The signalling receptor MCAM coordinates apical-basal polarity and planar cell polarity during morphogenesis

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The apical–basal (AB) polarity and planar cell polarity (PCP) provide an animal cell population with different phenotypes during morphogenesis. However, how cells couple these two patterning systems remains unclear. Here we provide in vivo evidence that melanoma cell adhesion molecule (MCAM) coordinates AB polarity-driven lumenogenesis and c-Jun N-terminal kinase (JNK)/PCP-dependent ciliogenesis. We identify that MCAM is an independent receptor of fibroblast growth factor 4 (FGF4), a membrane anchor of phospholipase C-γ (PLC-γ), an immediate upstream receptor of nuclear factor of activated T-cells (NFAT) and a constitutive activator of JNK. We find that MCAM-mediated vesicular trafficking towards FGF4, while generating a priority-grade transcriptional response of NFAT determines lumenogenesis. We demonstrate that MCAM plays indispensable roles in ciliogenesis through activating JNK independently of FGF signals. Furthermore, mcam-deficient zebrafish and Xenopus exhibit a global defect in left-right (LR) asymmetric establishment as a result of morphogenetic failure of their LR organizers. Therefore, MCAM coordination of AB polarity and PCP provides insight into the general mechanisms of morphogenesis.

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During morphogenesis, apical–basal (AB) polarity and planar cell polarity (PCP) are prerequisites for organizing cells into accurate structures such as tissues and organs. These two distinct patterning systems are composed of two different sets of proteins, but both concurrently operate during morphogenesis. However, the major question in morphogenesis, how cells couple these two polarity systems to simultaneously develop different morphologies, remains unclear.

Formation of multicellular rosette-like structures is a common intermediate process during morphogenesis in diverse species and is a well-studied example of cellular polarity-based processes. The zebrafish LR organizer of Kupfer’s vesicle (KV) and the posterior lateral line (PLL) system are typical rosette structures. Like adult cells in vertebrate, rosette cells also have ubiquitous organelles of cilia. Each rosette-like structure arises from the proliferation of a group of ciliated cells to form a spherical architecture of polarized cells enclosing a central lumen. Lumenogenesis requires AB polarity to dictate correct cellular arrangements and ciliogenesis needs JNK/PCP to regulate cilia assembly.

MCAM, also called CD146 or MUC18, is a signalling receptor and exerts its physiological functions primarily on embryonic development. However, our understanding of how MCAM regulates morphogenesis remains poorly understood. It has been well documented that the polarized distribution of MCAM on the leading edge of chemotaxing cells is vital for establishing cell polarity, but the underlying mechanisms are unclear. In these polarized cells, the concentration of calcium ions (Ca$^{2+}$) is spatially enhanced and polarized in the MCAM-enriched region. Ca$^{2+}$ is a crucial signalling element for activation of NFAT, a Ca$^{2+}$-dependent transcriptional response required for vertebrate development.

FGF signalling plays indispensable roles in the development of living organisms and primarily activates transcriptional factors for activator protein 1 (AP-1), forkhead box protein (FOXO) and NFAT. The chief intracellular substrates downstream from FGF signalling are FGR substrate 2 (FRS2) and PLC-γ. Upon FGF signals, FRS2 switches on AP-1 and FOXO, while PLC-γ turns on NFAT. The functional parallels between MCAM and FGF signalling in diverse processes, such as neural patterning, stem cell maintenance, angiogenesis, wound healing and epithelial–mesenchymal transition (EMT), hint that an unknown interplay between these two pathways exists.

The zebrafish KV plays central roles in LR asymmetric patterning and is an ideal model for investigation into the epithelial organ morphogenesis of metazoan development. Inspired by the involvement of FGF signalling in the morphogenesis of vertebrate LR organizer and in LR patterning, we hypothesized that MCAM may also exert similar morphogenetic functions as FGF signalling. Here, we have validated this hypothesis in vitro and in vivo levels. We demonstrate that MCAM coordinates two distinct patterning systems of AB polarity and PCP, mechanistically explaining how cell populations develop distinct morphologies synchronously. To achieve this coordination, MCAM concurrently activates NFAT and JNK to control AB polarity and PCP, respectively. Thus, our study suggests that MCAM coordination of AB polarity and JNK/PCP is a general mechanism for morphogenesis of organs/tissues during development.

**Results**

**MCAM is an independent receptor of FGF4.** MCAM is required for diverse developmental events and is a developmentally regulated signalling receptor with higher abundance in tissues/organs during embryogenesis relative to an adult stage. To decipher the mechanisms underlying MCAM functions in development, we sought to identify MCAM ligands that are necessary and sufficient for morphogenesis. We therefore performed a yeast two-hybrid assay by using the MCAM-GAL4-binding domain fusion protein as the bait to screen a human GAL4-activating domain fusion complementary DNA (cDNA) library.

The transmembrane domain of MCAM was deleted in the constructs to ensure nuclear localization of the MCAM bait proteins. The extra- or intra-cellular domains of MCAM were inserted in-frame into the pGBK7 expression vector to generate MCAM-1, MCAM-2 and MCAM-3 constructs (Fig. 1a; Supplementary Fig. 1). More than 1,000 prey were sequenced and analysed by BLAST searches, and an array of in-frame coded sequences was identified that represented potential MCAM-interacting proteins. Among them, FGF2, 4 and 8 were identified as prey for MCAM-2 (Fig. 1a).

Endogenous levels of MCAM protein are disparate in different cell lines, with high levels in embryonic or cancerous cells and low levels in most normal cell lines. We screened several cell lines to select one suitable for mimicking morphogenetic processes with high endogenous MCAM. The HEK293 cell line exhibits a high endogenous level of MCAM, comparable to that of several tumour cell types, and was thus selected for use in functional experiments of this investigation (Supplementary Fig. 2).

Co-immunoprecipitation (co-IP) experiments verified that FGF4 and MCAM formed a complex in HEK293 cells (Fig. 1b). Notably, none of the FGFRs (FGFR1–4) tested co-immunoprecipitated with MCAM (Supplementary Fig. 2). FGF4 belongs to the paracrine FGFs that signal FGFRs by forming a tripartite complex with FGFRs and heparan sulphate proteoglycan. To examine the potential requirement of HSPGs for interaction of MCAM with FGF4, we performed co-IP with samples of whole cell lysate that were pre-treated with heparinase to degrade endogenous HSPGs. We found heparinase treatment blocked the association of FGF4–FGFR1 but not that of FGF4-MCAM (Fig. 1b). These data thus provide solid evidence that MCAM is a genuine receptor of FGF4 and transmits FGF4 signals independently of HSPG and separately from FGFRs.

To confirm direct interaction and measure the binding affinity of FGF4 to MCAM, a surface plasmon resonance (SPR) assay was conducted. Results showed that FGF4 bound to MCAM with an affinity of 0.97 nM, which was higher than the values observed for FGF4 binding to FGFR1 and FGFR2 (Fig. 1c). FGF2 and 8 did not bind to MCAM although binding to FGFR1 and FGFR2 was detected (Supplementary Fig. 3).

