RESEARCH ARTICLE

The PDZ domain protein Mcc is a novel effector of non-canonical Wnt signaling during convergence and extension in zebrafish

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ABSTRACT

During vertebrate gastrulation, a complex set of mass cellular rearrangements shapes the embryonic body plan and appropriately positions the organ primordia. In zebrafish and Xenopus, convergence and extension (CE) movements simultaneously narrow the body axis mediolaterally and elongate it from head to tail. This process is governed by polarized cell behaviors that are coordinated by components of the non-canonical, β-catenin-independent Wnt signaling pathway, including Wnt5b and the transmembrane planar cell polarity (PCP) protein Vangl2. However, the intracellular events downstream of Wnt/PCP signals are not fully understood. Here, we show that zebrafish mutated in colorectal cancer (mcc), which encodes an evolutionarily conserved PDZ domain-containing putative tumor suppressor, is required for Wnt5b/Vangl2 signaling during gastrulation. Knockdown of mcc results in CE phenotypes similar to loss of vangl2 and wnt5b, whereas overexpression of mcc robustly rescues the depletion of wnt5b, vangl2 and the Wnt5b tyrosine kinase receptor ror2. Biochemical experiments establish a direct physical interaction between Mcc and the Vangl2 cytoplasmic tail. Lastly, CE defects in mcc morphants are suppressed by downstream activation of RhoA and JNK. Taken together, our results identify Mcc as a novel intracellular effector of non-canonical Wnt5b/Vangl2/Ror2 signaling during vertebrate gastrulation.

KEY WORDS: Convergence and extension, Non-canonical Wnt signaling, Zebrafish, Mutated in colorectal cancer (Mcc), Actin cytoskeleton

INTRODUCTION

Mutated in colorectal cancer (MCC) was first identified in 1991 as a candidate tumor suppressor gene for the autosomal dominant syndrome familial adenomatous polyposis (FAP) (Ashton-Rickardt et al., 1991; Kinzler et al., 1991b). FAP patients typically present hundreds to thousands of adenomas in the colon and rectum, but only a few of these lesions ever advance to colorectal cancer (CRC) (Rustgi, 2007). Shortly after the identification of MCC, more refined positional cloning efforts revealed that a second, closely linked gene (mcc), which encodes an evolutionarily conserved PDZ domain-containing putative tumor suppressor, is required for Wnt5b/Vangl2 signaling during gastrulation. Knockdown of mcc results in CE phenotypes similar to loss of vangl2 and wnt5b, whereas overexpression of mcc robustly rescues the depletion of wnt5b, vangl2 and the Wnt5b tyrosine kinase receptor ror2. Biochemical experiments establish a direct physical interaction between Mcc and the Vangl2 cytoplasmic tail. Lastly, CE defects in mcc morphants are suppressed by downstream activation of RhoA and JNK. Taken together, our results identify Mcc as a novel intracellular effector of non-canonical Wnt5b/Vangl2/Ror2 signaling during vertebrate gastrulation.

tumors, both the subcellular distribution and expression of Scrib are dysregulated (Zhan et al., 2008). These findings link polarity proteins to cellular transformation and raise the possibility of an alternate mechanism through which MCC exerts its tumor suppressor effect (Ellenbroek et al., 2012; Martin-Belmonte and Perez-Moreno, 2012). In addition, Mcc localizes to the leading edge of polarized cells and interacts in a PDZ-independent manner with the contractile protein myosin IIB (Pangon et al., 2012). Knockdown of MCC impairs lamellipodia formation and cell migration in Boyden chamber assays (Arnaud et al., 2009; Pangon et al., 2010, 2012). Lastly, we recently observed enriched MCC expression in a microarray screen comparing the differentiation of human embryonic stem cells in response to combinations of TGFβ-related growth factors (Teo et al., 2012). High levels of MCC coincided with the expression of cardinal gastrulation marker genes and, in the mouse embryo, Mcc is specifically expressed in the primitive streak – the site of epithelial-to-mesenchymal transformation in the pluripotent epiblast, where nascent mesoderm and definitive endoderm emerge during gastrulation (Young et al., 2011). Collectively, these findings suggest that MCC activity is intimately linked to the actin cytoskeleton and the regulation of cell shape and morphogenesis.

In an attempt to shed additional light on the disparate functions ascribed to MCC to date, we chose to isolate the zebrafish homolog of MCC and to thoroughly evaluate its expression and function. We find that mcc is expressed both maternally and zygotically and is unexpectedly required during gastrulation for convergence and extension (CE) movements that narrow the nascent germ layers dorsoventrally and finely coordinate their elongation from head to tail (Solnica-Krezel and Sepich, 2012; Tada and Heisenberg, 2012). Knockdown of mcc results in classic CE phenotypes, including a foreshortened body axis and reductions in anterior development, which often result from manipulating the non-canonical, β-catenin-independent Wnt signaling pathway. Epistasis experiments confirm that mcc lies downstream of wnt5b, as well as of ror2 and vangl2, which encode the Wnt5b cell surface receptor complex. In a series of gain-of-function experiments we further show that Mcc links extracellular Wnt5b signals to RhoA and JNK, which coordinate remodeling of the cytoskeletal network and morphogenesis. Mcc thus represents a hitherto uncharacterized intracellular regulator of Wnt/planar cell polarity (PCP) signaling in vertebrates.

RESULTS
mcc is required for convergence and extension during zebrafish gastrulation

Using a combination of database searches, phylogenetic alignments and 5′-RACE (see Materials and Methods), we identified a single mcc homolog near the end of zebrafish chromosome 5 (Fig. 1A). mcc encodes a 1012 amino acid protein that shares significant overall identity with its mammalian counterparts, particularly within the PDZ domains (Fig. 1A; supplementary material Table S1). Although two isoforms of Mcc differing only at their extreme N-termini are generated by alternative promoter usage in human and mouse (Young et al., 2011), we were unable to identify an mcc transcript encoding the shorter isoform 2 after exhaustive 5′-RACE and EST database searches. Given the length of the zebrafish Mcc polypeptide, we have therefore provisionally designated it as the longer isoform 1 (supplementary material Table S1).

