCHO1 is between residues 267 and 442 (Fig. 1a,b). Unlike ARH, the central segment of FCHO1 does not bind clathrin directly (Fig. 1b). FCHO2 does not associate with AP-2 (Supplementary Fig. S2a); the shorter central linker in FCHO2 shows less than 30% identity with FCHO1, pointing the absence of AP-2 interaction motif(s).

FCHO1 binds AP-2 through the globular appendage domain of the large $\alpha$ subunit, but some association with the $\beta_2$-subunit appendage is also evident (Fig. 1c). The contact site on the $\alpha$ appendage is the platform subdomain, because a W840A substitution abolishes binding (Fig. 1d); a sandwich subdomain disruption (Q782A; refs 25,26) has no effect on binding. Yet RNA interference suppression of AP-2, causing pronounced accumulation of dispersed transferrin receptors at the cell surface$^{7,28}$, is still compatible with surface-clustered FCHO1 or 2 puncta (Fig. 1e–l) also containing clathrin (Fig. 1m) and eps15 (Supplementary Fig. S2b), in line with these proteins being constituents of a pioneer unit$^{32,17}$. AP-2 depletion is seen on immunoblots (Fig. 1n). Thus, despite binding AP-2, the heterotrimer is not necessary for FCHO1 (or FCHO2) surface puncta. This is consistent with FCHO2 not contacting AP-2 directly and also supported by truncation analysis; whereas full-length GFP–FCHO1(1–889) and eps15(595–660) peptide efficiently competes with cytosolic Hrb, CALM and Dab2 in assays at lower concentrations than required to displace eps15R, intersectin or eps15 (Fig. 2g), all probably engage the FCHO1 $\mu$HD through a common or partly overlapping interaction surface, but with the scaffolds binding with higher apparent affinity. Numerous important accessory proteins and CLASPs thus converge on the FCHO1 $\mu$HD (Fig. 2h) and, therefore, similarly to the AP-2 appendage domains$^{68}$, this domain represents an endocytic hub.

**FCHO1 affects embryogenesis**

Misexpression of mammalian FCHO1 in zebrafish confirms the $\mu$HD importance. On microinjecting synthetic GFP–FCHO1(1–889) messenger RNA into one-cell-stage embryos, fluorescence is visible during the early gastrula period, $\sim5$ h post fertilization (hpf; Fig. 3a). Co-injection of a membrane-tethered red fluorescent protein (RFP) marker shows GFP–FCHO1 concentrates at the blastomere plasma membrane, but in discontinuous puncta as opposed to the uninterrupted RFP. The punctate GFP–FCHO1 pattern overlaps with endogenous AP-2 (Fig. 3b,c). By 24 hpf, microinjected ectopic mammalian FCHO1 produces a gain-of-function phenotype. Unlike controls, the FCHO1-expressing embryos are cyclopic and mildly ventralized, with small head regions, misshapen trunk somites, and loss of notochord tissue in severely affected embryos (Fig. 3d–j). Comparative *in situ* localization of transcripts for dorsally specified axial mesoderm, *no tail* and *sonic hedgehog*, confirms disruption of dorsal patterning. Strikingly, misexpression of similar amounts of a $\mu$HD-truncated *FCHO1*(1–609; $\Delta\mu$HD) mRNA leads to strong dorsalization (Fig. 3j,k). Embryos now show posteriorly shortened and twisted body axes and loss of the yolk extension and tail. The most severe resemble *C4/C5* category *snailhouse* (*bmp7*) and *swirl* (*bmp2b*) mutant embryos$^{33,34}$. Injection of *FCHO1* $\mu$HD(609–889) mRNA alone phenocopies *FCHO1*(1–609) overexpression (Fig. 3j,l), indicating that the dominant-negative effect derives from uncoupling the linked domains and that the normal operation of FCHO1 requires physical connection of the EFC and $\mu$HD regions. These reciprocal dysmorphic effects suggest that muniscins participate in early embryogenesis. In fact, because dorsoventral patterning is governed in part by Bmp signalling, Fcho1 may assist operation of Bmp receptors during gastrulation. The Smad1/5/8 transcription factors are direct phosphotargets of the type I Bmp receptor Alk8 (ref. 36), and phosphoSmad (pSmad1/5/8) localization is grossly misplaced dorsally in constitutively active (CA*)* alk8* mRNA-injected embryos (Fig. 3m,n). Likewise, ectopic FCHO1 expression drives notable, but weaker, dorsal expansion of nuclear pSmad1/5/8 localization in gastrulas (Fig. 3o–s), indicating hyperactive Bmp signalling.
A single gene encodes Fcho1 (LOC565812) and Fcho2 (ZDB-GENE-494) in zebrafish. The structural and topological features are conserved in the teleost proteins (Supplementary Fig. S3) and maternally deposited transcripts for both Fcho1 and Fcho2 are present; PCR with reverse transcription (RT-PCR) confirms the presence of maternally deposited transcripts for both Fcho1 and Fcho2 are still detectable and increase gradually through the segmentation period to 24 hpf. Both transcripts are still detectable and increase gradually through the onset of general zygotic transcription (Fig. 4n) is widened ventrally in shield-stage fcho1 morphants, with increased lethality at higher morpholino oligonucleotide concentrations. Morpholino oligonucleotide efficacy is verified by silencing of an appropriate fcho1–GFP reporter (Supplementary Fig. S4) and the phenotype is not markedly different in p53 morpholino oligonucleotide-co-injected embryos.37 Earlier in development, at the end of gastrulation (~10 hpf), the fcho1 morphants are oblong (Fig. 4h), typical of dorsalization.33,34 These morphological abnormalities accompany gene-expression pattern changes diagnostic for dorsalization: the positioning of the hindbrain marker krox20 reveals laterally expanded stripes when compared with controls, whereas, in the same embryos, expression of gata1, a marker of the ventrally specified erythroid lineage, is diminished (Fig. 4j–m). Also in line with expansion of dorsal-cell fates, regional mRNA expression of the dorsally positioned Bmp antagonist chordin (Fig. 4n) is narrowed, and the phenotype is not markedly different in p53 morpholino oligonucleotide-co-injected embryos.37 Earlier in development, at the end of gastrulation (~10 hpf), the fcho1 morphants are oblong (Fig. 4h), typical of dorsalization.33,34 These morphological abnormalities accompany gene-expression pattern changes diagnostic for dorsalization: the positioning of the hindbrain marker krox20 reveals laterally expanded stripes when compared with controls, whereas, in the same embryos, expression of gata1, a marker of the ventrally specified erythroid lineage, is diminished (Fig. 4j–m). Also in line with expansion of dorsal-cell fates, regional mRNA expression of the dorsally positioned Bmp antagonist chordin (Fig. 4n) is widened ventrally in shield-stage fcho1 morphants (Fig. 4o,p). Reciprocally, the lateral bmp4 expression field is variably