MCAM mediates vesicular transportation in response to FGF4. FGF4 acts as a chemotaxant during the morphogenesis of chick LR organizer. To evaluate the functions of MCAM or FGFRs on cellular behaviours responding to FGF4, we first conducted chemotaxis assays to mimic the process of cell polarity. FGFR2 and 8 were included as controls. Post 1 h addition of FGF2, 4 and 8, MCAM became enriched at the anterior edge of the chemotaxing cells in the direction of the FGF4 gradient. In contrast, FGFR1 was uniformly distributed on the polarized cell membranes in about 80% of observed cells (Fig. 1d). MCAM enrichment cannot be observed in the chemotaxing cells with the addition of FGF2 and 8 (Supplementary Fig. 4). Furthermore, in about 85% of chemotaxing cells, MCAM was co-localized with atypical (a) PKCζ and PAR3 (two apical markers) at the cellular leading edge in the direction of the FGF4 gradient (Fig. 1e; Supplementary Fig. 5). These results indicate that MCAM is a previously unidentified responsive receptor of FGF4 and that MCAM-enriched leading edge is equivalent to the apical surface of luminal organs.
To further understand how MCAM enriched at the anterior edge of the chemotaxing cells responds to FGF4, endogenous and exogenous MCAM and endogenous FGFR1 were tracked by live-cell imaging in a chemotaxis assay. After the addition of FGF4, endogenous MCAM became enriched at the anterior edge of the chemotaxing cells in the direction of the FGF4 gradient after an average time of 30 min in about 90% of tested cells (Fig. 1f; Supplementary Movie 1). Exogenous MCAM-RFP showed similar localization and trafficking patterns within 30 min in about 95% of tested cells. The direction of MCAM⁺ endocytic vesicular transportation was identical to that of cellular movement towards the FGF4 gradient within the period of monitoring (Fig. 1g; Supplementary Movie 2). In contrast, such movement was not observed for endogenous FGFR1 (Supplementary Movie 3). These results demonstrate that the asymmetric distribution of MCAM is mediated through endocytic vesicular transportation.

Loss of MCAM function impairs lumenogenesis. The core mechanisms underlying the biogenesis of luminal apical surfaces involve the asymmetric distribution of plasma membrane proteins through vesicular trafficking. We thus speculated that MCAM is involved in lumenogenesis. To test this hypothesis, we decided to use a three-dimensional (3D) in vitro model to mimic the lumenogenesis process from a single HEK293 cell.

Figure 1 | MCAM/FGF4-dependent apical surface biogenesis. (a) Upper panel, cartoon of MCAM-BD 1-3. Lower panel, yeast zygotes obtained after mating the bait strain containing pGBKT7-MCAM with the library strain containing pGADT7-FGF2, FGF4, and FGF8. BD, DNA-binding domain; AD, activation domain; +, positive control with p53-BD; and −, negative control with empty AD vector and BD-lambda. (b) Co-immunoprecipitation of MCAM/FGF4 and FGFR1/FGF4 with the protein lysate treated with or without the heparinase I and heparinase III (0.06 IU ml⁻¹). (c) Kinetic dissociation constant (K_D) of FGF4/MCAM, FGF4/FGFR1, or FGF4/FGFR2 complexes was measured using a surface plasmon resonance method. (d) Distribution of polarized MCAM and unpolarized FGFR1 on chemotaxing cells. The source concentration of FGF4 in the chemotaxis assay is 10 ng ml⁻¹. (e) Co-localization of endogenous MCAM and the apical marker aPKCζ in chemotaxing cells. Scale bar, 20 µm. (f,g) Time-lapse live-cells imaging of endogenous MCAM (f) or exogenous MCAM-RFP (g) at the leading edge of chemotaxing cells.
When cells transfected with MCAM-short hairpin (sh) RNA or control shRNA were plated to form cysts (Supplementary Fig. 6), most cells with control shRNA (85%) could form normal lumens. MCAM was localized at the apical surface of cyst lumens; its distribution pattern was similar with CDC42, a required protein in biogenesis of apical surfaces and an apical marker for cyst lumens. In contrast, MCAM-shRNA knockdown produced obvious defects in luminal biogenesis, with misshapen lumens and disorganized F-actin (an apical marker for cyst lumens) distribution observed in approximately 83% of cysts (Fig. 2a,b). These data indicate that MCAM is a crucial element for de novo synthesis of luminal tissues/organs.

Then, we questioned whether FGF4/MCAM signalling plays in vivo roles in morphogenesis. We first chose the zebrafish system as an in vivo model because of its high re-productivity and transparent embryos. There are two mcam genes in zebrafish, mcamα and mcamβ. Mcamα was the gene examined since it possesses high sequence similarity with the homologous genes in mammals. We initially analysed mcam mRNA distribution in zebrafish embryos by whole-mount in situ hybridization (WISH).

Results showed that mcam expression started around mid-gastrulation and was highly enriched in dorsal forerunner cells (DFCs) at 90% epiboly and bud stages, and in KV from the 1-somite stage to the 10-somite stages. At 21 h post-fertilization (hpf), mcam was expressed in the blood vasculature, heart, somites and eye field (Supplementary Fig. 7). The spatiotemporal distribution of mcam mRNA in DFC and KV was similar with that of fgf4, but differed from that of fgfr1 (Supplementary Fig. 8), implying that MCAM is a rational partner of FGF4 for KV development.

KV morphogenesis includes ciliogenesis and alignment of ciliated cells into luminal structures. To evaluate the specific roles of the investigated genes in KV morphogenesis, we
Exclusively knocked-down these genes in DFC/KV, but not in the rest of the embryo, and generated DFC-MO or mRNA embryos using the transgenic Tg(sox17: GFP) zebrafish line. In this line, the KV can be readily visualized during development owing to the expression of green fluorescent protein (GFP) driven by the promoter of the KV marker Sox17 (ref. 29). Specific knockdown of mcam in DFC/KV caused major morphological defects in the size and shape of the KV at the 10-somite stage (Fig. 2c,d). Labelling the KV lumen with an antibody against atypical protein kinase C (aPKC) confirmed that mcam knockdown caused about a 65% decrease in KV luminal volume (5.3 x 10^4 versus 1.8 x 10^4 μm^3; P < 0.001), without affecting the number of KV cells (Fig. 2e,f). In contrast, knockdown of fgf4 or fgfr1 in DFC had no effects on the luminal volume of KV (Fig. 2g). The reduction of luminal volume in DFC/mcam embryos was rescued by injecting mcam but not fgf4 or fgfr1 mRNA into DFC (Fig. 2h; Supplementary Fig. 9), ruling out the possible contribution of FGF signalling on MCAM-mediated AB polarity-driven lumenogenesis.

Depletion of mcam inhibits ciliogenesis. Depletion of fgf4 or fgfr1 causes severe shortening of cilia and the consequent disruption of fluid flow within the KV.5,30-32 Next, we found that the length of KV cilia also decreased by 35% in DFC/mcam MO embryos relative to DFC-control MO embryos (3.82 versus 5.85 μm; P < 0.001). This reduction in cilia length was rescued by DFC injection of mcam mRNA into DFC/mcam MO embryos. In addition, the cilia length was increased by 15% in DFC/mcam mRNA embryos compared to DFC-control MO embryos (6.77 versus 5.85 μm; P = 0.003) (Fig. 3a,b). Consistent with the unchanged KV cell number, these injections did not affect the numbers of cilia (Fig. 3c). The effects of cilia shortening caused by mcam knockdown were comparable to those caused by fgf4 or fgfr1 depletion (Fig. 3d). DFC injection of mcam mRNA cannot rescue the defects of cilia shortening in fgfr4 or fgfr1 DFC-morphants, which was rescued only by DFC-injection of fgf4 or fgfr1 mRNA (Fig. 3e,f). However, Mcam overexpression-induced increase of cilia length in wild-type embryos (Fig. 3b) was not found in fgf4 or fgfr1 depleted embryos (Fig. 3e,f). Thus, these data suggest that the effects of Mcam on cilia length are not entirely independent of FGF signals.