We next examined mcc expression by Q-PCR and whole-mount in situ hybridization (WISH). mcc expression levels are robust both maternally (2-256 cells) and zygotically, decreasing during segmentation [1-20 somites (s)] but increasing during the larval period (3 days and beyond) (Fig. 1B). Maternal mcc transcripts label...
all blastomeres during cleavage [2 h post fertilization (hpf)] and blastula (3 hpf) stages, and this broad distribution persists in all epiblast and hypoblast cells after the onset of zygotic transcription and initiation of gastrulation (6 hpf) (Fig. 1C). By 18 s, high levels of {mcc} are observed in the developing head structures, central nervous system and somites (Fig. 1C). Sections of larval stage embryos reveal {mcc} expression in endodermally derived organs such as the swim bladder, pancreas, liver and intestine (supplementary material Fig. S1A). We also cloned and analyzed {Xenopus laevis Mcc} and observed maternal and zygotic expression similar to that observed in zebrafish (supplementary material Fig. S1B,C).

We next performed {mcc} loss-of-function studies using two antisense morpholino (MO) oligonucleotides: one targeting the {mcc} ATG (MO1) and the other spanning the splice junction between intron 6 and exon 7 (MO2) (see Fig. 1A). Embryos injected with either MO display varying degrees of microcephaly/microphthalmia, often with a shift in head position and loss of tissue anterior to the eyes – a ‘hammerhead’ phenotype (Piotrowski et al., 1996) – tightly packed somites and an embryonic axis that is shorter and ventrally curved from anterior to posterior compared with wild-type controls at 1 day post fertilization (dpf) (Fig. 2A; supplementary material Fig. S2A). Anterior cell death is also occasionally observed after MO injection,

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Fig. 2. {mcc} acts downstream of {wnt5b} and {vangl2} to regulate convergence and extension. (A) Injection of ATG (MO1) or splice junction (MO2) MOs targeting {mcc} yields zebrafish embryos with a shorter and ventrally curved anteroposterior axis with tightly packed somites at 1 dpf. MO1 and MO2 positions are indicated in Fig. 1A. The black line with asterisk emphasizes the shortened yolk extension, and curved dotted line highlights the smaller eyes and reduced head development in {mcc} morphants compared with wild type (WT). The arrow indicates the region of anterior cell death. These defects are rescued by co-injection of mouse {Mcc} mRNA (see also supplementary material Fig. S2A). (B) Representative {wnt5b}, {gpc4} and {vangl2} morphants at 1 dpf. (C) Triple {ntl}, {hgg1} and {dlx3} WISH at 10 hpf. In wild-type embryos, {hgg1} marks the polster (p) that lies on the anterior neural plate (np) boundary expressing {dlx3}. {ntl} identifies the thin midline notochord (inset). In morphants, the neural plate is wider mediolaterally, the notochord is thickened and shortened, and the polster is abnormally elongated posteriorly. Co-injection of {Mcc} mRNA and either {mcc} MO1 or MO2 yields {hgg1/dlx3/ntl} expression patterns that are indistinguishable from those of wild type (see also supplementary material Fig. S2B). (D) Lateral images of shield stage embryos injected as indicated. EGFP-{Mcc} is exclusively cytoplasmic in both wild-type and {vangl2} morphant embryos. EGFP-{Vangl2} is principally membrane localized in wild-type embryos, but is also visible in cytoplasmic puncta. EGFP-{Vangl2} distribution is unaltered in {mcc} morphants. The N-terminal GFP-{Xenopus laevis Mcc} fusion (supplementary material Table S3) efficiently rescues zebrafish {mcc} morphants (data not shown). (E) Zebrafish {mcc} mRNA rescues loss of either {wnt5b} or {vangl2} but not {gpc4} at 1 dpf. Combining {mcc} mRNA and {wnt5b} MO results in three phenotypic classes, as evidenced by variable yolk (and body axis) extension and head position. (A-E) MO and mRNA concentrations are provided in supplementary material Table S3. (A-C,E) Phenotypic distributions are indicated as percentages, with scored embryo counts listed in supplementary material Table S4.
which is suppressed by co-injection of a p53 MO (Fig. 2A; supplementary material Fig. S2A) (Robu et al., 2007). Importantly, mcc morphants are almost entirely rescued by co-injection of mRNA encoding mouse Mcc (Fig. 2A; supplementary material Fig. S2A). We also performed a series of control injection experiments to confirm that neither an mcc mismatch MO (MO1*) nor irrelevant nls-lacZ mRNA results in phenotypes similar to mcc morphants (supplementary material Fig. S2A).

Because altered dorsoventral (DV) patterning can profoundly disturb gastrulation movements (Langdon and Mullins, 2011), we performed WISH for cardinal dorsal (gsc and chd) and ventral (gata2 and vent) marker genes and observed that their expression patterns are identical in wild-type and mcc morphant embryos at shield stage (6 hpf) (supplementary material Fig. S3B). This result prompted us to consider that mcc morphant phenotypes are more likely to be the result of impaired cell movement and morphogenesis, and we further noted that at 1 dpf mcc morphants are strikingly similar to classic zebrafish mutants with impaired CE. These mutants typically display a broadened and shortened body axis by the end of gastrulation, which can be readily visualized by simultaneously assessing the expression of three marker genes: dlx3 (dlx3b – ZFIN), no tail (ntl; brachyury – ZFIN) and hatching gland 1 (hgg1; cathepsin Lb – ZFIN). dlx3 labels the border of the anterior neural plate, whereas ntl identifies the thin midline notochord and hgg1 the anterior prechordal plate (or polster) (Fig. 2C). We compared the expression of these three markers in mcc morphant embryos with that in null mutants or MO-injected embryos for a number of genes known to play crucial roles in CE. These include wnt5b (ppt), wnt11 (slb) and wnt11-related (wnt11r), which encode non-canonical Wnt ligands, and gpc4 (kny) and vangl2 (tri, stbm), which encode a cell-surface proteoglycan and multi-pass transmembrane protein, respectively (Fig. 2B; supplementary material Fig. S2B) (Gao, 2012; Solnica-Krezel and Sepich, 2012). In wnt5b, gpc4 and vangl2 morphants at 10 hpf, the neural plate is predictably much wider than in the wild type, the notochord is shorter and much thicker, and the polster lags posteriorly (Fig. 2C). Similar defects are also observed in both wnt11 and wnt11r homozygous mutant embryos (supplementary material Fig. S2B). In mcc morphants, dlx3, ntl and hgg1 expression patterns are altered identically to mutants with loss of CE genes and are efficiently restored to the wild-type pattern by co-injection of mouse Mcc mRNA (Fig. 2C; supplementary material Fig. S2B). Lastly, for some CE genes, both gain- and loss-of-function manipulations impair gastrulation movements (Gao et al., 2011; Jessen et al., 2002; Park and Moon, 2002) and, consistent with this, we observed that mcc overexpression also results in CE defects that are superimposable with loss of mcc (supplementary material Fig. S2B). Taken together, these results indicate that mcc is required for normal CE during zebrafish gastrulation.