Figure 5 The Fcho1–Alk8 association. (a–d) Representative images of live 24 hpf embryos after injection of 0.5 or 5 ng alk8 morpholino oligonucleotide (MO), 0.5 ng fcho1 morpholino oligonucleotide or 0.5 ng each of alk8 + fcho1 morpholino oligonucleotides. The defective ventral tail fin is bracketed (green). Scale bar, 250 µm. (e) Quantification of single- and double-morphant phenotypes at 24 hpf. Colour-coding and classifications in a–e are as in Fig. 4a–g. (f) Coomassie-stained gel and replicate blots from a pulldown assay using Alk8–Myc-overexpressing HeLa cell lysate and 150 µg immobilized GST or 50 or 150 µg human (Hs) GST–FCHO1 µHD or D. rerio (Dr) GST–Fcho1 or GST–Fcho2 µHDs. Alk8 (arrowheads in pellet (P) fractions) immunoblot with anti-Myc. Note that both bands of expressed Alk8–Myc, probably differentially N-glycosylated forms, bind to the FCHO1/Fcho1 µHD. Non-specific bands (asterisks) are indicated. (g) Pulldown assay using wild-type (WT) or CA’ Alk8–Myc-overexpressing HeLa cell lysate and 150 µg immobilized GST or GST–Dr Fcho1 µHD. (h) Pulldown assay using Alk8–Myc-overexpressing HeLa cell lysate and immobilized GST or GST–Hs FCHO1 µHD. Addition of the eps15(595–660) competitor peptide (5 or 25 µM) is indicated. Uncropped images of blots are shown in Supplementary Fig. S8.

Danio rerio Fcho1 and Fcho2 in dorsoventral patterning

A single gene encodes Fcho1 (LOC565812) and Fcho2 (ZDB-GENE-050522-228) in zebrafish. The structural and topological features are conserved in the teleost proteins (Supplementary Fig. S3) and maternally deposited transcripts for both Fcho1 and Fcho2 are present; PCR with reverse transcription (RT-PCR) confirms the presence of the appropriate gene-specific amplicons whereas in situ hybridization shows localization in four-cell embryos through shield stage (when gastrulation begins) at ~ 6 hpf (Supplementary Fig. S3). After the onset of general zygotic transcription (~3 hpf), both fcho1 and fcho2 transcripts are still detectable and increase gradually through the segmentation period to 24 hpf. Both fcho1 and fcho2 messages are broadly distributed but, by 24 hpf, regional differences are apparent; expression patterns are therefore overlapping but not identical.

Injecting an initiation-codon-targeting fcho1 antisense morpholino oligonucleotide (Supplementary Fig. S4) at the one-cell stage causes a strong dorsalized phenotype at 24 hpf, where structures originating from the dorsal side predominate, with accompanying loss of ventral tissues (Fig. 4a–g). Again, severely affected embryos mirror snailhouse and swirl mutants. The phenotype is dose dependent, with increased lethality at higher morpholino oligonucleotide concentrations.
Figure 6 Fcho1 operates in a genetic Bmp-to-Smad signalling pathway. (a–f) Group survey views of comparative morphology of control embryos and embryos injected with 25 pg bmp2b with 100 pg bmp7 mRNA, 5 ng each of fcho1/2-p53 morpholino oligonucleotides, bmp2b+bmp7 mRNA along with the fcho1/2-p53 morpholino oligonucleotides, 200 pg CA* smad1 mRNA, or the CA* smad1 mRNA along with the fcho1/2-p53 morpholino oligonucleotides at 24 hpf. Scale bar, 250 μm. (g) Quantification of mRNA-injected gain-of-function (ventralized; black-bracketed) and morphant (dorsalized; orange-bracketed) phenotypes. Ventralized phenotypes are classified as normal (pale blue), slight (V1, grey), weak (V2, indigo), moderate (V3, violet), strong (V4, light green) and severe (V5, green). The relevant unit for injected mRNA is pg and that for morpholino oligonucleotide is ng. (h–m) Representative images of the range and classification of ventralization phenotypes induced by injection of 25 pg bmp2b with 100 pg bmp7 mRNA at the one-cell stage. Colour-coding as in g. Scale bar, 250 μm. (n) Schematic representation of the linear Bmp-dependent signalling pathway including the relative positioning of Fcho1.

