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2 TITLE: CARMIL3 is important for cell migration and morphogenesis during early development
3 in zebrafish

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5 SHORT TITLE: CARMIL3 and early development

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26 Abstract

27 Cell migration is important during early animal embryogenesis. Cell migration and cell
 28 shape are controlled by actin assembly and dynamics, which depend on capping proteins,
 29 including the barbed-end heterodimeric actin capping protein (CP). CP activity can be
 30 regulated by capping-protein-interacting (CPI) motif proteins, including CARMIL (capping
 31 protein Arp2/3 myosin-I linker) family proteins. Previous studies of CARMIL3, one of the three
 32 highly conserved CARMIL genes in vertebrates, have largely been limited to cells in culture.
 33 Towards understanding CARMIL function during embryogenesis *in vivo*, we analyzed
 34 zebrafish lines carrying mutations of *carmil3*. Maternal-zygotic mutants show impaired
 35 endodermal migration during gastrulation, along with defects in dorsal forerunner cell (DFC)
 36 cluster formation, affecting the morphogenesis of Kupffer's vesicle (KV). Mutant KVs are
 37 smaller and display decreased numbers of cilia, leading to defects in left/right (L/R) patterning
 38 with variable penetrance and expressivity. The penetrance and expressivity of the KV
 39 phenotype in *carmil3* mutants correlated well with the L/R heart positioning defect at the end
 40 of embryogenesis. This first *in vivo* animal study of CARMIL3 reveals its new role for
 41 CARMIL3 during morphogenesis of the vertebrate embryo. This role involves migration of
 42 endodermal cells and DFCs, along with subsequent morphogenesis of the KV and L/R
 43 asymmetry.

45 Introduction

46 *CARMIL Regulation of Actin Assembly via Capping Protein*

47 CARMILs are one family of capping-protein-interacting (CPI-motif) proteins, reviewed in
 48 (Edwards et al., 2014). Vertebrates, including zebrafish, have three conserved CARMIL-
 49 encoding genes, called *CARMIL1*, *CARMIL2* and *CARMIL3* in humans (Stark et al., 2017;
 50 Stark and Cooper, 2015). The zebrafish genes, *carmil1*, *carmil2* and *carmil3*, have distinct
 51 spatial and temporal expression patterns during development (Stark and Cooper, 2015). In
 52 human cultured cells, available evidence indicates that the gene products have distinct
 53 subcellular locations and functions, even within one cell type (Lanier et al., 2015; Liang et al.,
 54 2009; Stark et al., 2017). The functions of *CARMIL1* and *CARMIL2* include cell migration,
 55 macropinocytosis, lamellipodial activity and cell polarity.

56 In contrast to this information for CARMIL1 and CARMIL2, relatively less is known about

the *CARMIL3* gene. In mouse, the *CARMIL3* protein localizes to developing synapses and spines in neurons, where it recruits actin capping protein. Depletion of *CARMIL3* protein in neurons leads to defects in spine and synapse assembly and function (Spence et al., 2019). In breast and prostate cancer patients, elevated expression of *CARMIL3* correlates with poor outcomes, and mouse tumor models reveal a role for *CARMIL3* in epithelial-mesenchymal transition, cadherin-based cell adhesions, and cell migration and invasion (Wang et al., 2020).

Cell Migration and Morphogenesis in Early Vertebrate Development

Early vertebrate embryogenesis sees massive cell rearrangements that establish and shape the three germ layers, mesoderm, endoderm and ectoderm during gastrulation. The most deeply positioned endoderm gives rise to the gut and other alimentary organs. At the onset of zebrafish gastrulation, mesendodermal progenitors are located at the margin of a cup-shaped blastoderm that covers the animal hemisphere of a large yolk cell (Warga and Kimmel, 1990). The mesodermal and endodermal lineages soon separate, and internalized endodermal cells initially disperse on the yolk surface as individuals via a random walk to almost completely cover the yolk cell (Pézeron et al., 2008). Concurrently, mesoderm and ectoderm spread around the yolk cell in the process of epiboly (Warga and Kimmel, 1990). Later during gastrulation, endoderm cells migrate towards the dorsal midline along trajectories that are biased either animally/anteriorly (for cells in animal hemisphere) or vegetally/posteriorly (for cells in vegetal hemisphere), thus simultaneously elongating the nascent endoderm along the AP axis (Schmid et al., 2013). Endodermal cell migration during gastrulation depends on Rac1-regulated actin dynamics (Woo et al., 2012).