To provide additional evidence for a crucial role of Mcc in orchestrating CE, we asked whether its function is evolutionarily conserved between Xenopus and zebrafish, as both species employ similar cell behaviors to establish their body plans (Solnica-Krezel and Sepich, 2012; Tada and Heisenberg, 2012). We find that injection of an ATG-targeting MO at the Xenopus two-cell stage results in weakly penetrant gastrulation (stage 11) and neurulation (stage 19) phenotypes (supplementary material Fig. S2C). Gastrulation defects include delayed blastopore closure and, in rare cases, exogastrulation. At neurula stages, embryos unilaterally injected with an ATG-targeting MO display neural tube closure defects with an expanded and flattened neural plate, which suggests failed cellular intercalation, and is a phenotype wholly consistent with the widened dlx3 expression in zebrafish mcc morphants (Fig. 2C; supplementary material Fig. S2C). Mcc overexpression results in embryos with a foreshortened body axis with a characteristic buckling (dorsal flexure) that is strikingly reminiscent of the phenotypes observed after injection of Xenopus Vangl2 (Xstbm) mRNA, which, as in zebrafish, encodes a key regulator of CE in dorsal mesoderm and neural ectoderm (supplementary material Fig. S2D) (Darken et al., 2002; Goto and Keller, 2002).

MCC has previously been reported to antagonize canonical Wnt signaling in CRC cell lines (Fukuyama et al., 2008). We evaluated whether Mcc can inhibit β-catenin-dependent activation of the –0.8 kb Xenopus Siamois luciferase reporter, and found that Mcc overexpression has no effect on reporter activation – neither synergy nor inhibition – upon co-injection of mRNA encoding either the canonical Wnt8 ligand, which normally suppresses dorsal organizer genes and patterns ventrolateral mesoderm, or its effector β-catenin (Baker et al., 2010) (supplementary material Fig. S2E). We further confirmed by WISH that Brachyury (pan-mesodermal), Gsc (dorsal organizer) and Hex (nascent endoderm) are expressed normally at stage 10.5 in both morphant and Mcc-overexpressing Xenopus embryos (supplementary material Fig. S2F). Taken together, these data support a model whereby depletion of Mcc in Xenopus does not impair the early establishment of DV pattern but disrupts gastrulation movements independently of the canonical Wnt signaling pathway.

mcc is downstream of wnt5b, ror2 and vangl2

Immunolocalization studies in a variety of cell types show that MCC is principally a cytoplasmic protein (Fukuyama et al., 2008; Matsumine et al., 1996; Pangon et al., 2010; Senda et al., 1999). We determined the subcellular distribution of Mcc in the gastrulating zebrafish embryo by injecting mRNA encoding an N-terminal GFP-Mcc fusion protein and imaging GFP fluorescence at shield stage. GFP-Mcc was uniquely membrane localized with conspicuous cytosolic puncta (Fig. 2D). These findings qualify Mcc as a potential intracellular effector of the Vangl2 transmembrane protein. To test this hypothesis, MOs targeting vangl2 as well as wnt5b and gpc4 were co-injected with zebrafish mcc mRNA and phenotypic classes scored. At 1 dpf, overexpression of mcc efficiently complements loss of either wnt5b or vangl2, as 70% of the embryos showed wild-type-like development (Fig. 2E). By contrast, mcc mRNA fails to rescue loss of wnt11 or gpc4, with the latter giving rise to two phenotypic classes that are likely to represent the variability in targeting gpc4 with MOs (Fig. 2E; supplementary material Fig. S2G). In addition, no alteration in the membrane localization of GFP-Vangl2 was observed in mcc morphants (Fig. 2D), and GFP-Mcc was consistently cytoplasmic upon Vangl2 depletion (Fig. 2D). Based on these data, we propose that Mcc acts downstream of Wnt5b and Vangl2 during zebrafish CE.