reduced ventrally, consistent with suppression of positive feedback of bmp expression by Bmp signalling in the ventral region (Fig. 4q,r; ref. 38). During segmentation, the fcho1 morphants have laterally broadened somitic mesoderm (Fig. 4s,t) that could reflect delayed/abnormal convergence–extension movements. Defective convergence probably reflects a second function of the Bmp gradient...
Figure 7 AP-2 morphants are unlike fcho1/2 double morphants. 
(a–e) Representative survey views of comparative morphology of 10 ng control, ap2a1 or fcho1/2+p53 morphants at 14 (a, b) and 19 (c–e) hpf. Extruded-yolk (arrowheads) and exploded/disintegrating (asterisk) embryos are shown. Scale bars, 250 μm. (f) Quantification of ap2a1 morpholino oligonucleotide rescue with mouse (Mm) αc-subunit (Ap2a2) mRNA. The ap2a1 phenotypes are classified as normal (pale blue), weak (white), moderate (orange), strong (pink) and arrested (black). See Supplementary Fig. S7d–h for representative images of the phenotypic classes. (g–i) mRNA expression patterns of Fgf signalling genes in control (g), ap2a1 (h) or fcho1 (i) shield- or 30% epiboly-stage morphants. The zones of expanded expression (brackets) are indicated: D, dorsal; V, ventral. Scale bar, 250 μm. (j) Restoration of dusp6 expression in 30% epiboly (~5 hpf) ap2a1 morpholino oligonucleotide embryos by co-injection of mouse Ap2a2 mRNA. The zone of expansion without rescue (brackets) is indicated. (k–m) Fgf-dependent GFP expression in live embryos injected with 10 ng control, ap2a1 or fcho1+fcho2+p53 morpholino oligonucleotides. Delaminating ectoderm, broadly expanded GFP expression (arrowheads) in ap2a1 and diminished ventral tissue (bracket) in fcho1/2 morphants are shown. A, anterior; P, posterior; mhb, mid-hindbrain boundary; r4, rhombomere 4; Kv, Kupfer's vesicle. An elliptical fcho1 morphant (orange arrow) is shown in m. Scale bar, 250 μm. (n–p) mRNA expression profiles of early patterning signalling components in control (n), ap2a1 (o) or fcho1 (p) shield- or 30% epiboly-stage morphants. Arrowheads and brackets show zones of expanded expression. Scale bar, 250 μm.
in the gastrula: proper locomotion of cells to the midline by modulating cell adhesion\textsuperscript{49}.

Administering full-length FCHO1 mRNA together with the fcho1 morpholino oligonucleotide increases morphologically normal 24 hpf embryos to 67\% from 12\% with the morpholino oligonucleotide alone (Fig. 4u–y). There is more than 5 base pairs (bp) mismatch with the FCHO1 RNA (Supplementary Fig. S4), excluding the possibility that the co-injected transcript titrates out the fcho1 morpholino oligonucleotide. Injection of the ΔμHD-encoding (1–610) transcript does not similarly correct development in fcho1 morphants, indicating that the basis for the dorsovenral patterning defect is Fcho1 insufficiency and that the intact protein is necessary for proper function (Fig. 4x,y).

Because FCH01 and FCHO2 are functionally redundant\textsuperscript{12}, a translation-blocking fcho2 morpholino oligonucleotide was also analysed. The fcho2 morphants differ strikingly from those of fcho1, despite globally similar expression profiles during early embryogenesis (Supplementary Fig. S5). The largely distinct developmental defects of the morphants indicate that Fcho1 and Fcho2 regulate separate events during zebrafish gastrulation and segmentation, but may also reflect temporal and regional differences in the expression of the two paralogues. There is evidence of weak overlapping function during embryogenesis: although fcho1/2 morpholino oligonucleotides combined do not provoke a gross phenotype distinct from fcho1 morpholino oligonucleotide alone, the krox20 lateral expansion, elliptical morphology and spectrum of dorsalized embryos is slightly more severe in fcho1/2 + p53-injected embryos than in fcho1+p53-treated ones (Supplementary Fig. S5l–r).

**Fcho1 binds to Alk8**

As the general fcho1 morpholino oligonucleotide phenotype mirrors Bmp antagonism, we investigated interaction between Fcho1 and Alk8, an early dorsovenral patterning type I receptor for Bmp2b–Bmp7 (refs 14,40,41). A genetic link between fcho1 and alk8 is revealed by co-injection of morpholino oligonucleotides targeting the two transcripts at levels (0.5 ng) that, alone, do not produce many dorsalized embryos (Fig. 5a–e). When combined, mildly dorsalized (C2) embryos result with defective ventral tail-fin development that resembles 5 ng alk8 morphants, suggesting that Alk8 and Fcho1 operate along the same pathway. This conclusion is directly substantiated biochemically. C-terminally Myc-tagged Alk8 in detergent lysates from transfected HeLa cells is bound by GST–FCHO1 μHD dose-dependently, along with the other binding partners (Fig. 5f–h). Also, Alk8–Myc interacts with the immobilized cognate GST–Fcho1 μHDs (~55\% identical), whereas neither the Fcho2 (Fig. 5f), FCHO2 nor SGIP1 μHD (Supplementary Fig. S6) bind similarly, despite equivalent eps15 interactions. The strong, differential interaction of the Fcho1/FCHO1 μHD with Alk8 is clearly consistent with the different phenotypes in fcho1 or fcho2 morpholino-injected embryos. There is no obvious preference of the Fcho1 μHD for binding to CA\textsuperscript{μ} Alk8\textsuperscript{Q204D} mutant\textsuperscript{41} over the wild-type receptor (Fig. 5g), whereas the related Alk1 interacts with the μHD comparably weakly (Supplementary Fig. S6). The μHD interaction with Alk8 is similar to Hrb binding and both are displaced by the eps15(595–660) peptide at low concentrations (Fig. 5h); so Alk8 does not seem to use a tyrosine-based sorting signal to engage the Fcho1 μHD.

Injecting bmp2b + bmp7 mRNA elicits strong ventralization, although co-injection with fcho1/2 morpholino oligonucleotides results in genetic suppression, with primarily dorsalized embryos resulting (Fig. 6). Ectopic mRNA encoding a phosphomimetic CA\textsuperscript{μ} Smad1 also strongly ventralizes, even in the presence of fcho1/2 morpholino oligonucleotides (Fig. 6e–g). These epistasis-type experiments place Fcho1 within a genetically specified sequence for Bmp signalling (Fig. 6n). Overall, our data imply that Fcho1 positively regulates ventral specification during embryogenesis by bridging Alk8 and the clathrin machinery, consistent with expanded pSmad1/5/8 staining in FCHO1 mRNA-injected embryos.