Depletion of mcam disrupts KV function. Zebrafish KV is a fluid-filled organ and the directional nodal flow in KV plays a central role in controlling LR asymmetric development later on.32 To observe whether or not Mcam is implicated in KV flow formation, mcam knockdowns in DFC were conducted. In contrast to the persistent counter-clockwise movement of fluorescent beads in the KV lumen of control morphants, a consistent directional flow was absent in the KV of DFC/mcam MO embryos (Fig. 3g; Supplementary Movies 4 and 5).

**Figure 3 | Mcam depletion in DFC disrupts ciliogenesis and direction of KV fluid flow in zebrafish.** (a) KV cilia labelled with an antibody against acetylated tubulin after DFC injection of embryos with control or mcam MO, mcam mRNA or a combination of mcam MO and mRNA. Scale bar, 20 μm. (b) Mean cilia length in (a) is statistically analysed. Data are presented as mean ± s.e.m. One-way ANOVA with Tukey’s post-test. **P < 0.001 and ***P < 0.001. (c) Mean cilia number (mean ± s.e.m.) of embryos in a was determined. (d) Comparison of cilia length in embryos with DFC injection of mcam, fgfr1 or fgfr1 MO. Data are presented as mean ± s.e.m. One-way ANOVA with Tukey’s post-test. ***P < 0.001. (e,f) Failure of mcam mRNA to rescue fgfr4 MO (e) or fgfr1 MO (f) induced reduction in cilia length. Data are presented as mean ± s.e.m (n = 3) and analysed using one-way ANOVA with Tukey’s post-test. ***P < 0.001 and NS, not significant. (g) Direction of KV fluid flow tracked with fluorescent beads in control and DFC/mcam MO embryos. Scale bar, 20 μm. (h) Randomized expression of left-side-specific spaw in DFC/mcam MO morphants. Scale bar, 250 μm. (i) Quantitative analysis of embryos with normal (left-sided), reversed (right-sided), cardio bifida (bilateral) and absent expression. n, number of embryos (b, f, i).
Spaw, the earliest left-sided expressed gene, relays nodal flow-delivered signals in KV to initiate a downstream LR signalling cascade in zebrafish. To assess the position of Mcam in the signalling cascade for LR determination, we analysed the expression of spaw and its target genes, including pitx2, lefty1, and lefty2 in DFC and MO embryos. Mcam knockdown in DFC perturbed the normal left-sided expression of spaw and pitx2 in the lateral plate mesoderm, and induced expression of lefty1 and lefty2, the markers for the left part of the dorsal diencephalon and heart primordium, respectively (Fig. 3h,i; Supplementary Fig. 10). These data indicate that, similar to FGF signalling, Mcam is also required at an earlier stage of LR development when the nodal flow in KV becomes emergence.

Mcam plays conserved roles in LR asymmetry development. KV is a transient organ to control LR asymmetric development. To observe effects of Mcam in LR determination, the positions of visceral organs were visualized with heart (cmel2) and liver/pancreas (foxa3) markers by WISH. Mcam deficiency in DFC caused a severe disruption of cardiac jogging, an early feature of cardiac LR asymmetry, as well as a randomization of the heart position (Fig. 4a). Abnormal right jogging (6.7%), no jogging (60.0%) and cardia bifida (6.8%) were observed along with normal left jogging (Fig. 4b). LR asymmetry in the liver, gut, and pancreas was also affected by mcam deficiency (Fig. 4c), with Mcam knockdown in DFC perturbing the normal left-sided expression of spaw and pitx2 in the lateral plate mesoderm, and induced expression of lefty1 and lefty2, the markers for the left part of the dorsal diencephalon and heart primordium, respectively (Fig. 3h,i; Supplementary Fig. 10). These data indicate that, similar to FGF signalling, Mcam is also required at an earlier stage of LR development when the nodal flow in KV becomes emergence.

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**Figure 4 | Mcam regulation of left-right asymmetric development in zebrafish and Xenopus.** (a-d) Developing organs in DFC morphants were visualized by WISH using RNA probes of cmlc2 (a, heart) and foxa3 (c, endoderm). Quantitative analysis of embryos with normal, reversed, bilateral and absent asymmetry is shown in b,d, n, number of embryos. The left schema in a shows embryo positions in the embryo (A, anterior; P, posterior; L, left; and R, right). In c, t (green arrow) = liver, p (red arrow) = pancreas and g (blue arrow) = gut. h.p.f., hours post-fertilization. Scale bars in a, 100 µm. (e) Xmcam-MO (1 pM) caused situs defects at stage 45, in Xenopus embryos including heterotaxia (ht) and situs inversion (si) compared with the wild type (wt) situs solitus. Heart looping and position is outlined by red dots or red arrow, respectively. Gut coiling is outlined by light blue dots. Position of gall bladder is indicated by green auto-fluorescence and a green arrow. Scale bar, 1 mm. (f) Normal left-sided expression of heart (cmel2) and liver/pancreas (foxa3) markers by WISH. Mcam deficiency in DFC caused a severe disruption of cardiac jogging, an early feature of cardiac LR asymmetry, as well as a randomization of the heart position (Fig. 4a). Abnormal right jogging (6.7%), no jogging (60.0%) and cardia bifida (6.8%) were observed along with normal left jogging (Fig. 4b). LR asymmetry in the liver, gut, and pancreas was also affected by mcam deficiency (Fig. 4c), with Mcam knockdown in DFC perturbing the normal left-sided expression of spaw and pitx2 in the lateral plate mesoderm, and induced expression of lefty1 and lefty2, the markers for the left part of the dorsal diencephalon and heart primordium, respectively (Fig. 3h,i; Supplementary Fig. 10). These data indicate that, similar to FGF signalling, Mcam is also required at an earlier stage of LR development when the nodal flow in KV becomes emergence.

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blastomeres, also shortened cilia length relative to control MO-injected embryos (Supplementary Fig. 11). The positions of the gall bladder, heart, and gut looping were inverted (situs inversus) or developed discordantly (heterotaxia) (Fig. 4e,f). These results suggest that MCAM plays conserved roles in LR organizer morphogenesis as well as in LR asymmetric patterning during vertebrate development.