In mice, Wnt5a, Vangl2 and Ror2 exhibit extensive genetic interactions and their individual null mutant phenotypes show significant similarities (Gao et al., 2011; Ho et al., 2012). Moreover, mature mouse Wnt5a, which is ~85% identical to zebrafish Wnt5b, has been shown to bind the cysteine-rich extracellular domain of the Ror2 receptor tyrosine kinase, which induces Ror2-Vangl2 complex formation and phosphorylation of the Vangl2 N-terminus (Gao et al., 2011; Oishi et al., 2003). To determine if Ror2 function is required for CE in zebrafish, we cloned zebrafish ror2, examined its expression during development and performed knockdown studies with two splice junction MOs (Fig. 3A). Like mcc, ror2 is expressed maternally and zygotically, with transcripts labeling all blastomeres during cleavage and blastula stages and becoming ubiquitous during gastrulation (Fig. 3B,C). Knockdown of ror2 results in CE
Fig. 3. ror2 is required for convergence and extension in zebrafish and acts upstream of mcc. (A) Schematic of the ror2 locus, with the position of splice junction MOs (MO1 and MO2) indicated. (B) Q-PCR analysis of ror2 expression in developing zebrafish. Maternal ror2 transcripts are detected from 2-256 cells, with dynamic expression over the next 3 days. Data are normalized to β-actin. Error bars indicate s.e.m. epi, epiboly; s, somite. (C) ror2 WISH at the indicated stages. (D) Injection of ror2 MO1 results in CE defects at 10 hpf, as assessed by ntl, dlx3 and hgg1 triple in situ hybridization; these defects are robustly rescued at 10 hpf and 1 dpf by mouse Ror2 mRNA. The solid black line with asterisk emphasizes the shortened yolk extension at 1 dpf, and the curved dotted line highlights the smaller eyes and head in ror2 morphants compared with wild type. np, neural plate; p, polster; n, notochord. (E) Co-injection of ror2 MO1 and zebrafish mcc mRNA results in ostensibly normal embryos at 1 dpf. (F,H) Membrane GFP-labeled notochord (F) and dorsal ectoderm (H) cells of wild type and mcc, ror2 or vangl2 morphants as indicated at 90-95% epiboly (9.5-10 hpf). Dorsal view with anterior upwards. The notochord boundary in F is marked by vertical dashed yellow lines. Note the extra columns of cells in the morphant notochords. Dorsal ectoderm cells selected for morphometric analysis are outlined in H in white. The number of cells analyzed (n) and length-to-width (LWR) ratios (expressed as mean±s.e.m.) are indicated in the Rose diagrams, which depict cell orientation relative to the embryonic anterior-posterior axis (vertical dashed line). (G,I) The percentage of mediolaterally aligned cells with a longitudinal axis oriented ±20° with respect to the embroyonic mediolateral axis (horizontal dashed line in the Rose diagrams). MO and mRNA concentrations are provided in supplementary material Table S3, with scored embryo counts listed in supplementary material Table S4.
defects similar to loss of \textit{wnt5b}, \textit{vangl2} or \textit{mcc} at both 10 hpf and 1 dpf, with no impact on DV marker gene expression at shield stage (Fig. 3D; supplementary material Fig. S3A,B). Co-injection of mouse \textit{Ror2} mRNA with either \textit{ror2} MO1 or MO2 results in efficient rescue, with >80% of the embryos displaying a wild-type-like phenotype (Fig. 3D; supplementary material Fig. S3A). Consistent with a role for \textit{ror2} in CE, overexpression yields phenotypes largely overlapping with that due to loss of \textit{ror2} (supplementary material Fig. S3A). Lastly, we established that \textit{mcc} lies downstream of \textit{ror2} by co-injecting \textit{ror2} MO1 or MO2 with zebrafish \textit{mcc} mRNA. Nearly 90% of the embryos show rescue (Fig. 3E; supplementary material Fig. S3A).

During CE, cells elongate, become oriented mediolaterally and intercalate, which results in axial elongation (Solnica-Krezel and Sepich, 2012; Tada and Heisenberg, 2012). We reasoned that if \textit{mcc} indeed operates downstream of \textit{Ror2} then \textit{mcc} and \textit{ror2} morphants should display similar defects in CE cell behaviors. Using \textit{in vivo} confocal microscopy we analyzed the cell shape and elongation of notochord and dorsal ectoderm cells in \textit{Tg(β-actin:mgfp)} uninjected control and \textit{ror2} MO- and \textit{mcc} MO-injected embryos (Cooper et al., 2005). The regulation of mediolateral cell polarity by \textit{vangl2} has been reported previously, and we thus included \textit{vangl2} morphants as a reference for our comparative morphometric analyses (Jessen et al., 2002). Consistent with the widened and shortened notochords revealed by \textit{ntl} WISH at 10 hpf (Fig. 2C, Fig. 3D; supplementary material Fig. S2B, Fig. S3A), \textit{mcc}, \textit{vangl2} and \textit{ror2} morphants have extra columns of cells within their notochords – a phenotype that is indicative of defective mediolateral intercalation (Fig. 3F). In the notochord of uninjected \textit{Tg(β-actin:mgfp)} embryos, cells are elongated with a strong mediolateral bias: 72% of cells are oriented mediolaterally along their long axis within ±20° are perpendicular to the embryonic midline and with an average length-to-width ratio (LWR) of 1.96±0.49 (n=78) (Fig. 3F,G). By contrast, \textit{mcc} and \textit{vangl2} morphant notochord cells are significantly less elongated [LWR=1.59±0.32 (n=111; P<0.001) and LWR=1.54±0.31 (n=146; P<0.001), respectively] and exhibit less mediolateral bias in their orientation (42% and 45%, respectively) (Fig. 3F,G). Similarly, dorsal ectoderm cells are less elongated in \textit{mcc} and \textit{vangl2} morphants [LWR=1.47±0.26 (n=155; P<0.001) and LWR=1.50±0.27 (n=165; P<0.001), respectively] compared with control embryos [LWR=1.73±0.38 (n=118)] (Fig. 3H). In wild-type dorsal ectoderm, 64% of cells are oriented mediolaterally, in contrast to 43% and 37% in \textit{mcc} and \textit{vangl2} morphants, respectively (Fig. 3I). Interestingly, the percentage of mediolaterally aligned notochord cells [60% with LWR=1.59±0.32 (n=103; P<0.001)] and dorsal ectoderm cells [52% with LWR=1.56±0.26 (n=105; P<0.05)] in \textit{ror2} morphants is intermediate between the control and \textit{mcc} and \textit{vangl2} morphant embryos (Fig. 3F-I). This finding is consistent with the slightly less severe \textit{ror2} morphant phenotype at 1 dpf (Fig. 3D; supplementary material Fig. S3A) when compared with both \textit{mcc} and \textit{vangl2} morphants (Fig. 2A,B; supplementary material Fig. S2A), and potentially reflects compensation by \textit{ror1}, which is expressed during CE (our unpublished observations); \textit{Ror1} and \textit{Ror2} are known to display functional redundancy in other systems (Ho et al., 2012; Nomi et al., 2001). Taken together, the similar phenotypic defects in mediolateral cell polarity among \textit{mcc}, \textit{ror2} and \textit{vangl2} morphants provides additional compelling evidence that \textit{Mcc} operates downstream of the \textit{Wnt5b/Ror2/Vangl2} signaling axis during zebrafish CE.