**AP-2 loss of function**

FCHO1/2 are argued to be master actuators of endocytosis, remodelling the initial membrane patch and recruiting eps15 and intersectin before AP-2 (ref. 12). If FCHO1/2 are compulsory for clathrin-coated vesicle formation, one testable prediction is that the early dorsalizing effect of fcho1/2 morpholino oligonucleotides is wholly reflective of clathrin-mediated endocytosis dysfunction in developing embryos. Similarly to Fcho1/2, in *D. rerio* the AP-2 α subunit is encoded by a single gene (*ap2α1*) and is maternally deposited. The transcript is broadly expressed in blastula–gastrula–segmentation-stage embryos (Supplementary Fig. S7a–c), similarly to fcho1/2.

Translation-blocking *ap2α1* morpholino oligonucleotide (Supplementary Fig. S4) produces pleomorphic early patterning and axis formation abnormalities and many embryos do not survive to 24 hpf (Fig. 7a–e, Supplementary Fig. S5). Phenotypes of surviving morphants at 24 hpf are classified from mild to arrested (Supplementary Fig. S7d–i). In the most severely affected embryos, the epiboly movement of blastomeres toward the vegetal pole slows and blastopore closure with incomplete epiboly results in dysmorphic bulging of the yolk (Fig. 7b,d). This defect persists beyond bud stage (10 hpf) and yolk rupture typically follows 14–19 hpf (Fig. 7d). Surviving arrested embryos are not developmentally delayed because there is no further morphological progression by 24 hpf (Supplementary Fig. S6h). *ap2α1 + p53* morpholino oligonucleotide–co-injected embryos still arrest, whereas simultaneous injection of the *ap2α1* morpholino oligonucleotide and mouse *Ap2α2* mRNA reverts the wild-type morphology (Fig. 7f). A second *ap2α1* and an AP-2 μ2-subunit-targeting morpholino oligonucleotide both cause an analogous phenotype. Epiboly arrest may be due, in part, to defective yolk-cell endocytosis as the blastoderm margin moves toward the vegetal pole\textsuperscript{45}. Later, in surviving embryos, the ectoderm delaminates and the gross morphology is indicative of severe patterning defects (Fig. 7l, Supplementary Fig. S6f–h), consistent with the severe effect of μ2 morpholino oligonucleotide in early Xenopus embryos\textsuperscript{43}.

A survey of standard target genes for early development\textsuperscript{35} shows serious expression abnormalities. When gastrulation begins Fgf is normally produced from the dorsal organizer and weakly along the margin, but in *ap2α1* (but not fcho1) morpholino oligonucleotide embryos *fgf8* transcripts are expanded towards both the ventral and animal poles (Fig. 7g–i). Improper *fgf8* transcription is evident at 30\% epiboly (~4.7 hpf). At this stage, there is also precocious hyperactivation of the phosphatase Dusp6, an Fgf target gene\textsuperscript{44}. Abnormal and mislocalized production of *dusp6* in *ap2α1* morphants persists to shield stage and, strikingly, expands far beyond the zone of *fgf8* ligand expression. Co-injecting mRNA for AP-2 α subunit restores the normal *dusp6* expression pattern (Fig. 7j). Expansion of Fgf signalling range in AP-2-compromised embryos is consistent.
Figure 8 AP-2–clathrin coats persist in FCHO1 and FCHO2 siRNA-treated HeLa and BS-C-1 cells. (a) Biochemical validation of FCHO1 transcript silencing in GFP–FCHO1-expressing HeLa cells by immunoblot analysis of lysates from mock or FCHO1 siRNA-transfected cells. An ON-TARGETplus SMARTpool (siRNAp; Dharmacon) was used. Replicate immunoblot with an anti–α-tubulin mAb to verify equivalent loading of lysates. (b) HeLa cells subject to mock, FCHO1 or FCHO2 siRNAp silencing and also ectopically expressing GFP–FCHO1 (along with RFP to mark co-transfected cells) were fixed and stained with Hoechst 33258. Representative, colour-separated or merged, confocal optical microscopy sections are shown. Note that both the FCHO1 and FCHO2 siRNAp sets efficiently quench the GFP–FCHO1 fluorescence. Scale bar, 10 μm. (c) Biochemical verification of FCHO2 siRNA, or siRNA-mediated silencing in HeLa cells by immunoblotting. SDS–polyacrylamide gel electrophoresis resolved lysates from cells subjected to the indicated treatments were immunoblotted with antibodies against either GFP or FCHO2 and β-tubulin as a loading control. HC, heavy chain. Notice that the FCHO2 siRNAp suppresses both the transfected GFP–FCHO2 and the endogenous protein in these cell populations highly efficiently. (d,e) Representative single confocal optical microscopy sections of HeLa cells subjected to mock (d) or combined FCHO1 + FCHO2 siRNAp (e) followed by fixation and immunodetection of the endogenous AP-2 α subunit using mAb AP.6. Note that, although the roughly regular patterning and surface density of AP-2–clathrin structures is diminished on knockdown of FCHO1/2, AP-2-positive puncta still persist, and generally seem to increase in size and are more irregularly deposited (arrowheads). (f) Biochemical verification of FCHO1 + FCHO2’s siRNAp, or siRNA-mediated silencing in BS-C-1 cells by immunoblotting. Notice, again, that the Stealth (Invitrogen) FCHO2 siRNAp very effectively extinguishes the endogenous FCHO2 in these populations of cells. Mock transfected HeLa cells are included for comparison. (g–j) Representative single confocal optical microscopy sections of clathrin LCa–GFP-expressing BS-C-1 cells subjected to mock (g) or combined FCHO1 siRNAp + FCHO2 siRNAp (h) followed by fixation and immunodetection of the endogenous AP-2 α subunit using mAb AP.6. (i,j) A colour-separated enlarged region corresponding to the boxed area in h. Surface AP-2-positive puncta clearly persist in these FCHO1/2-silenced BS-C-1 cells as well (arrowheads). Note that only a subset of clathrin-labelled structures is AP-2 positive in both the mock and FCHO1/2-silenced cells, as clathrin normally assembles on other intracellular structures as well. Scale bar, 10 μm. Uncropped images of blots are shown in Supplementary Fig. S8.
with endocytosis shaping and maintaining this morphogen gradient in the zebrafish gastrula\textsuperscript{35,36}. Clear Fgf signalling and phenotypic differences between ap2a1 and fcho1/2 + p53 morphants are apparent in live transgenic Tg(Dusp6:d2EGFP)\textsuperscript{96} reporter embryos\textsuperscript{47} at the six-somite stage (Fig. 7k–m). As the ap2a1 morpholino oligonucleotide embryos are still round, suppressing AP-2 expression must cause further cellular/regional defects because Fgf receptor hyperactivation typically induces dorsalization\textsuperscript{46,48}. Importantly, the GFP reporter rules out the possibility that abnormal activation of Fgf signalling underlies the fcho1 morpholino oligonucleotide dorsalization.