On the dorsal side there is a small cluster of dorsal forerunner cells (DFCs) that travel vegetalward in advance of the spreading germ layers, which later during segmentation will form an epithelial ciliated vesicle known as Kupffer's Vesicle (KV), the left-right (L/R) organizer of zebrafish (Amack and Yost, 2004). At the end of epiboly, DFCs form multiple rosette-like epithelial structures whose focal points are enriched for apical proteins. During segmentation, these rosettes arrange into a single rosette lined by a lumen with cilia at the apical membrane of the cells, thereby forming the KV (Oteíza et al., 2008). L/R patterning in zebrafish depends on the motile cilia in the KV that generate an asymmetric fluid flow (Gokey et al., 2015; Gokey et al., 2016; Sampaio et al., 2014). Mutations that affect the shape and size of the KV or that affect the number or length of cilia in the KV can impair robust L/R patterning (Amack, 2014).

To investigate the function of CARMIL3 in vertebrate development, we examined phenotypes resulting from the disruption of the gene encoding CARMIL3 in zebrafish. We found defects in endodermal cell migration, DFC migration and clustering, KV morphogenesis, the number of cilia in the KV, and L/R asymmetry.

Materials & Methods

Zebrafish lines and husbandry

Animal protocols were approved by the Institutional Animal Care and Use Committees at University of Iowa and Washington University. At University of Iowa, zebrafish were maintained as described previously (Xu et al., 2011) and embryos were obtained by natural spawning and staged according to morphological criteria or hours post fertilization (hpf) at 28°C or 32°C unless otherwise specified, as described previously (Kimmel et al., 1995). At Washington University, zebrafish and embryos are maintained at 28.5°C using the standard operating procedures and guidelines established by the Washington University Zebrafish Facility, described in detail at <http://zebrafishfacility.wustl.edu/documents.html>. The following zebrafish lines were used in this study: AB*/Tuebingen, *Tg(sox17:EGFP)* (Mizoguchi et al., 2008), *carmil3^{sa19830}* (*lrrc16b^{sa19830}*), and *carmil3^{stl413}*.

Zebrafish line *carmil3^{sa19830}* (*lrrc16b^{sa19830}*) was obtained from the Zebrafish International Resource Center (Eugene, OR) (described at <https://zfin.org/ZDB-ALT-131217-14950>) (Kettleborough et al., 2013). The mutation was created by *N*-ethyl-*N*-nitrosourea (ENU) treatment of adult males, and the mutated gene has a G to T conversion at an essential splice site of intron 27-28, which introduces multiple stop codons beginning at amino acid residue 832 (Kettleborough et al., 2013). Failure to splice at this site is predicted to change amino-acid residue 831 from E to D, with the next codon being ochre TAA, and thus truncating the protein to 832 residues from its normal length of 1384 residues. Zebrafish line *carmil3^{stl413}* was produced via TALEN-mediated mutagenesis (Boch et al., 2009; Moscou and Bogdanove, 2009). TALEN sequences used were 5'-TGACAAGACATCAATCAAGT and 5'-TTTGCCACTCTTGTCTCTG (corresponding to bases 72063-72082 and 72101-72120, respectively, of the sequence for the genomic locus FQ377660). TALEN cleavage led to several different independent mutations in founder fish. The largest deletion was of an 11-bp segment of exon 2, 5'-ACGTATCAAAG. This deletion eliminates the Alu1 restriction site found in exon 2, and the deletion leads to a premature stop codon at amino-acid residue 101. Fish carrying this mutation were selected for outcrossing and further study.