**Mcc binds the Vangl2 cytoplasmic tail**

The Vangl2 C-terminal tail terminates with the type I PDZ interaction motif ETSV, which has previously been shown to mediate its direct interaction with the PDZ protein Scrib (Belotti et al., 2013; Kalyoncu et al., 2010; Montcouquiol et al., 2006). In addition, the Vangl2 cytoplasmic tail contains a second, non-canonical PDZ interaction motif that involves (at least) serine 464, which is mutated to asparagine (S464N) in the classic Loop-tail mouse (Kibar et al., 2001). This unconventional motif facilitates the interaction between Vangl2 and the Dishevelled (Dvl) family of PDZ domain-containing proteins (Belotti et al., 2013; Torban et al., 2004). We therefore investigated whether epitope-tagged versions of Vangl2 and Mcc directly interact using pull-down assays in HEK293 cells. Full-length HA-Vangl2 indeed efficiently precipitates FLAG-Mcc (Fig. 4A). Deleting either four (A4) or 12 (A12) C-terminal residues of Vangl2 does not impact its ability to pull down FLAG-Mcc, whereas removal of the Vangl2 cytoplasmic tail (A243-521, indicated as ΔC) entirely eliminates interaction with Mcc (Fig. 4A). We also performed a parallel series of control immunoprecipitations and confirmed that Vangl2Δ4 cannot bind endogenous human SCRIB in HEK293 cells (supplementary material Fig. S4A) (Belotti et al., 2013; Montcouquiol et al., 2006). This result supports a direct interaction between Vangl2 and Mcc in HEK293 cells, since HA-Vangl2Δ4 and Vangl2Δ12 consistently pull down FLAG-Mcc equally as well as wild-type Vangl2. In addition, Vangl2Δ4 and Vangl2Δ12 efficiently bind \textit{Xenopus} ΔDIX-Dvl, which is an N-terminal Dvl truncation that retains its single, central PDZ domain (supplementary material Fig. S4B) (Park and Moon, 2002). Taken together, these data argue that Mcc binds the non-canonical PDZ interaction motif within the Vangl2 cytoplasmic tail in a manner similar to Dvl. We however cannot formally exclude the possibility that another, unidentified PDZ domain-containing protein in HEK293 cells bridges Mcc to the non-canonical PDZ interaction motif within the Vangl2 cytoplasmic tail.

We next evaluated the contribution of the individual PDZ domains within Mcc to its interaction with Vangl2 using a series of Mcc deletion constructs (Fig. 4B). In HEK293 cells, HA-Vangl2 efficiently precipitates truncated versions of Mcc lacking either PDZ1 or both PDZ1 and PDZ2, but poorly pulls down a deletion construct retaining PDZ1 and PDZ2 but missing Mcc ΔC-terminal residues of Vangl2 does not impact its ability to pull down FLAG-Mcc, whereas removal of the Vangl2 cytoplasmic tail (A243-521, indicated as ΔC) entirely eliminates interaction with Mcc (Fig. 4A). We also performed a parallel series of control immunoprecipitations and confirmed that Vangl2Δ4 cannot bind endogenous human SCRIB in HEK293 cells (supplementary material Fig. S4A) (Belotti et al., 2013; Montcouquiol et al., 2006). This result supports a direct interaction between Vangl2 and Mcc in HEK293 cells, since HA-Vangl2Δ4 and Vangl2Δ12 consistently pull down FLAG-Mcc equally as well as wild-type Vangl2. In addition, Vangl2Δ4 and Vangl2Δ12 efficiently bind \textit{Xenopus} ΔDIX-Dvl, which is an N-terminal Dvl truncation that retains its single, central PDZ domain (supplementary material Fig. S4B) (Park and Moon, 2002). Taken together, these data argue that Mcc binds the non-canonical PDZ interaction motif within the Vangl2 cytoplasmic tail in a manner similar to Dvl. We however cannot formally exclude the possibility that another, unidentified PDZ domain-containing protein in HEK293 cells bridges Mcc to the non-canonical PDZ interaction motif within the Vangl2 cytoplasmic tail.

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We also performed complementation experiments by co-injecting mRNA encoding a series of mouse Mcc truncations (Fig. 4B) and mcc MO1 and assaying for rescue of CE defects at 1 dpf. Eliminating just one of the three Mcc PDZ domains (PDZΔ1) fails to complement knockdown of \textit{mcc}, as morphants show characteristic CE phenotypes (Fig. 4E). These findings reveal that the interaction between Vangl2 and Mcc \textit{in vivo} more stringently depends upon the presence of (at least) PDZ1 than has been revealed by simple overexpression studies in HEK293 cells. Moreover, deleting the Mcc C-terminus (MccΔC) also fails to rescue loss of \textit{mcc} (Fig. 4D). This result implicates the existence of...
PDZ domain-containing protein(s) that bind Mcc and are required for its downstream function, and one leading candidate is Scrib.

In zebrafish, *scrib* and *mcc* expression patterns largely overlap, and *in vitro* Scrib and Mcc physically interact, colocalize and display promigratory activities in Boyden chamber assays (Arnaud et al., 2009; Belotti et al., 2013; Wada et al., 2005). However, overexpression of mouse *Scrib* can neither rescue depletion of *mcc* nor loss of *wnt5b* and *ror2* (supplementary material Fig. S5A), which suggests that Scrib is not a central effector of Wnt5b/Ror2 signals during zebrafish CE. Conversely, co-injection of *mcc* mRNA and *scrib* MO results in embryos with minor CE defects that strongly resemble those of the *landlocked* (*llk*) zebrafish mutant, which involves the *scrib* gene, or of *scrib* morphants (Li et al., 2013; Wada et al., 2005) (supplementary material Fig. S5B). We did
however observe an unexpected genetic interaction between  
and  after co-injection of MOs targeting both genes at doses 
that do not ordinarily result in CE defects. Double  
 morphants show a highly penetrant (73%) and severe CE phenotype—a result that is strikingly similar to the strong genetic interaction 
between  and  that results from the simultaneous 
knockdown of  and overexpression of  mRNA in fish (supplementary material Fig. S5C) (Wada et al., 2005). Based on 
these findings, we favor a model whereby  and  act in parallel pathways that govern similar intracellular 
processes during fish (Montcouquiol et al., 2003; Murdoch et al., 2003).