Other body-plan patterning pathways are anomalous in ap2a1-silenced embryos when gastrulation starts: distribution of chordin, bozozok, goosecoid and squint transcripts is abnormal (Fig. 7n–p). Thus, AP-2 seems to function generally in multiple developmental pathways during early development, as seriously defective body-patterning information results without a functional adaptor. Because the effect on transducing Fgf and other inductive signals in ap2a1 morphants is distinct from how fcho1/2 morpholino oligonucleotides alter the fate map, we conclude that AP-2 function is not invariably dependent on upstream Fcho1/2 during embryogenesis.

**DISCUSSION**

Proper embryonic patterning depends on precise temporal integration of spatially complex signalling events. Endocytosis modulates signalling by constantly adjusting surface protein abundance\textsuperscript{49}, and here we show that this process is vital for zebrafish development. Our results are consistent with Fcho1 operating with Alk8 and Bmp2b–Bmp7 heterodimers to signal ventral fates. Because FCHO1/2 are incontestably clathrin-coat constituents, the simplest model is that clathrin-mediated endocytosis promotes Bmp signalling. The precise basis for this remains to be elucidated; one possibility is that Fcho1/2 with Alk8 shapes the ventral Bmp gradient, as does type I Bmp receptor Thick veins in Drosophila\textsuperscript{90}. Thick veins, the Drosophila Decapentaplegic receptor, binds directly to another intersectin-binding EFC-domain protein, Nervous wreck\textsuperscript{31}. Yet Nervous wreck has a C-terminal SH3 domain substituted for the μHD, so the basis for the interaction cannot be structurally similar, and, critically, the consequence of the association is negative feedback on Decapentaplegic signalling. It thus seems remote that muniscins govern routing of Alk8 receptors to lysosomes, because Fcho1 positively modulates Bmp signalling in zebrafish embryos.

Receptors can require previous internalization and recycling to gain signalling competence\textsuperscript{32}. Another possibility then is that, by promoting uptake of uncomplexed type I receptors (Alk8), the level of type I–II functional heterodimers is optimized. Alternatively, Fcho1 could be involved in forming Bmp gradients by decreasing available receptor/ligand in dorsally positioned cells, but that would give rise to ventralization, not dorsalization, on morpholino oligonucleotide injection. More likely, clathrin-mediated endocytosis provides access to an endosomal signalling station for cytosolic propagation and refinement of the Bmp signal. Spatial separation between TGF-β/BMP receptor activation at the plasma membrane and signal propagation from endosomes is apparent\textsuperscript{35,36}. In cultured cells, the extent of activin-triggered Smad phosphorylation decreases when internalization is blocked\textsuperscript{35}. Optimal phosphorylation of R-Smads by TGF-β receptors requires SARA, an endosome-associated PtdIns3P-binding protein\textsuperscript{36–39}. The R-Smad-binding FYVE-domain protein Endofin plays an analogous role during BMP signalling\textsuperscript{40}. Fcho1 could then drive the delivery of Alk8 to endosomes for an encounter with Endofin.

Zebrafish are characterized by very rapid developmental progression; tissue-specific cell phenotypes are evident during gastrulation (>6 hpf) and a recognizable body plan and differentiating organs apparent before 12 hpf (ref. 61). Perhaps Fcho1/2 has been previously missed because the time constraints for signal propagation in other systems are dissimilar to D. rerio early development. An association between FCHO1 and ACVR1, the mammalian Alk8 orthologue, has been mapped by high-throughput affinity-capture proteomics\textsuperscript{42}. Another hallmark of zebrafish embryogenesis is brisk and expansive cell movements. Maximal signal transmission from an endosomal extension of the cell surface may enable proper ‘memory’ signalling in cells that have moved away from the initial, fate-determining morphogen gradient\textsuperscript{43}.

On the basis of morpholino oligonucleotides recapitulating maternal-effect phenotypes\textsuperscript{23}, it is clear that the translational-silencing approach ablates maternal-gene expression. Why then are fcho1, fcho2 and ap2a1 morphants not more severely affected? Targeted gene disruption of the AP-2 μ2 subunit is lethal\textsuperscript{36}, but ap2a1 morpholino oligonucleotide in zebrafish enables visualization of early embryonic processes before dysmorphic arrest due to protein insufficiency. This is because morpholino oligonucleotide-based silencing cannot obstruct maternal deposited protein. Substantial clathrin-coat machinery must be provisioned in the oocyte, as massive cortical granule exocytosis following fertilization is balanced by compensatory clathrin-dependent endocytosis\textsuperscript{45}. Clathrin-coated buds are also seen at the cleavage furrow during the initial rounds of cell division following fertilization\textsuperscript{46}. Still, an early and severe developmental defect on ap2a1 silencing is fully consistent with AP-2 μ2-subunit morphants in Xenopus\textsuperscript{45}. Strikingly, the phenotypic outcomes of extinguishing AP-2 or Fcho1/2 transcripts are not identical. If Fcho1/2 are obligatory for the nucleation of endocytic clathrin coats, this is unexpected. One possibility is that the perdurance of the muniscins is greater than that of the AP-2 heterotetramer in early embryos. However, simultaneous RNA interference silencing of both FCHO1 and FCHO2 in either HeLa or BS-C-1 cells does not cause loss of AP-2-positive clathrin-coated structures at the cell surface (Fig. 8). This is fully consistent with the very mild phenotype in syp1Δ yeast\textsuperscript{15,17,18}.