122 *carmil3^{sa19830}* and *carmil3^{stl413}* were genotyped by restriction enzyme digestion of PCR
 123 amplicons containing the mutations. For *carmil3^{sa19830}* the forward primer was 5'-
 124 AGCAGAGTGTCTTTCTCCAC, and the reverse primer was 5'-
 125 GATCGAGGTTGGAGGTGAAC. MseI digest distinguished WT (191 bp band) from *sa19830*
 126 mutant (bands at 123 bp and 38 bp). For *carmil3^{stl413}* the forward primer was 5'-
 127 AGAATAGTGTAATCCACTCATTTCACCG, and the reverse primer was 5'-
 128 AGGCAGGTGTGAATACCTTTAAAGTCTTCA (corresponding to bases 71935-71965 and
 129 72249-72278, respectively, of the sequence for the genomic locus FQ377660). AluI digestion
 130 produced two bands at 157 bp and 187 bp from WT genomic DNA, and a single band at 333
 131 bp from the mutant. Mutant founders were outcrossed into WT AB fish to produce
 132 heterozygous fish, which were fully viable, and they were mated to produce maternal and
 133 maternal-zygotic homozygous mutants. Alternatively, genotyping of *carmil3^{sa19830}* mutants
 134 was performed by Transnetyx (Cordova, TN) using real-time PCR with allele-specific probes.

135 *Protein expression and purification*

136 Capping protein (CP, mouse alpha1beta2) was expressed and purified as described
 137 (Johnson et al., 2018). Glutathione-S-transferase (GST)-tagged CP binding region (CBR)
 138 fragments of human CARMIL1a (E964-S1078, plasmid pBJ 2411), human CARMIL3 (S955-
 139 S1063, plasmid pBJ 2449), zebrafish Carmil3 (S943-N1040, plasmid pBJ 2451) and
 140 zebrafish Carmil3 CPI-mutant (S943-N1040, with point mutations H944A and R966A, plasmid
 141 pBJ 2452) were expressed from pGEX-KG vectors in *E. coli* BL21 Star (DE3). The fusion
 142 proteins were affinity-purified on Glutathione Sepharose® 4 Fast Flow (GE Healthcare), and
 143 then bound to POROS GoPure XS (Applied Biosystems) in 20 mM NaH₂PO₄, 100 μM EDTA,
 144 1 mM NaN₃, 5 mM DTT, 4M urea (pH 7.5). After elution with a gradient to 1 M NaCl in the
 145 same buffer, the purified GST-CBR fragments were concentrated and stored at -70°C.

146 *Actin polymerization assays*

147 Pyrene-actin polymerization assays were performed as described (Carlsson et al.,
 148 2004). Pyrene-labeled and unlabeled gel-filtered rabbit muscle actin stocks were mixed to
 149 produce a total actin monomer concentration of 1.5 μM in the cuvette. Pyrene-actin filament
 150 seeds were prepared as described (Ramabhadran et al., 2012). CP at 5 nM and GST-CBR at
 151 varied concentrations were added at the start of the experiment (0 sec). Pyrene-actin
 152 fluorescence was measured using time-based scans on a steady-state fluorometer
 153 (QuantaMaster, PTI, Edison, NJ) with excitation at 368 nm and emission at 386 nm.

154 *Whole-mount RNA in situ hybridization (WISH)*

155 Digoxigenin-labeled antisense RNA probes for *myl7* (cardiac myosin regulatory light
156 chain) (Ye and Lin, 2013; Yelon et al., 1999), *sox17* (sex determining region Y-box 17)
157 (Alexander et al., 1999; Hu et al., 2018), *southpaw* (*spaw*) (Long et al., 2003; Panizzi et al.,
158 2007) were synthesized by *in vitro* transcription. Staged embryos were fixed in 4% fish fix
159 solution (4% paraformaldehyde, 4% sucrose, 0.1 M phosphate buffer pH 7.2, 0.12 mM CaCl₂)
160 at 4°C overnight. Fixed embryos were manually dechorionated and dehydrated with a series
161 of methanol washes. WISH was performed as described (Thisse and Thisse, 2008).