Lastly, serine phosphorylation of MCC has been observed at 
residues 115, 118 and 120 and also at amino acid 827 (position −1) 
within the C-terminal ETSL PDZ interaction domain (Pangon et al., 2010, 2012). We generated several versions of mouse MCC isoform 2 
in which these serine residues were mutated to non-phosphorylatable 
alanine to address the requirement of phosphorylation for MCC function (supplementary material Fig. S4C). Phosphomutant mRNAs 
were then co-injected with  and rescue of CE defects assayed at 1 dpf. Mutation of residues 115, 118 and 120 resulted in 
robust rescue (83%), roughly equivalent to the levels achieved with wild-type MCC mRNA (Fig. 2A; supplementary material Fig. S2A, 
Fig. S4C). By contrast, introduction of the S827A mutation alone or 
in combination with  and  failed to complement loss of  
(supplementary material Fig. S4C). This finding aligns well

with our rescue results after deletion of MCC C-terminal residues 
including ETSL (MccΔ in Fig. 4B) and with published reports that 
emphasize the importance of S827 for robust interaction with Scrib in vitro (Arnaud et al., 2009). Taken together, these data argue that 
MCC is a direct intracellular effector of Wnt5b/Ror2/Vangl2 signaling 
and works to coordinate CE intracellularly through its C-terminal 
 
Mcc is upstream of RhoA and JNK

Biochemical and genetic evidence has shown that Wnt5b/Vangl2/Ror2 engages the non-canonical, β-catenin-independent intracellular 
effectors Rho and c-Jun N-terminal kinase (JNK) (Angers and Moon, 
2009; Gao, 2012; Niehrs, 2012). We examined whether Rho and/or 
JNK act downstream of MCC during CE. We co-injected mRNAs 
encoding either an activated form of the Rho family GTPase RhoA* 
(RhoA-G14V) or JNK* (MKK7B2Jnk1a1) and  and scored 
for CE defects at 1 dpf (Lei et al., 2002). Both activated RhoA* 
and JNK* resulted in significant rescue (76-85%) (Fig. 4F). Similar results 
were observed when  or  mRNA was co-injected with 
either  or  MO (supplementary material Fig. S4D,E). In addition, Wnt5a/Ror2 has been shown to directly regulate 
transcription of  
, which encodes a transmembrane cell adhesion molecule involved in CE, via the JNK signaling cascade 
(Medina et al., 2004; Schambony and Wedlich, 2007; Unterseher 
et al., 2004). Consistent with this, we observed weak  
 expression in the widened and shortened posterior adaxial and paraxial mesoderm of both  and  morphants when compared with wild-type embryos (supplementary material Fig. S4F).

We also individually injected a truncated version of Ca²⁺/ 
calmodulin-dependent kinase II (CAMKII) that activates the non-
canonical Wnt-Ca²⁺ pathway or two versions of  
denoted ΔDEP and ΔDIX, that selectively transduce canonical 
and non-canonical Wnt signals, respectively (Tada and Smith, 
2000; Westfall et al., 2003). Neither Wnt-Ca²⁺ activation nor 
overexpression of ΔDEP or ΔDIX was able to restore normal CE in

DISCUSSION

Non-canonical Wnt signaling is a crucial regulator of the collective 
regional cell migrations and cell intercalations that underlie CE. Prior 
Studies on Wnt5b and Vangl2 along with our present analysis of 
Ror2 establish an essential role for the Wnt5b/Ror2/Vangl2 
signaling module in coordinating CE in (Gao, 2012). How these signals are interpreted intracellularly and communicated to the actin cytoskeleton to drive morphogenesis is as yet poorly 
described. We find that the PDZ domain-containing protein 
MCC physically associates with the Vangl2 C-terminus via an 
unconventional PDZ interaction motif and serves as an obligate 
intermediate between extracellular Wnt5b and its downstream 
targets RhoA and JNK. This pathway, which we present as a simple ‘linear’ model in Fig. 5, is supported not only by our present 
findings but also from extensive genetic studies of Wnt5a, Vangl2 
and Ror2 in mice (Gao et al., 2011; Ho et al., 2012). Downstream of 
MCC, it remains unclear whether JNK and RhoA act in the same or

Fig. 5. Linear model of MCC function in Wnt/PCP signaling. Wnt5b binds 
Ror2 and triggers complex formation between Ror2 and Vangl2 and 
phosphorylation of the Vangl2 N-terminus, possibly by Casein kinase I (CK1α) 
(adapted from Gao et al., 2011). MCC associates with the Vangl2 C-terminus 
via an unconventional PDZ interaction motif N-terminal to the extreme type I 
PDZ motif ETSV, and relays receptor activation through an unknown PDZ 
protein (X) to RhoA and JNK, which then coordinate rearrangement of the actin 
cytoskeleton.
parallel pathways or cross-regulate one another (dashed arrows in Fig. 5). Nevertheless, vangl2, ror2 and mcc morphants are efficiently rescued by the activation of either pathway (Fig. 4C).

In Xenopus, evidence does however exist for an alternative model whereby Wnt5b/Ror2 activates JNK in a manner apparently independent of Vangl2 to directly regulate the expression of the cell adhesion gene Pape (Schambony and Wedlich, 2007). Consistent with this, we indeed observe reduced pape levels in the posterior paraxial mesoderm of ror2 morphant zebrafish embryos (supplementary material Fig. S4F) and robust rescue of their CE phenotypes at 1 dpf with constitutively activated JNK* mRNA (supplementary material Fig. S4E). We also observe pape downregulation in mcc morphants, rescue of ror2 morphants with mcc mRNA and rescue of mcc morphants with JNK* mRNA (Fig. 3E, Fig. 4F; supplementary material Fig. S4F). These findings support a direct model whereby the cytoplasmic pool of Mcc, through an unknown mechanism, bridges Wnt5b/Ror2 receptor activation to JNK and the transcriptional regulation of pape. In addition, Pape itself has been shown to stimulate RhoA, which is sufficient to activate JNK (Unterseher et al., 2004). This complex regulation of JNK, both as an upstream regulator of pape transcription and a downstream effector of its function via RhoA (Schambony and Wedlich, 2007; Unterseher et al., 2004), potentially provides an explanation for our observation that constitutively activated RhoA* mRNA also rescues ror2 morphants (supplementary material Fig. S4E). Although we did not examine pape expression in vangl2 morphants, downregulation of posterior pape similar to that observed in ror2 and mcc morphants was previously reported by Henry et al. (2000) in 10-somite stage zebrafish vangl2 mutants. We also show that vangl2 morphants are moderately rescued (72%) by JNK* mRNA injection, with a phenotype that is obviously less wild-type-like (e.g. shorter yolk and tail extension, intermediate anterior development) than that of mcc morphants injected with JNK* mRNA (Fig. 4F; supplementary material Fig. S4D). Taken together, these findings raise the possibility that the transcriptional regulation of (at least) the Wnt5b target pape is governed by both Vangl2-dependent, as illustrated by our linear model (Fig. 5), and Vangl2-independent inputs.