The structure, dynamics\textsuperscript{2,12,67} and extensive interactions shared by FCHO1 and FCHO2 suggest that muniscins advance clathrin endocytosis. This may explain the weak additive effect of fcho2 morpholino oligonucleotide on the fcho1 morphant phenotype, reflecting a degree of functional overlap between the paralogues. If part of a pioneer module\textsuperscript{2,12}, how do clathrin coats persist without these factors? A topological feature of the clathrin interactome is redundancy\textsuperscript{2,6}. Surplus connections make the network tolerant to perturbations; AP-2 can be largely extinguished in cultured cells but rapid uptake of certain cargo continues\textsuperscript{27,28}. As the μHD engages other pioneer scaffolds (Fig. 2h), collective establishment of this preponderance of links, even without Fcho1/2, permits positioning of AP-2 at the first encounter zone of a bud site. Cargo-selective defects are instead manifest, highlighting that all known μHD-bearing proteins are involved in gathering cargo\textsuperscript{15}. □
We are indebted to our many colleagues for providing reagents that were essential for various experiments. B.W. and M.T. provided intellectual input, contributed to experimental design and advised on data interpretation. L.M.T. conceived and directed the overall project and wrote the manuscript with comments from all the authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

The full-length human FCHO1 and FCHO2 cDNAs were obtained from Open Biosystems. The sequences encoding full-length FCHO1, FCHO2 (1-609), FCHO1 (422-609), FCHO1 (422-609) full-length and FCHO2 (1-609) were PCR amplified and cloned into pCRII or pGEX-4T-1 for expression of either an N-terminal GFP or GST fusion protein. The full-length human FCHO1 and FCHO2 (1-609) and zebrafish Fcho2 (2-610) were similarly cloned into pCS2+ for mRNA synthesis or transient transfections. 5′- and 3′-regions of the full-length zebrafish fcho1 mRNA were first amplified by RT-PCR from 1,000-cell-stage zebrafish embryos using a set of PCR primers designed from EST clones flanking 5′ and 3′ regions of the zebrafish transcript. The amplified DNA was subjected to agarose gel electrophoresis and exon-specific regions at the 5′ and 3′ ends of the mRNA were sequenced and the sequence deposited in GenBank (accession number JN412732).

Cell culture and transfections. HeLa Sf6 cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM l-glutamine at 37 °C in an atmosphere of 5% CO₂. BS-C-1 cells stably expressing GFP-tagged clathrin light chain a were provided by T. Kirchhausen. The BS-C-1 cells were similarly grown in DMEM, 10% FCS, Cells were fixed with 4% paraformaldehyde in 10% normal goat serum, 0.2% saponin, in PBS. For transferrin binding and uptake assays, cells were incubated in DMEM, 25 mM HEPES, 0.5% BSA, for 1 h at 37 °C to remove bound transferrin. Alternatively, lysates from parental HeLa cells or HeLa cells expressing (from pCS2+) full-length 1-889 or truncated 1-609 (hompo sapiens) FCHO1, or Myc-tagged wild-type Apk8 or CA1-Apk, were prepared from transiently transfected (24-48h) cells collected by trypsinization. After washing, cells pellets were solubilized for 30 min in 25 mM HEPES-KOH (pH 7.2), 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EDTA, 2 mM EGTA and 2 mM dithiothreitol (assay buffer) supplemented with 1% Triton X-100, 1 mM PMSE and Complete Protease Inhibitor Cocktail (Roche). Lysates were centrifuged at 20,000 × g for 5 min before use in binding assays.

Zebrafish maintenance, morpholinos and mRNA injections. The Oregon AB+ and Tg(Drap6:2ZEGFP)61 strains were maintained under standard conditions at the University of Pittsburgh in Medicine in accordance with institutional and Federal guidelines for care, and maintenance of experimental animals and with University of Pittsburgh Institutional Animal Care and Use Committee approval. Embryos from natural matings were obtained and developmentally staged14. The sequences of the various morpholinos used in this study, custom synthesized by Gene Tools, are listed in Supplementary Table. The translation-blocking ATG-specific fcho1 morpholino was designed on the basis of the specific 5′ sequence of zebrafish embryonic fcho1 transcript mapped by 5′ RACE. A standard control morpholino morfno2k and a p53 morpholino oligonucleotide were obtained from Gene Tools as detailed elsewhere25. The desired concentrations of the appropriate morpholinos were microinjected at 5 nl per embryo into the yolk at one-cell stage. Capped mRNAs for misexpression and rescue experiments were transcribed in vitro from linearized pCS2+ constructs using an SP6 mMessage mMACHINE kit (Ambion) and microinjected at 1 nl into the blastomere at the one-cell stage. After injections, the embryos were incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.01% methylene blue) at 28 °C until the desired stage.

Whole-mount in situ hybridization and immunofluorescence. The ES clones for fcho1 (GenBank ALR920021) and apk2 (GenBank ALR920300) in pBluescript SK+ were obtained from Open Biosystems, linearized and directly used for riboprobe synthesis. For fcho2, an 880-bp fragment encoding the C-terminal end of the gene was PCR amplified (using fcho2 T7 sense and antisense primers) from the zebrafish fcho2 EST clone (GenBank BC095680). All other probes used in this study (no tail, sonic hedgehog, bmp4, chordin, krox20, gata1, goosecoid, bavzozok, squint, fgfr, fgfr1 and dusp6) were made similarly by linearizing corresponding vectors containing a T7 RNA polymerase promoter, using appropriate restriction enzymes as described elsewhere67,68. Riboprobe synthesis for in situ hybridization was carried out using digoxigenin RNA labelling mix and T7 RNA polymerase (Roche) according to the manufacturer’s recommendations. Zebrafish embryos at appropriate developmental stages were fixed in 4% paraformaldehyde at 4 °C overnight, washed once in PBS and stored in methanol at –20 °C. Whole-mount in situ hybridizations were carried out by standard procedures69. Embryos were washed several times in PBS and mounted in glycerol. Alternatively, live embryos, either within or removed from the chorion, were mounted in 3% methylecellulose and overlaid with E3 containing 0.016% tricine (pH 7.0). Oriented embryos were viewed using a Leica MZ16FA stereo fluorescence microscope with a ×1 (numerical aperture 0.14) objective and brightfield fluorescence images collected with a QImaging Retiga-EXI Fast 1394 digital camera.