162 *Whole-mount Immunofluorescence*

163 Staged embryos (10-14 somites) were manually dechorionated and fixed in Dent's
164 fixative (80% methanol: 20% DMSO) at room temperature for a minimum of 2 hrs. Antibody
165 staining was performed in PBDT (1% BSA, 2% goat serum, 2% DMSO, 0.1% Triton X-100 in
166 PBS) as described (Topczewski et al., 2001; Ye and Lin, 2013). The following antibodies
167 were used: mouse anti-acetylated tubulin (clone 6-11b-1, Sigma-Aldrich, diluted 1:2000) and
168 goat anti-mouse Alexafluor 488 (Invitrogen, diluted 1:2000).

169 *Microscopy and Image Analysis*

170 WashU: For Figure 4 D-G, fluorescence images were collected on a spinning disk
171 confocal microscope (Quorum, Canada) using an inverted Olympus IX-81 microscope, a
172 Hamamatsu EMCCD camera (C9100-13) and Metamorph acquisition software. For Figures
173 2A, 5, and 6, WISH images were collected on a Nikon Macroscope with a Nikon AZ100
174 objective, a 1x lens N.A. 0.1, and a 4x lens N.A. 0.4.

175 Iowa: For still epifluorescence images, live or fixed embryos were mounted in 2%
176 methylcellulose and photographed using a Leica DMI 6000 microscope with a 5×/NA 0.15
177 objective or a 10×/NA 0.3 objective. For ISH images in Figure 3, embryos were mounted in
178 80% glycerol/PBS and photographed using a Leica M165FC Stereomicroscope with a Leica
179 DFC290 Color Digital Camera.

180 For time-lapse imaging of endodermal cells in Figure 2 C-G and Figure 4 A-C,
181 *Tg(sox17:EGFP)* embryos were embedded in 0.8% low-melting agarose in a dorsal-mount
182 imaging mold as previously described (Ye et al., 2015). Time-lapse imaging was taken in the
183 dorsal region of endoderm at 25°C, at 5-minute interval with a 5×/NA 0.15 objective on an
184 inverted Leica DMI 6000 microscope. Images were processed and cell tracking analyzed

185 using ImageJ (Schneider et al., 2012). Data were exported to Excel where cell migration
186 speed, paths, direction were determined as previously reported (Lin et al., 2005).

187 *Statistical Analysis*

188 Data were compiled from two or more independent experiments and were presented
189 as the mean $\pm$ SEM or $\pm$ SD as indicated in figure legends. Statistical analyses were
190 performed in GraphPad Prism (GraphPad Software) using unpaired two-tailed Student's *t*-
191 tests with unequal variance. The numbers of cells and embryos analyzed in each experiment
192 and significance levels are indicated in graphs and/or the figure legends.

193

194 **Results**

195 As part of our interest in how actin assembly and actin-based motility contributes to
196 morphogenesis and cell movement during development, we examined the role of CARMIL3.
197 The CARMIL family of proteins regulate the heterodimeric actin capping protein (CP) that
198 controls polymerization of actin filaments at barbed ends, *in vitro* and in cells (Stark et al.,
199 2017). To advance our understanding of the functions of the three CARMIL isoforms
200 conserved across vertebrates, we first assayed biochemical activities of zebrafish Carmil3 in
201 comparison to human CARMIL3 and CARMIL1. Second, we altered the activity of the
202 zebrafish *carmil3* gene, previously known as *lrrc16b* and *si:ch211-204d18.1*, by creating
203 and/or interrogating lines carrying homozygous loss-of-function mutations.