The type 1 PDZ interaction domain of Mcc is required for its function, as constructs missing the extreme Mcc C-terminus (MccΔC) or harboring the S827A mutation fail to rescue mcc morphants (Fig. 4E; supplementary material Fig. S4C). This finding predicts the existence of a second PDZ protein (depicted as X in the model in Fig. 5) that binds to and acts downstream of Mcc. One such candidate is Arhgef11 (also known as PDZ-RhoGEF), a PDZ domain-containing guanine exchange factor for RhoA whose downstream target is Rho-dependent kinase (ROCK) (Panizzi et al., 2007). Physical association between Mcc and Arhgef11 might provide a platform for ROCK-dependent myosin II activation and remodeling of the cytoskeleton. Support for such a mechanism comes from the colocalization of MCC and myosin II in human mammary epithelial cells (Arnaud et al., 2009). In addition, zebrafish arhgef11 and mcc expression overlap during gastrulation and arhgef11 morphants display a ventral flexure similar to, but an overall phenotype generally less severe than, other Wnt/PCP morphants, probably owing to compensation by the highly related arhgef12 (Panizzi et al., 2007). Thus, it will be interesting to establish formally whether Arhgef11 and Mcc physically interact during zebrafish CE.

A second candidate PDZ protein downstream of Mcc is membrane-associated guanylate kinase (Magi3). Magi3 contains six PDZ domains and forms a ternary complex with Vangl2 and the Wnt receptor Frizzled 4 (Fzd4) in vitro that strongly activates JNK (Yao et al., 2004). (The Fzd4 C-terminus also contains a type I PDZ interaction motif.) This raises the possibility that, in the context of the gastrulating embryo, Mcc links Ror2/Vangl2 directly to Fzd. In zebrafish, knockdown of fzd7a/b results in CE defects largely overlapping with those caused by loss of wnt5b, vangl2, ror2 and mcc (Quesada-Hernandez et al., 2010). Despite this phenotypic similarity, Vangl2 and Fzd7 have recently been shown to differentially regulate the dynamic changes in the extracellular matrix that accompany zebrafish gastrulation: Vangl2 controls fibronectin assembly in a Prickle1a-dependent manner, whereas Fzd7 and its co-receptor Gpc4 antagonize fibronectin fibrillogenesis through cadherin-mediated cell adhesion (Dohn et al., 2013). Although it is formally possible that Fzd7 serves as a Vangl2/Ror2 co-receptor for Wnt5b signals, it remains to be determined whether Mcc or candidate Mcc-binding proteins such as Magi3 function downstream of Fzd7. Moreover, it is important to note our result that mcc overexpression fails to rescue gpc4 morphants (Fig. 2E), which weakens the case for a role for Mcc downstream of Fzd7/Gpc4.

Lastly, our data show that, at least in the context of zebrafish CE, Scrib is highly unlikely to be a component of the Mcc interactome. When scrib mRNA was co-injected with wnt5b, ror2 or mcc MOs, we observed no rescue of CE defects (supplementary material Fig. S5A). In addition, the phenotype of mcc morphants is much more severe than either maternal zygotic lik mutants or scrib ATG morphants (Li et al., 2013; Wada et al., 2005). Finally, overexpression of mcc neither exacerbates nor rescues the weak scrib morphant phenotype (supplementary material Fig. S5B). If, however, low doses of both mcc and scrib MOs, which ordinarily produce no phenotype on their own, are co-injected, there is a strong and highly penetrant (73%) genetic interaction (supplementary material Fig. S5C). Considering these results together, we interpret the failure to rescue and this strong genetic interaction to indicate that Vangl2/Mcc and Vangl2/Scrib act in parallel pathways to impact similar cellular processes during CE.

We previously reported that Mcc null mice are viable and fertile (Young et al., 2011). This dramatic difference between the zebrafish knockdown and mouse loss-of-function phenotypes most likely originates from species-specific differences in early Mcc/mcc expression and/or in the unique cell movements that each organism deploys during gastrulation and formation of the body axis (Solnica-Krezel and Sepich, 2012). It is also formally possible that a redundant gene encoding an uncharacterized PDZ protein compensates for Mcc loss in mice. Extensive genetic interactions have been demonstrated among Wnt5a, Ror2 and Vangl2 null alleles during diverse mammalian CE process, such as neural tube closure, inner ear hair cell polarity and limb elongation (Gao, 2012). It will be interesting to determine the phenotypic consequences of introducing the Mcc null allele into these sensitized genetic backgrounds. Lastly, Mcc is expressed in the crypts of the adult mouse intestine and recent evidence both in mouse and human supports a tumor suppressor role for Mcc in CRC (Kohonen-Corish et al., 2007; Starr et al., 2009). Our findings in zebrafish therefore suggest that disrupted or attenuated non-canonical Wnt signaling also plays an important role in the pathogenesis of cancer (McDonald and Silver, 2009).

MATERIALS AND METHODS

Isolation of zebrafish and Xenopus Mcc homologs

The Ensembl Zv9 mce entry ENSDARG00000087450 lacks an ATG-containing exon. 5'-RACE was thus performed on RNA extracted at 24 hpf using the FirstChoice RLM-RACE kit (Ambion). The ATG-containing exon 1 corresponds to a 970 bp 5'-RACE product and encodes the

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N-terminal 242 amino acids (purple in Fig. 1A schematic). The GenBank accession number for zebrafish mcc isoform 1 is KC904239. Primers are listed in supplementary material Table S2.

Full-length Xenopus laevis Mcc was assembled by PCR using EST sequences from IMAGE clones 8319627 and 3378448 and 5′ and 3′ sequence from BJ065474 (Xenbase Clone ID XL083806). ESTs were aligned to Xenopus tropicalis Mcc (NM_001142123). Given that X. laevis and X. tropicalis Mcc encode 847 and 846 amino acid proteins, respectively, which are roughly the size of human MCC and mouse Mcc isoform 2, we have provisionally designated the frog versions as isoform 2 (supplementary Table S1). The full-length X. laevis Mcc isoform 2 was deposited in GenBank (accession KC454032). Primers are listed in supplementary material Table S2.