For immunofluorescence, embryos fixed overnight in 4% paraformaldehyde at 4 °C were permeabilized in 1% Triton X-100, blocked in 10% sheep serum, 1% DMSO, 0.1% Triton X-100, in PBS and probed overnight with rabbit anti-pSmad 2/3 (1:500; 1:1000) from Cell Signaling Technology or GD1/1 (1:100) antibody. The immunizing peptide for the GD1/1 antibody (GD1LNLGPPPV) is 100% conserved in the human and mouse FCHO2 sequence. For imaging of the mouse anti-rabbit Alexa 488- or Cy5-conjugated antibody (1:500; Molecular Probes) and 33258 nuclear stain before mounting for confocal microscopy.
For GFP-FCHO1/AP-2 immunofluorescence, embryos at 5 hpf were embedded in 1% low-melting-point agarose in E3 medium on poly-d-lysine-coated dishes (MatTek) with the animal pole oriented toward the glass bottom. For pSmad 1/5/8 immunofluorescence, embryos at 7 hpf were embedded with dorsal shield side toward the right. Images were acquired on an Olympus Fluoview1000 confocal microscope using a UPlanSApo ×20 (numerical aperture 0.75) or a UPlanFLN ×40 (numerical aperture 1.3) oil objective. Data were acquired using the FV10-ASW software. Similar procedures were used for immunofluorescence analysis of cultured mammalian cells.

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Figure S1 FCHO1 and FCHO2 at cell surface endocytic structures.

(a) Representative confocal optical section of HeLa cells transiently transfected with GFP-FCHO1 and stained with an anti-AP-2 (α subunit) mAb AP.6. Low (arrowheads) and high overexpressing (arrows) cells are indicated. Scale bar: 10 μm. (b-e) Confocal optical section of HeLa cells transiently transfected with GFP-FCHO1 and stained for EEA1 and APPL1 on endosomes. Panel c–e are from a different field with separate color channel pairs (d and e) compared with the merged image (c). (f-i) Cofocal optical section of immunofluorescent colocalization of endogenous FCHO2 with AP-2 (α subunit) in HeLa cells. Insets (i) show enlargements of the boxed region. (j) Architectural relationship of the muniscin family. Hs, Homo sapiens; Sc, Saccharomyces cerevisiae. (k) Coomassie-stained gel of supernatant (S) and pellet (P) fractions from a liposome sedimentation assay titrating the EFC domain of FCHO1 (residues 1-275) or FCHO2 (residues 1-303) with synthetic liposomes as indicated. Molecular mass standards (kDa) are shown.
Figure S2 FCHO1 and FCHO2 interactions with AP-2 and surface clathrin coats. (a) Coomassie-stained gel and replicate blot of supernatant (S) and pellet (P) fractions from a pull-down assay using brain cytosol and immobilized GST, the indicated GST-FCHO1 and GST-FCHO2 fusions or GST-ARH as a positive control. Immunoblotted with anti-clathrin heavy chain (HC) and AP-1/2 b1/b2-subunit antibodies. Molecular mass standards (kDa) are shown and the position of the intact, recombinant GST-FCHO2 (1-809) (arrowhead) and major degradation product (asterisk) is indicated. (b-e) Mock or AP-2 a-subunit siRNA-treated HeLa cells transfected with GFP-FCHO2 as indicated were incubated with 25 μg/ml Alexa568-labeled transferrin at 37°C for 10 min before fixation. Representative confocal images show AP-2 silencing leads to diffuse, featureless surface transferrin as opposed to the endosomally clustered ligand in mock RNAi-treated cells. Nonetheless, eps15 still colocalizes with the GFP-FCHO2 surface puncta in AP-2 siRNA silenced cells. (arrowheads). Non-silenced cells (asterisks), identified by perinuclear transferrin accumulation in endosomes, are indicated. Insets (c-e) provide color-separated and merged enlargements of boxed regions. Scale bar: 10 μm. (f) Immunoblot analysis of lysates from HeLa cells transfected with the indicated GFP-FCHO1 constructs. Duplicate immunoblots probed with either anti-GFP or anti-FCHO1 EFC domain antibodies as indicated to confirm the synthesis of the various GFP-tagged FCHO1 constructs. Duplicate immunobots probed with either anti-GFP or anti-FCHO1 EFC domain antibodies as indicated to confirm the synthesis of the various GFP-tagged FCHO1 constructs. (g-i) Representative, color-separated and merged confocal optical sections of transfected HeLa cells showing the intracellular localization of full-length (1-889; panels taken from Fig. 1o) (g) or central, AP-2 interacting FCHO1 regions (265-467) (h) or 265-609 (i) fused to GFP. Intracellular distribution compared with endogenous AP-2 detected with an a-subunit mAb AP.6. Scale bar: 10 μm.
**Figure S3** *fcho1* and *fcho2* expression in *Danio rerio* embryos. (a) Diagram of the domain organization and primary sequence identity of *D. rerio* (*Dr*) Fcho1 and Fcho2 proteins compared with the *H. sapiens* (*Hs*) FCHO1/2 orthologues. (b) RT-PCR analysis of control β-actin, *fcho1* and *fcho2* transcript-specific amplicons from RNA isolated from embryos at the indicated developmental stages. Molecular standards (bp) are shown. (c–d) Whole mount *in situ* analysis of *fcho1* (C) and *fcho2* (D) transcript localization in wild-type Oregon AB* strain embryos at the indicated developmental stages. The animal (An) and vegetal (Vg) poles and dorsal (D) and ventral (V) axes are indicated. Note that the expression pattern of *fcho2* is very different from that reported in the ZFIN database for *fcho2* ([http://zfin.org/cgi-bin/webdriver?MIval=aa-fxallfigures.apg&OID=ZDB-PUB-010810-1&fxallfig_probe_zdb_id=ZDB-EST-020816-12](http://zfin.org/cgi-bin/webdriver?MIval=aa-fxallfigures.apg&OID=ZDB-PUB-010810-1&fxallfig_probe_zdb_id=ZDB-EST-020816-12)). We do not observe the reported restricted expression in the hypoblast, polster and, later, in the hatching gland. Our antisense probe is derived from the coding region of the *fcho2* gene as opposed to the 3’ untranslated region used in the high-throughput screening. Scale bars: 250 μm.
**Figure S4** MO target sequences and activity. (a, c, e) Design of the 5’ nucleotide sequence of the fcho1-N-GFP, fcho2-N-GFP and ap2a1-N-GFP fluorescent reporter constructs with the MO target sequence and initiation ATG codon(s) highlighted relative to the GFP ATG start codon (boxed). Note that the GFP reporters only contain the MO target sequences for the relevant gene, not the entire protein-encoding sequence, and that the extent of base pair mismatch with the mammalian rescue mRNA sequence is indicated. (b, d, f) Wide-field fluorescence images of live embryos at the indicated stages after injection of 100 pg GFP, fcho1-N-GFP, fcho2-N-GFP or ap2a1-N-GFP mRNA and 5 ng control, fcho1, fcho2 or ap2a1 MO as indicated. Prominent yolk autofluorescence in 24 hpf embryos is indicated (asterisks). All three MOs specifically silence strongly the appropriate GFP reporter expression. Scale bar: 250 μm.
**Figure S5** The *fcho2* MO phenotype. (a-f) Representative images of the phenotype of *fcho2* (6 ng MO) and *fcho2*+p53 (6 ng of each MO) morphants compared with control (6 ng MO). Necrosis (arrowheads) and zoomed images of notochord and trunk somite (broken line) regions (c, d, f) are shown. Unlike *fcho1* morphants, at 24 hpf, *fcho2* MO-injected embryos have an undulating notochord, malformed somites and conspicuous necrosis in the head/brain region. The latter is due to knockdown-associated p53 activation as it is prevented by coinjection of a p53-silencing MO, while the wavy notochord, rounded somite and diminished dorsal and ventral tail fin persist. Scale bar: 250 μm (a-c) or 125 μm (d-f). (g-h) Images of typical *chordin* transcript expression pattern at shield stage in 10 ng control and 10 ng *fcho2* MO-injected embryos. Both animal pole (left panel) and lateral Right panel) views shown for comparison. D, dorsal; V, ventral. Relative expression zones (arrowhead in animal and bracket in lateral views) shown. Scale bar: 250 μm. (i-m) Representative mRNA expression patterns of both *krox20* (bracket) and *gata1* (arrows) in control (10 ng; i), *fcho2*+p53 (10 ng each; j, k) or *fcho1*+ *fcho2*+p53 (10 ng each; l, m) injected 5-somite-stage morphants. Individual lateral view: i, k, m; group survey dorsal view: j, l. (n-q) Representative gross morphology of live, 6-somite stage morphants highlighting the elongated shape (orange arrows) with *fcho1* MO. Scale bar: 250 μm. (r) Quantitation of single and double morphant phenotypes at 24 hpf.
**Figure S6** Selective engagement of Alk8-myc by the FCHO1/Fcho1 μHD.