204

205 *CARMIL3 inhibits actin capping activity of CP*

206 The biochemical activities of the vertebrate CARMIL1 and CARMIL2 isoforms with
207 respect to CP and actin have been studied in mouse and human systems (Stark et al., 2017).
208 The ~100-aa capping protein binding region (CBR) of CARMIL1 and CARMIL2 are known to
209 bind directly to CP and partially inhibit its actin capping activity, via an allosteric mechanism.
210 Comparative studies of CARMIL3 had not been previously done and so we performed them
211 here.

212 We prepared recombinant proteins corresponding to the CBR region of CARMIL3 from
213 human and zebrafish, and we tested their ability to inhibit the capping activity of mouse non-
214 sarcomeric CP (α 1 β 2) (Edwards et al., 2013). In actin polymerization assays seeded
215 with barbed ends of actin filaments, CP inhibited actin polymerization (Fig. 1A). A human

216 CARMIL3 (C3) CBR fragment partially reversed the inhibitory effect of CP, with a potency
 217 slightly greater than that of human CARMIL1 (C1) (Fig. 1A). Zebrafish Carmil3 (C3) CBR also
 218 inhibited CP (Fig. 1B), with a potency approximately half that of human CARMIL3. Specific
 219 point mutations known to impair the ability of human CARMILs to interact with CP (Edwards
 220 et al., 2013; Lanier et al., 2015) were also created in the CBR fragment of zebrafish Carmil3
 221 (C3-HRAA). The mutant CBR failed to inhibit CP activity (Fig. 1 B). Thus, the CBR region of
 222 CARMIL3, from both human and zebrafish, is able to inhibit the capping activity of CP, in a
 223 manner consistent with the direct-binding mechanism described for other CARMILs (Stark et
 224 al., 2017). This result supports the hypothesis that functional properties of CARMIL3 in
 225 vertebrates may involve direct interaction with CP.

226

227 **Figure 1.** CARMIL3 inhibits capping protein (CP) activity in actin polymerization assays, with
 228 pyrene-actin fluorescence (arbitrary units) plotted vs time. A) Human CARMIL3 (C3) inhibited
 229 the ability of CP to cap barbed ends of actin filaments. Black points, labeled “Seeds,” are
 230 from a control with

231 monomeric actin and
 232 filamentous actin seeds.
 233 Pink points, labeled “CP,”
 234 correspond to a sample to
 235 which CP was added. Red
 236 and blue curves contain
 237 CP plus the indicated
 238 concentrations of human
 239 CARMIL1 or CARMIL3
 240 CBR fragment. B)
 241 Zebrafish CARMIL3 (C3,
 242 blue curves) inhibited
 243 capping by CP. In
 244 comparison to human
 245 CARMIL3 (C3, red curves),
 246 the inhibitory activity of
 247 zebrafish CARMIL3 was
 248 slightly less than that of

human CARMIL3, based on similar concentrations as indicated. A mutant form of zebrafish CARMIL3 (C3-HRAA, green curve) containing two point mutations at conserved residues of the CP-binding CPI motif, failed to block CP activity, as expected.

252

253 *Genomic mutations of zebrafish carmil3: Creation and characterization*

254 A *carmil3* mutant line from the Zebrafish Mutation Project (Kettleborough et al., 2013),
255 *carmil3^{sa19830}*, carries a single-nucleotide change at an essential splice site, predicted to
256 truncate the protein to 832 residues from its WT length of 1384 residues. We created a
257 second *carmil3* mutant line by TALEN-mediated mutagenesis, *carmil3^{stl413}*; this mutation
258 produces an 11-bp deletion within exon 2 that causes a frameshift followed by a nonsense
259 mutation, predicted to produce a truncated protein of 101 residues. Heterozygous and
260 homozygous zygotic mutants of both alleles were viable as embryos and adults and did not
261 present any overt phenotypes. When fish homozygous for either of the two alleles were
262 crossed, the resulting embryos, deficient in both maternal and zygotic *carmil3* function,
263 MZ*carmil3^{sa19830/sa19830}* (thereafter MZ*carmil3^{sa19830}*) or MZ*carmil3^{stl413/413}* (thereafter
264 MZ*carmil3^{stl413}*), also completed epiboly and gastrulation and developed into morphologically
265 normal embryos.