MCAM activates NFAT as an anchor of PLC-γ. Next, we explored the molecular mechanisms underlying the roles of MCAM in lumenogenesis and ciliogenesis. To identify whether MCAM modulates the PLC-γ-initiated FGF signalling pathway, the NFAT reporter encoding consensus sequence recognized by a NFAT transcription factor was generated. MCAM knockdown drastically suppressed the activation of NFAT (by 45%; γ<0.008) and these reductions were rescued by reconstitution of MCAM (Fig. 5a; Supplementary Fig. 12). There were no direct interactions between MCAM with FRS2 or growth factor receptor-bound protein (Grb) 2, which are critical intermediate signalosomes between activated FGFR and ERK–c-Fos–AP1 or PI3K–PKD–FOXO pathways. The activation of AP-1 or FOXO upon FGF4 stimulation was significantly inhibited in cells with FGFRs knockdown, compared with those cells with MCAM knockdown. In contrast, the effects of MCAM depletion on NFAT activation are higher than those of FGFR1–4 depletion (Fig. 5b; Supplementary Figs 13–15).

In addition, the NFAT upstream elements of PLC-γ1, PLC-γ2, and IP3R1 were also identified as candidate MCAM-interacting proteins in the yeast two-hybrid screen. These interactions were further confirmed by co-IP (Fig. 5c), suggesting that MCAM is a component of the IP3 receptor complex and works together with IP3R to promote intracellular Ca2⁺ release. Our findings thus consolidate the previous observations that MCAM resides in the endoplasmic reticulum (ER) and elevates intracellular Ca2⁺ levels at the MCAM-enriched regions11.

Recruitment of PLC-γ to the cell membrane is a prerequisite step for the catalysis of membrane-bound PIP2 as well as the subsequent Ca2⁺ release from ER30. We found that MCAM knockdown impaired PLC-γ1 and –γ2 anchoring to cell membranes in both the resting and activated status (that is, in the absence or presence of FGF4). By contrast, FGFR1–4 knockdown had no effects on PLC-γ recruitment onto cell membranes in either status (Fig. 5d,e). These results, together with previous findings that MCAM recruits Fyn kinase to activate PLC–γ33, not only demonstrate that MCAM can independently turn on PLC–γ, but also explain why the knockdown effects of MCAM on NFAT activation are higher than those of FGFRs.

MCAM drives lumenogenesis through NFAT pathway. To evaluate the roles of NFAT activation on morphogenesis, we first blocked NFAT activity in the in vitro 3D cyst cultures with 1 μM of 11R-VIVIT (MAGPHPVIVITGPHEE), a specific peptide inhibitor of NFAT34. Statistical analysis indicated that NFAT blockage significantly disrupted lumen formation (Supplementary Fig. 16), which was similar to that of MCAM knockdown (Fig. 2a,b). Thus, these in vitro data
Figure 6 | MCAM controls lumenogenesis by activation of NFAT. (a) Detecting the expression of RFP-HA and VIVIT-RFP-HA in HEK293 cells by IB. (b) VIVIT selectively inhibiting NFAT reporter activity. Jurkat cells were co-transfected with NFAT-Luc (left panel) or NF-κB-Luc (right panel) reporter plasmid, and with RFP and RFP-VIVIT expression plasmids. Twenty-four hours after transfection, cells were left untreated or were stimulated for 6 h with phorbol 12-myristate 13-acetate (PMA; 20 nM) and ionomycin (1 mM) (P + I). (c) Calcineurin dependence of NFAT and NF-κB reporter activity. Jurkat cells were transfected with NFAT-Luc (left panel) or NF-κB-Luc (right panel) reporter plasmid. Twenty-four hours after transfection, cells were left unstimulated or were stimulated for 6 h with P + I in the absence or presence of 1 μM cyclosporin (CsA). (d) Both RFP-tag and VIVIT-RFP were localized and expressed in DFC after microinjection of RFP and RFP-VIVIT mRNA into DFC of zebrafish KV. DIC means digital image of contrast. (e, f) Messenger RNAs were injected into zebrafish DFC of Sox17:GFP transgenic embryos, which were harvested at the 10 s stage. Lumen cells were labelled with an antibody against αPKCζ (red). Data are presented as mean ± s.e.m. One-way ANOVA with Tukey’s post-test. ***P value < 0.001 and NS = not significant. (g, h) KV cilia were labelled with an antibody against acetylated tubulin after injection of the indicated mRNAs. Data are presented as mean ± s.e.m. and analysed using unpaired student’s t-test. The NS means not significant. (i) Quantitative analysis of heart joggings after injection of the indicated mRNAs into DFC of Sox17:GFP zebrafish embryos. Normal (left), reversed (right) and absent (no) jogging were calculated. Scale bar, 20 μm. n, number of observed embryos (e–i).

indicate that NFAT is a critical player for AB polarity-driven lumenogenesis.

To further evaluate the in vivo roles of NFAT in lumenogenesis, we constructed a plasmid containing the VIVIT sequence. The expression of the red fluorescent protein (RFP)-tagged VIVIT blocked activation of an NFAT reporter but not of an NF-κB reporter in Jurkat cells stimulated with phorbol ester (PMA) plus ionomycin (Fig. 6a,b), although both reporters were equivalently sensitive to the inhibition of calcineurin with cyclosporine A (CsA; Fig. 6c).

Similar to observations in DFCmcam MO embryos (Fig. 2), DFC injection of VIVIT mRNA caused about a 60% decrease in KV luminal volume in these zebrafish morphants, as compared to DFCmRNA embryos (5.3 x 10⁴ versus 2.1 x 10⁴ μm³; P < 0.001; Fig. 6d). The volume reduction in zebrafish DFCVIVIT mRNA embryos could not be rescued by injecting DFC with mcam or PLC-γ1 mRNA (Fig. 6f). In contrast, blocking NFAT in DFC did not affect cilia length (Fig. 6g,h). Therefore, these in vitro and zebrafish in vivo data demonstrate that NFAT is the effective cascade downstream from the MCAM/PLC-γ pathway to fulfill lumenogenesis.

Consequently, blocking NFAT in zebrafish DFC resulted in a severe disruption of cardiac jogging. Abnormal right jogging (20.1%, n = 136), no jogging (10.3%, n = 53) and cardia bifida (10.8%, n = 30) were observed along with normal left jogging in zebrafish embryos (Fig. 6i). The high similarities in defects of
mediated coordination of cell polarity during morphogenesis. See text for details.

Then we explored which signalling cascade downstream from MCAM constitutively activates JNK to determine ciliogenesis. We found that MCAM overexpression induced a robust increase in JNK activity with or without FGF2, 4 or 8 stimulation, which was suppressed by blocking MCAM via MCAM knockdown (Fig. 7a). Subsequently, we examined the activation status of the key kinases upstream of these two transcriptional factors. We found that MCAM overexpression inhibited reporter’s activation of AP-1 (by 25%; \(P = 0.045\)) and FOXO (by 30%; \(P = 0.008\); Fig. 7a). Subsequently, we examined the activation status of the key kinases upstream of these two transcriptional factors. We found that MCAM overexpression induced a robust increase in JNK activity with or without FGF2, 4 or 8 stimulation, which was suppressed by blocking MCAM.

**MCAM constitutively activates JNK to determine ciliogenesis.** Then we explored which signalling cascade downstream from MCAM is responsible for ciliogenesis. The MCAM knockdown inhibited reporter’s activation of AP-1 (by 25%; \(P = 0.045\)) and FOXO (by 30%; \(P = 0.008\); Fig. 7a). Subsequently, we examined the activation status of the key kinases upstream of these two transcriptional factors. We found that MCAM overexpression induced a robust increase in JNK activity with or without FGF2, 4 or 8 stimulation, which was suppressed by blocking MCAM.