(a) Coomassie-stained gel and replicate blots of supernatant (S) and pellet (P) fractions of a pull-down assay using Alk8-myc overexpressing HeLa cell lysate and immobilized GST, human (Hs) GST-FCHO1, FCHO2 or *D. rerio* (Dr) Fcho2 μHDs. Immunoblots probed with anti-myc and the indicated endocytic protein specific antibodies. (b) Pull-down assay using Alk8-myc overexpressing HeLa cell lysate and immobilized GST, human (Hs) GST-FCHO1, FCHO2 or *M. musculis* (Mm) SGIP1 μHDs. (c) Pull-down assay using Alk8-myc or Alk1-myc overexpressing HeLa cell lysates and immobilized GST or *D. rerio* (Dr) GST-Fcho1 μHD.
Figure S7 Expression and translational silencing of the AP-2 adaptor α subunit gene (ap2a1) in D. rerio embryos. (a) Schematic depiction of the subunit architecture of the heterotetrameric AP-2 adaptor. (b) RT-PCR analysis of control β actin and ap2a1 transcript-specific amplicons from RNA isolated from embryos at the indicated developmental stages. Molecular standards (bp) are shown. (c) Whole mount in situ analysis of ap2a1 transcript localization in wild-type AB* strain embryos at the indicated developmental stages. The animal (An) and vegetal (Vg) poles and dorsal (D) and ventral (V) axes are indicated. Scale bar: 250 μm. (d-h) Representative examples of surviving morphant phenotypic range at 24 hpf after injection with 4 ng control or 4 ng ap2a1 MO. The delaminating dorsal ectodermal tissue defects (arrows) are indicated. I. Quantitation of indicated surviving ap2a1 MO phenotypes at 24 hpf. J-L. Morphology at 24 hpf of 4 ng control or 4 ng ap2a1 MO injected embryos and with resultant no tail mRNA expression patterns. Scale bar: 250 μm.
Figure 2c

Figure 2d

Figure 2f

Figure 2g

Figure S8 continued
**Figure 5f**

eps15

Alk8-myc

**Figure 5f**

Dab2

Intersectin

Hrb

CALM

**Figure 5g**

eps15

Alk8-myc

**Figure 5h**

eps15

Alk8-myc

Hrb
Figure S8a

Figure S8b

Figure S8c

Figure S8d

Figure S8c

Figure S8d

Figure S8d

Figure S8d

Figure S8 continued