Embryo injection and culture
Zebrafish and Xenopus embryos and adults were produced, grown and maintained according to protocols approved by the Biological Resource Centre (BRC) Institutional Animal Care and Use Committee (IACUC) at the Biopolis, Singapore (IACUC protocols #130817, #120708 and #120709). Zebrafish embryos were produced by natural mating, staged according to morphology and injected at the 1-cell stage (Westerfield, 2007). wnt11 (sib) and wnt1r1 mutant zebrafish were obtained from the Zebrafish International Research Center (ZIRC). Fertilization, culture and microinjection of Xenopus embryos were performed as previously described (Harland, 1991). All Xenopus injections were performed at the 2-cell stage, and both blastomers were injected (10 nl per cell). Sense-capped mRNA was synthesized using the mMessage mlMachine system (Ambion). MO and mRNA concentrations as well as details of pCS2+ expression vectors are listed in supplementary material Table S3.

Whole-mount in situ hybridization (WISH) and quantitative PCR (Q-PCR)
WISH was performed as described (Harland, 1991; Thisse and Thisse, 2008). CDNA fragments for zebrafish ntl, dlx3, hsg1, mcc, gsc, ash, gata2, vent, pape and rotor or Xenopus Mcc, Brachyury, Gsc, Twist and Hex were used as templates for antisense riboprobe synthesis (supplementary material Table S2). For Q-PCR, total RNA from various embryonic stages was extracted using Trizol (Invitrogen), treated with DNase I (Qiagen) and reverse transcribed with Multiscribe RT (Applied Biosystems). cDNA corresponding to 25 ng of the initial total RNA input was used for Q-PCR (Applied Biosystems 7500 Fast-Real-Time System). Data were normalized corresponding to 25 ng of the initial total RNA input was used for Q-PCR reverse transcribed with Multiscribe RT (Applied Biosystems). cDNA used as templates for antisense riboprobe synthesis (supplementary material Table S1). The full-length Xenopus Mcc isoform 2 (supplementary Figure 1) was cloned into the pCS2+ expression vector (Ambion) and sequenced. Primers are listed in supplementary material Table S2.

Luciferase reporter assay
Two-cell stage Xenopus embryos were injected with 50 pg Siamois (S01234) and 10 pg Renilla (Promega) luciferase reporter DNAs in combination with 200 pg Wnt8 or β-catenin and 750 pg Mcc mRNAs (Brannon et al., 1997). RNA concentrations were equalized to 950 pg total RNA by co-injecting lacZ mRNA. For luciferase assays, six samples containing lysates from six embryos, lysed at stage 11, were used for each measurement with the Dual-Luciferase Reporter Assay Kit (Promega).

Western blot and immunoprecipitation
HEK293 cells were transfected with various expression constructs using Lipofectamine 2000 (Life Technologies) (supplementary material Table S3), cultured for 24 h and lysed in 50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40 with protease inhibitors (Calbiochem). Lysates were incubated with antibodies overnight at 4°C followed by a 3 h incubation with Protein A Dynabeads (Life Technologies) at 4°C. Immunoprecipitations were washed four times in lysis buffer and subjected to standard western analysis. Antibodies used in this study were rabbit anti-HA (Cell Signaling 3724 at 1:12,000), mouse anti-FLAG M2 (Sigma F1804 at 1:1000), rabbit anti-Myc (Abcam ab9106 at 1:150,000) and mouse anti-Scrib (Santa Cruz sc-55543 at 1:250).

Confocal imaging and cell polarity analyses
GFP-Mcc mRNA was injected into one-cell stage zebrafish embryos, whereas GFP-Vangl2 was injected at the 2- to 4-cell stage to ensure mosaic GFP expression and clearly visible cell borders. Fluorescent images were taken on a Zeiss LSM 510 META inverted system at 40× under oil immersion (numerical aperture, 1.35) at 6 hpf/shield stage. Live Tg(β-actin:mgfp) wild-type or MO-injected embryos were mounted at 9.5 hpf in 0.7% low-melting agarose in zebrafish E3 embryo medium and imaged with an Olympus FV1000 inverted confocal microscope with total internal reflection fluorescence at 40× magnification (Cooper et al., 2005). Measurements and analyses of LWRs and mediolateral alignment were carried out as previously described (Jessen, 2012; Weiser and Kimelman, 2012). LWRs were calculated at the position of greatest cell diameter. Image analysis was performed in ImageJ with Fit Ellipse. Rose diagrams plotting cellular orientation were drawn using Rose.NET (Todd A. Thompson).

Processing of embryos for photography and histology
Zebrafish embryos were processed for wisc WISH, post-fixed in 4% paraformaldehyde, paraffin embedded according to standard protocols, sectioned at 9 µm and photographed. Stage 25 and stage 35 Xenopus embryos were processed for WISH, cleared in a solution of benzyl benzoate and benzy alcohol (2:1) and photographed. Stage 18 and stage 35 Xenopus embryos were embedded in a mixture of gelatin and albumin in PBS with 25% glutaraldehyde, vibratome sectioned at 25 µm (Leica VT1000S) and photographed.

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Competition interests
The authors declare no competing financial interests.

Author contributions
T.Y. performed and interpreted most zebrafish experiments, including microinjection of mRNA and MOs, phenotypic scoring of resulting embryos, WISH and paraffin embedding and sectioning; Y.P. performed subcloning, sequence analysis and Q-PCR, designed and tested MOs, and contributed to the analysis of manipulated zebrafish embryos; E.K.T., A.S. and M.L. performed subcloning, built assorted constructs and carried out all transfection and western experiments; S.T. and S.O. performed all confocal imaging and morphometric analyses of manipulated zebrafish embryos; P.W., J.S.-L., C.Y.L., W.S.T. and Y.A. carried out all studies described in the supplementary data; A.B. and K.S. served as advisors and collaborators throughout the duration of the project; and N.R.D. conceived the project, interpreted experiments and wrote the manuscript with input from all authors.

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Supplementary material
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