**Figure 7 | MCAM-dependent JNK activation regulates ciliogenesis.** (a) Luciferase activity measured 30 h post transfection. AP-1-Luc or FOXO-Luc reporter vectors were co-transfected with either MCAM RNAi or control RNAi. Data (mean \(\pm\) s.e.m.) were analysed with unpaired student’s t-test. \(*P < 0.05\) and \(*\*P < 0.01\). (b) Phosphorylation and expression of JNK following overexpression of MCAM by transfection with pCS2-MCAM-HA or blockage of MCAM by its functional antibody AA98. (c,d) DFC-specific knockdown of jnk1 did not affect lumen formation in Sox17:GFP zebrafish embryos. Lumen cells were labelled with an antibody against aPKC (red). Scale bar, 20 \(\mu\m). Data (mean \(\pm\) s.e.m.) were analysed with unpaired student’s t-test. The NS means not significant. (e,f) DFC-specific knockdown of jnk1 shortened KV cilia. The cilia were labelled with acetylated tubulin antibody. Scale bar, 20 \(\mu\m). Data (mean \(\pm\) s.e.m.) were analysed using one-way ANOVA with Tukey’s post-test. \(**P < 0.001\). (g,h) Randomized expression of left side-specific lefty1 in the DFC\(jnk1\ MO\) morphants. Scale bar, 100 \(\mu\m). (i) Quantitative analysis of embryos with normal (left-sided), reversed (right-sided), cardiac bifida (bilateral) and absent expression. n, number of embryos (d,f,h,i). (j) MCAM is localized at the zebrafish PLL system. Embryos of Et (gata2:EGFP) mp189b zebraline at the 28 h.p.f. stage were collected and stained with MCAM antibody. Scale bar, 15 \(\mu\m). (k) A model of MCAM-mediated coordination of cell polarity during morphogenesis. See text for details.
with MCAM’s functional antibody of AA98 (Fig. 7b). In contrast, overexpression or blockage of MCAM had no effects on the change of ERK, p38 and PDK phosphorylations. ERK phosphorylation was completely dependent on the presence of FGFs, but not on the abundance of MCAM (Supplementary Fig. 17). These results thus demonstrate that MCAM constitutively activates JNK, independent of FGF signals.

We next asked whether jnk is linked with Mcam-regulated KV morphogenesis or ciliogenesis. WISH data with zebrafish embryos indicated that like mcam, jnk1 mRNA expression appeared to be ubiquitous, including in zebrafish KV (Supplementary Fig. 18). The KV co-expression of Mcam and JNK manifests the spatial rationality that Mcam can access and activate JNK during KV morphogenesis. Next, we found that the DFC knockdown of jnk1 in zebrafish did not affect KV luminal volume (Fig. 7c,d). Notably, DFC knockdown of jnk1 resulted in a reduction of KV cilia length by 17% relative to DFC control MO zebrafish embryos (4.62 versus 3.85 μm; P<0.001). Such reduction was rescued by the DFC injection of jnk1 mRNA into those morphants (Fig. 7e,f). The jnk1 knockdown consistently induced randomized expression of spaw and its target genes, including pitx2, lefty1 and lefty2, and caused the significant defect of LR asymmetry in zebrafish embryos (Fig. 7g–i; Supplementary Fig. 19). These results demonstrate that constitutive activation of JNK by MCAM is crucial for the determination of cilia length as well as LR asymmetry.

Polarized rosettes are common intermediates during morphogenesis of diverse organs, such as the zebrafish KV, the vertebrate pancreas, as well as the neural stem cell niche. We then asked whether or not the functions of Mcam in morphogenesis are only limited to KV rosette formation. Beyond KV, the zebrafish PLL system is another widely used model to investigate the cell polarity-driven morphogenesis. Next, we used the transgenic zebrafish Et (gata2;EGFP) mp189b line to trace the development of the PLL system. Labelling endogenous Mcam with immunostaining in this line demonstrates that Mcam is also localized at the PLL system (Fig. 7j and Supplementary Fig. 20), suggesting that Mcam-mediated coordination of AB polarity and PCP is a broadly utilized mechanism during morphogenesis (Fig. 7k).

**Discussion**

A major challenge in understanding morphogenesis is to decipher mechanisms underlying the coordination of AB polarity and PCP using genuine tissues. Here we reveal how MCAM simultaneously couples these two distinct patterning systems for lumen formation and cilium growth at the molecular, cellular and in vivo levels. Thus, this study sheds light on the mechanisms by which cell populations synchronously develop distinct morphologies during organ formations.

The unsolved central question in comprehending mechanisms of AB polarity is to decode the nature of in vivo polarity cues. In this study, at both molecular and cellular levels, we demonstrate that FGFR4 acts as a spatial cue for biogenesis of apical surface. Thus, in light of this study, morphogens with chemotactic activity may have the potential to serve as in vivo spatial cues for the establishment of AB polarity. Furthermore, the cooperation of MCAM/FGFR4 in AB polarity suggests that MCAM could be crucial in FGFR4-executed morphogenetic events.

Another open question in cell polarity is to identify the critical polarity complexes that regulate polarized endocytosis. The machinery of endocytic transportation is composed of secretory organelles, including the ER, Golgi complex and endosome. Our findings that MCAM interacts with IP3Rs of the ER, and mediates endocytic vesicular transportation strongly suggest that MCAM is a crucial player in the apical polarization of membrane proteins.

Although it has been well established that PIP2 plays a predominant role in the generation of intracellular asymmetry and an apical surface, the upstream signals initiating PIP2 asymmetry remain elusive. Our findings that MCAM can independently anchor PLC-γ, which is also anchored onto the cellular membrane by its interaction with PIP2, suggest that MCAM, PLC-γ, and PIP2 could form a ternary complex. Therefore, such findings suggest the possibility that the apical distribution of MCAM on a cellular surface could contribute to the origination of PIP2 apical asymmetry.

Formation of cellular polarity requires the orchestration of intracellular signalling events. Our study implies that the FGFR4-MCAM-NFAT axis represents an essential signalling pathway for AB polarity as well as lumenogenesis. PLC-γ is a shared signalling nexus that acts downstream from more than 100 cell surface receptors. In this regard, PLC-γ-mediated NFAT activation, elicited from signalling receptors other than MCAM, relies on at least partially, MCAM-mediated PLC-γ activation. Thus, MCAM may be the long-sought-after upstream receptor of NFAT that integrates divergent intracellular Ca2+ signals with NFAT-dependent transcriptional response, for lumenogenesis.

It is well established that JNK activation is dependent on outside environmental signals. Thus, our finding that MCAM constitutively activates JNK is a novel scenario, which facilitates the understanding of MCAM as a requirement in JNK/PCP-dependent ciliogenesis. Meanwhile, this constitutive activation of JNK by MCAM can also explain why there is increasing evidence linking MCAM to a wide spectrum of JNK-involved morphogenetic processes.

To our knowledge, our findings provide the first in vivo evidence on how two distinct patterning systems are integrated in genuine tissues and will facilitate better understanding of the key molecular and cellular players of morphogenesis. Therefore, this study is expected to shed light on the general mechanism by which cell populations coordinate two distinct cell polarities during morphogenesis.

**Methods**

**Antibodies.** AA1 (1:1,000) and AA98 (1:1,000), both of which are murine anti-MCAM monoclonal antibodies generated in our laboratory, recognize the first V domain and the last C2 domains in the extracellular region of MCAM, respectively. Additional information for all commercial antibodies is as following.

- Anti-FGFR3 (1:1,000, ab139938) antibody was purchased from Abcam limited.
- Anti-HA-Tag (1:30,000, #2276), anti-Myc-Tag (1:30,000, #2276), anti-VEGF1 (1:1,000, #9740), anti-PLC-γ1 (1:1,000, #2822), anti-PLC-γ2 (1:1,000, #3872), anti-JNK (1:1,000, #9252), anti-phospho-JNK (1:1,000, #925S),-ERK (1:1,000, #9107), anti-phospho-ERK (1:1,000, #4370), anti-phospho-KC (1:1,000, #9107), anti-phospho-P38 (1:1,000, #511), anti-PDK (1:1,000, #3061), anti-phospho-ERK (1:1,000, #566), Alexa Fluor 488 Phalloidin (1,000, 8878), and the secondary antibodies of donkey anti-rabbit Alexa Fluor 555 (1,000, 8953S) antibodies were from Cell Signaling Technology. Anti-FGFR3 (1:2,000, ab10830), anti-FGFR8 (1:2,000, ab181492), anti-CDC42 (1:500, ab187643) and anti-PKCζ (1,000, ab19291) antibodies were from Abcam.
- Anti-MCAM (1:2,000, LS-C71062) antibodies were from Lifespan.
- Anti-MCAM antibody (1:2,000, KM8082) was from Tianjin Sungene Biotech Co., Ltd. The anti-RFP-Tag (1:2,000, KM8082) was purchased from Biorbyt limited.
- Anti-FGF3 (1:2,000, orb39033) antibody was purchased from Biorbyt limited.

**Protease inhibitor, Protein G PLUS-Agarose, and PhosSTOP (phosphatase inhibitor) Protein G PLUS-Agarose, and PhosSTOP (phosphatase inhibitor)**

**Reagents.** Chemicals such as ionicomycin, phosphol 12-myristate 13-acetate, cyclosporine, heparinase I and heparinase III were from Sigma. All cell culture media were from Gibco and all transfection reagents were from Invitrogen. Protease inhibitor, Protein G PLUS-Agarose, and PhosSTOP (phosphatase inhibitor)**

**Figure Legends**

**Fig. 7. MCAM regulates KV morphogenesis.** (A) Schematic illustration of KV and PIP2 asymmetry. (B) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (C) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (D) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (E) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (F) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (G) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (H) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (I) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (J) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (K) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (L) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (M) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (N) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (O) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (P) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (Q) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (R) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (S) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (T) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (U) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (V) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (W) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (X) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (Y) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV. (Z) Immunostaining of MCAM (green), IP3R1 (red) and IP3R2 (white) in KV.
inhibitor cocktail) were from Roche. The Matchmaker Gold Yeast Two-Hybrid System was purchased from Clontech. The Dual-Luciferase System (E1910) was from Promega. The membrane isolation kit was from Thermo Scientific. Heparen Sulfate Proteoglycan (HSPG, PA656H50u) was from USCN Life Science Inc. Human serum albumin recombinant was from California Bioscience. The recombinant FGF4 (AF-100-31) was from Peprotech, and FGF8a (4745-F8-050) was from R&D. The recombinant FGF2 (10014-HNAE), FGFR1/Fc (10616-H30H), FGFR2/Fc (10824-H3M), MCAM/Fc (10115-H20I), and IgG1 Fc (10702-HNAH) were from Sino Biological Inc.

Cell lines with culture conditions and treatments. All of the cell lines were obtained from ATCC, and were authenticated by single nucleotide polymorphism testing and mycoplasma contamination testing. The HEK293 (human embryonic kidney 293, CRL-1573) cell line and the human liver cancer cell line HepG2 (HB-8065) were maintained in EMEM with 10% FBS. The human malignant melanoma cell line A375 (CRL-1619) was maintained in DMEM with 10% FBS. The breast cancer cell line MDA-MB-231 (HTB-26) was maintained in EMEM with 10% FBS and 0.1 mM of nonessential amino acid (NEAA). The MCF-7 (HTB-22) breast cancer cell line was maintained in EMEM with 5% FBS, 0.1 mM of nonessential amino acid (NEAA), and 10% FBS. The human colon cancer cell line HT-29 (HTB-38) was maintained in McCoy’s 5a medium modified with 10% FBS. The Jurkat cells (TIB-152) were maintained in RPMI-1640 medium.

The following are the primers for construction of MCAM-RFP: forward, 5' gggatcccCTAGCCCCGGCTCTCCGGCTCC-3', reverse, 5' gccgaattcATGGGCCTGGACTTGGACACCA-3'. The human colon cancer cell line HT-29 (HTB-38) was maintained in McCoy’s 5a medium modified with 10% FBS. The Jurkat cells (TIB-152) were maintained in RPMI-1640 medium.

The following are the primers for construction of MCAM-RFP: forward, 5' gggatcccCTAGCCCCGGCTCTCCGGCTCC-3', reverse, 5' gccgaattcATGGGCCTGGACTTGGACACCA-3'. The yeast strain Y2HGold, which expresses bait protein from the plasmids of pGBKT7-MCAM-1, can be found in Supplementary Fig. 21.

Immunoﬂuorescence staining and confocal microscopy. Cells were cultured on μ-Slide ibidi chambers. After 24 h, cells were exposed to a FGF4 gradient with a concentration of 10 μM. Cells were then permeabilized by treating them with PBS and then blocked with 3% BSA in PBS for 1 h. Cells were then incubated with primary antibodies against MCAM (1:100), FGFR1 (1:50), and PAR3 (1:100) in PBS, with 3% BSA overnight at 4 °C. Cells were then washed three times with PBS/0.1% Tween 20 and then incubated for one hour with fluorescence-conjugated secondary antibody at a dilution of 1:300 at 37 °C. After 45 min, the cells were washed with PBS for 15 min and then counterstained with DAPI for 5 min. A confocal laser scanning microscope (Olympus FLUOVIEW FV1000) with a mounted Olympus IX81 digital camera was used for image acquisition.

Chemotaxis assay and live-cell imaging. Chemotaxis experiments were carried out in μ-Slide ibidi chambers according to the manufacturer’s instructions, with modifications as described earlier. A HEK 293 cell suspension was diluted to 3 × 10^5 cells per ml. Six microliter of cell suspension was applied onto one of the filling ports of the μ-Slide using a 20 μl pipetter, and 6 μl of air was aspirated from the opposite filling port. The slides were then placed in a sterile 10 cm Petri dish with a wet tissue around the slide and were transferred to a 37 °C incubator. After 45 min, the cells attached, all plugs were gently removed from the filling ports and both reservoirs were filled with 45 μl of chemotactrant-free medium. For chemotactrant application, one of the filling ports was filled with 18 μl of FGF4 (10 ng ml^-1) source solution by removing 18 μl of chemotactrant-free medium from the other port on the same side of the device. All the ports were then closed with plugs. Cell migration was recorded by mounting the μ-Slide onto the stage of an inverted microscope (Eclipse Ti, Nikon), which was fitted with a 37 °C incubator (5% CO2). Movies were taken using the CSU-X1 spinning disk confocal scanner (UltraView VoX, Perkin-Elmer), a × 60 lens (Nikon), and the Volocity 6.0 software.

Surface plasma resonance measurements. The binding kinetics between the soluble FGF2, FGF4 or FGF8 to MCAM, FGFR1 or FGFR2, were analysed at room temperature on a Biacore T100 machine (GE Healthcare) with CM5 chips (GE Healthcare) were coated to a density of 15 000 resonance units (RU) using the ZNEA chemical coupling method. The binding measurements were performed on Biacore T100 with the following buffer of 1 x PBS with 0.05% Tween 20 was used for all measurements. For surface plasma resonance (SPR) measurements, two chips were used. Recombinant human chimeric proteins of MCAM-Fc, FGFR1/Fc, FGFR2/Fc or Grf1 Fc, were immobilized on the CM5 chip using 1μg ml^-1 of IgG1 Fc and PBS with 0.05% Tween 20 were used either as the negative chimeric-protein or the blank solvent control. SPR chips were treated using the same protocol; immobilization was conducted using N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl carbodiimide, and neutralization was carried out with 1.0 M ethanolamine. Upon completion of data collection for each sensor surface, the system was regenerated with washing with 10 mM glycine-HCl. A series of concentrations were used for the designed experiments. Kinetic and equilibrium parameter (Kd) values were calculated by fitting the raw sensor gram with the 1:1 binding model using the Biacore T100 evaluation software.

Co-immunoprecipitation and immunoblotting. The HEK293 cells were collected with 5 mM EDTA in PBS and lysed for 1 h at 4 °C in a lysis buffer (containing 10 mM Tris–HCL, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.5 mM sodium vanadate, 1% NP-40, a protease inhibitor cocktail and a phosphor-stop cocktail, pH 7.5). After removal of cell debris by centrifugation for 15 min at 14,000 g, proteins (~50 μg) were pre-cleaned using normal mouse IgG and Protein G PLUS-Agarose. The pre-cleaned supernatants were incubated overnight with antibodies at 4 °C. The next day, the cell lysates were further incubated with Protein G PLUS-Agarose for three hours at 4 °C. The immunoprecipitates were rinsed three times with a washing buffer (1 M NaCl, 1% NP-40, 50 mM Tris–HCL, pH 7.5) and analysed through immunoblotting (IB) with the desired antibody. The IB was carried out using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and polyvinylidene difluoride membranes were visualized by the ECL immunoblot detection system. All uncropped western blots can be found in Supplementary Fig. 21.

Growth of cysts in a three-dimensional matrigel. Cyst cultures in the matrigel were carried out according to an established protocol as following. The matrigel was first thawed at 4 °C overnight, and then the dishes (Nest, with 15 mm diameter) were coated with 120 μl of Matrigel. The culture dishes were incubated for 15–30 min at 37 °C that allow the Matrigel to gel. A single cell suspension with 4 × 10^3 cells per ml in 2% Matrigel was made. The 1.200 μl of cell suspension was plated onto the Matrigel-coated surface. The cultures were maintained for 5–7 days, replacing the Matrigel-medium mixture every two days until cysts with lumen formed. The nuclei of cysts were counterstained with DAPI (0.5 μg ml^-1) in PBS for 5 min at room temperature. Photos were taken with a confocal laser scanning microscope (Olympus FLUOVIEW FV1000) with an Olympus IX81 digital camera.

Fish handling. The zebrafish lines of Tubingen strain, Tg(sox17: GFP)^s870 and Tg(gata2:eGFP)^mp18995 were maintained in a breeding facility. Embryos were raised in an E3 buffer at 26–30 °C. Zebrafish experiments were conducted in an ethical manner as approved by the Animal Care and Use Committee of Tsinghua University. Embryo stages, according to the established criteria, are indicated in the relevant figures and legends. WISH was performed according to standard procedures. For DFCMO experiments, a volume of 1 nl fluorescent MO was injected into embryo yolks at the 500–1000 cell stage, and embryos were selected by fluorescence microscopy for MO enrichment as the established standard.

RNA probes of zebrafish. Digoxigenin-labelled RNA probes of zebrafish mcam24, fgf4, fgf1, foxa3 (ref. 47), cmll2 (ref. 48), southpaw24, pitx2 (ref. 50), lefty1 (ref. 51) and lefty2 (ref. 51) were synthesized by in vitro transcription using T7 or SP6 RNA polymerase.

Antisense morpholinol oligonucleotides. Morpholinol oligonucleotides (MOs) were synthesized by Gene-Tools, LLC (Corvallis, OR). The sequences of MOs used in the zebrafish were as follows: mcam MO25, 5'-AGGACGTCGGGTTAGGCTTAATTCCT-3'; mcam MO5's mis control23, 5'-AGGCGTGCGGAGTACCTTGGTC-3'; fgf4 MO25, 5'-GCTAACGTGTTCTGATGTCTGAG-3'; fgf1 MO25, 5'-GACGACGGTGAATCTCTGACT-3'; m27 MO (designed based on the GenBank sequence AB030900), 5'-ACGTGATCCTGGGACCAGAAG-3'; mkl MO 5's mis control, 5'-ACGTAGTCTCCTGCTAGTCCAT-3'; mkl MO 5's mis control, 5'-ACGTGATCCTGGGACCAGAAG-3'; mkl MO 5's mis control, 5'-ACGTGATCCTGGGACCAGAAG-3'.

RNA probes of zebrafish. Digoxigenin-labelled RNA probes of zebrafish mcam24, fgf4, fgf1, foxa3 (ref. 47), cmll2 (ref. 48), southpaw24, pitx2 (ref. 50), lefty1 (ref. 51) and lefty2 (ref. 51) were synthesized by in vitro transcription using T7 or SP6 RNA polymerase.
**Kupffer’s vesicle flow analysis.** Zebrafish embryos at the 6 to 8 somite stage were dechorionated and mounted in a 1% low melt agarose. Fluorescent beads (0.25 μm) were injected into each embryo and detected with a ×40 lens. Metamorph software (Universal Imaging Corp.) was used to track the injected beads to calculate bead velocity.

**Xenopus laevis handling and RNA probe of Xenopus laevis.** Xenopus laevis frogs (Nasco, Atlanta, GA, USA) were stimulated to lay egg by injection of human chorionic gonadotropin. Embryos were obtained by in vitro fertilized and dejellied with 3.2 μM DL-Dithiothreitol, pH 8.9, and cultured in 0.1 × 0.1 × 0.3 cm modified Ringer’s (MMR; 0.1 M NaCl, 20 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM Hepes, pH 7.8, 0.1 mM EDTA pH 8.0). All animal work received ethics approval from the Animal Care and Use Committee of Institute of Biophysics, Chinese Academy of Sciences.

For classification of organ situs defects, embryos at stage 45 were anesthetized in 0.15% MS222 (Sigma-Aldrich, St Louis, MO, USA). 0.1 × MMR and recorded under stereo microscope (Olympus SZX16, Japan). Situs defects were classified into three types, complete inversion of organ situs (situs inversus), random organ transposition (heterotaxia) and situs solitus (wild type) according to deviation from the normal position and morphology5,4.

Probes for Xenopus laevis mcam and Xnr1 (ref. 55) were synthesized with linearized pcDNA3.1+ HA-Xmcam (a full-length cDNA described as below) and pcDNA3 Xnr1 (a 388 bp fragment of the 5′-coding region) as templates, respectively.

**Synthesis of mRNAs.** In vitro synthesis of mRNAs was performed using the mMessage mMachine Kit (Ambion) from the following linearized pcDNA3+ HA plasmids: Z-mcam forward, 5′-acctgagcATGACCTACCCAGGCTCTG-3′; and Z-mcam reverse, 5′-gg actgatTTCCATGGTATCTTTTT-3′. Z. mfps forward, 5′-acctgagcATGATGTCCTGAGCCTC-3′; and Z. mfps reverse, 5′-gg actgatGAATTCGTTGGAACAGC-3′. Z. npc1 forward, 5′-acctgagcATGTCCTGAGCCTC-3′; and Z. npc1 reverse, 5′-ggactgacGCGCCGGTGGAGT-3′. Z. jnk1 forward, 5′-acctgagcAGAATCTGGGCTGGC-3′; and Z. jnk1 reverse, 5′-ggactgacCTGGGCTGGC-3′. Z. aggctc reverse, 5′-ggactgacCTGGGCTGGC-3′; and Z. aggctc forward, 5′-ggactgacGCGCCGGTGGAGT-3′. The mRNA was injected into zebrafish or Xenopus embryos with 3.2 ng/μl.

**Gene silencing with siRNA and shRNA.** The concentration of each siRNA is indicated in the respective figures, and the inhibition of endogenous MCAM RNA, without affecting exogenous transcripts. The coding sequence of the RFP-tagged NFAT inhibitor, RFP-VIVIT peptide (MAGPHPVIVITGPHEE), was inserted into the plasmid of pCS2 TA-3 linearized pCS2 TA-3 plasmid. The mRNA was injected into zebrafish or Xenopus embryos with 3.2 ng/μl.

The coding sequence of the GFP-tagged NFAT inhibitor, GFP-VIVIT peptide (MGAGPHVIVITGFPEE), was inserted into the plasmid of pcDNA3.1+ HA. The linearized pcDNA3+ HA-VIVIT-RFP plasmid was used as the template for in vitro synthesis of the GFP-VIVIT mRNA. VIVIT forward 5′-acctgagcATGCGCTGCCCAGAGCGCT-3′ and VIVIT reverse 5′-ggactgacGCGCCGGTGGAGT-3′. The mRNA was injected into zebrafish or Xenopus laevis with a 250 pg dose of mRNA for each embryo.

**Silencing gene with siRNA and shRNA.** SiRNAs were purchased from GenePharma. The siRNAs against FGFR1, FGFR2, FGFR3 and FGFR4 were designed to target the luciferase-coding regions. The siRNAs against mCAM were designed to target at its 3′-untranslated region in such way that it results in the specific inhibition of endogenous MCAM RNA, without affecting exogenous transcripts. The concentration of each siRNA is indicated in the respective figures, and the transfection followed the manufacturer’s instructions of Lipofectamine RNAi MAX. The siRNA sequences used were as follows: CAMC-1 #1 sense is 5′-CAGGGUGAUAUGCCUAUAGU-3′; CAMC-2 #1 sense is 5′-CAGGGUGAUAUGCCUAUAGU-3′; CAMC-3 #1 sense is 5′-CAGGGUGAUAUGCCUAUAGU-3′. The mRNA was injected into zebrafish or Xenopus laevis with a 250 pg dose of mRNA for each embryo.

**Luciferase reporter assay.** Cells (1 × 10^5) were transfected with 0.2 μg of reporter constructs and 4 μg of pRL-TK (Promega) using Lipofectamine 2,000 reagent (Invitrogen). Cells were transfected with MCAM-siRNA or pCS2 TA-3#1 siRNAs, or co-transfected with MCAM-siRNA and HA-tagged MCAM-containing vectors for overexpression of exogenous MCAM. After 24h of transfection, cells were treated with or without FGF10 for 48h. Both firefly and Renilla luciferase activities were measured using a dual-luciferase reporter assay system in a Glomax multi-detection system luminometer (Promega). Firefly luciferase activity was normalized against Renilla luciferase activity, which was detected by co-transfection with pRL-TK in all reporter experiments. The luciferase activity was normalized against luciferase activity of each transfected with the pG3L-E control vector. The relative luciferase activities were expressed as fold increase over the paired control cells. The pG3L-E-NFAT, AP-1, FOXP or NF-κB plasmids were constructed by inserting consensus sequences to the respective transcription factor binding sites, and are indicated as following: pG3L-E-NFAT65, 5′-AggAAA AACG-3′; pCS2 TA-3 AP-1, 5′-AGTATTCACTG-3′; pG3L-E-FOXO4, 5′-GTA AACA-3′; pG3L-E-NFκB69, 5′-gggACCTTCC-3′.

**Zebrafish immuno-staining.** The embryos were collected at desired stages and theirchorions were removed. They were then fixed with 4% paraformaldehyde overnight at 4°C. The fixed embryos were dehydrated with sequentially graded PBST/PBST series (3, 1.1 and 1.3) and 100% methanol. They were stored at ~20°C for more than 30 min. After that, the embryos were rehydrated with sequentially graded methanol/PBST series (3, 1.1 and 1.3) and 100% PBST. The PBST was removed with 1 μl of PBST (pH 8.0) and the embryos were boiled in this EDTA solution for 10 min. They were then cooled slowly to room temperature. The embryos were washed with PBST and PBS-Trition X-100 (0.5%) sequentially. The embryos were blocked with a 3% BSA-PBST solution for at least an hour, and then incubated with anti-MCAM (1:100) overnight at 4°C. After washing them three times with PBST, the embryos were incubated with the fluorescence labelled secondary antibody (1:200) overnight at 4°C. The nuclei were stained with DAPI (1 μg ml⁻¹). After washing three times with PBST, the embryos were mounted with coverslips and photographed with a confocal microscope.

**Statistical analysis.** Statistical analysis was performed in SPSS. Data are shown as mean ± s.e.m. All data sets were tested for normal distribution with normality tests before proceeding with parametric or non-parametric analysis. Unpaired Student’s t-test was applied to data sets with normal distributions. When appropriate in case of multiple comparisons, one-way analysis of variance (ANOVA) with Tukey post-test or two-way ANOVA with Bonferroni post-test was applied. Statistical evaluation of experiments represented by bar graphs was performed using Pearson’s chi-square tests. Where possible to avoid subjective assessments.

**Data availability.** The DNA sequence of Xenopus mcam has been deposited in GeneBank under the accession code KJ913668.1. The authors declare that all the data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request.

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Author contributions

Z.W., A.M. and X.Y. designed the study and interpreted the data and wrote the paper with input from all authors. Q.G. and J.Z. performed all the experiments, with the exception of Xenopus laevis handling performed with X.W., Y.L. and R.H. X.L. conducted immunostaining of zebrafish. Antibody production and purification were performed by F.W., D.Y. and J.F.

Additional information

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