266

267 *Migration of endodermal cells*

268 To assess potential subtle or transient developmental defects, we analyzed the
269 expression of germ layer markers in MZ*carmil3* mutants at mid-gastrulation by whole mount
270 *in situ* hybridization (WISH). We examined the positions of endodermal cells and DFCs,
271 marked by expression of *sox17*, over time during gastrulation. In embryos fixed and stained
272 by whole-mount *in situ* hybridization (WISH) at midgastrulation (70-80% epiboly), we noted
273 that the leading edge of the vegetally migrating endodermal cells in MZ*carmil3^{sa19830}* mutant
274 embryos lagged behind that of WT embryos (Fig. 2A). To quantify the effect, we measured
275 the distance from the front edge of the endodermal cells to the edge of the aggregation of
276 DFCs, as diagrammed on the right side of Figure 2B. The distance was larger for the
277 MZ*carmil3^{sa19830}* mutant, compared to WT embryos (Fig. 2B), indicating that *carmil3* is
278 important for endodermal migration. The mean distance values were 67 μ m (95% confidence
279 interval of 60-74, n=61) for WT embryos and 142 μ m (95% confidence interval of 135-150,
280 n=55) for mutant embryos. The difference between the two sets of data had a two-tailed P

281 value of <0.0001 in an unpaired t test. These data were combined from clutches obtained on
282 separate days.

283

284

285

286 **Figure 2.** Pattern of endodermal cell migration in *carmil3* mutant embryos compared to WT
287 embryos. Panels A and B are results from *sox17* staining of embryos at 70-80% epiboly. A.
288 Representative images illustrating the patterns observed in WT compared to MZ*carmil3*^{sa19830}
289 mutant embryos. Red dotted line indicates the margin of the endoderm, used to measure
290 migration distance. B. Endodermal cell migration distribution, measured as illustrated in the
291 schematic and the images of panel A. In the plot, each data point corresponds to one
292 embryo. Values for the distributions were as follows (mean ± s.d.): WT 74 ± 25 (N=50),
293 MZ*carmil3*^{sa19830} mutant 144 ± 28 (N=44). Asterisk (*) indicates p value of <0.0001 in
294 Student's *t*-test. Panels C through G are results from epifluorescence time-lapse experiments
295 performed on MZ*carmil3*^{sa19830} or MZ*carmil3*^{sa19830} mutant embryos, each carrying
296 *Tg(sox17:EGFP)/+*. (C and E) Snapshots at 80% epiboly stage from the time-lapse movie,
297 with the tracked cells labeled. (C' and E') Snapshots at 95% epiboly stage, with the migration
298 tracks of endodermal cells from the 80% to 95% epiboly stage superimposed. Scale bar:
299 100µm. (D, F) Migration tracks delineate routes of endodermal cells. Solid magenta squares
300 denote the endpoint of migration. (G) Total speeds, net speeds, and migration persistence.
301 The number of cells analyzed is indicated in the first graph. Asterisk (*) indicates p value of
302 <0.0001 in Student's *t*-test.

303

To test this observation directly in living embryos, we then examined the migration of the endodermal cells by conducting fluorescence microscopy time-lapse analyses using a transgenic line expressing eGFP fluorescent protein from the *sox17* gene promoter *Tg(sox17-GFP)* (Fig. 2) (Mizoguchi et al., 2008). Cell tracking revealed that in control embryos (heterozygous *carmil3^{sa19830/+}*), endodermal cells migrate toward the vegetal pole and converge dorsally along fairly straight paths (Schmid et al., 2013) (Fig. 2C-D). In contrast, in *carmil3*-deficient mutants (*MZcarmil3^{sa19830}*) the endodermal cells took less direct paths (Fig. 2E-F). Further cell tracking analyses showed that both cell movement, i.e. (total speed: movements in all directions) and migration efficiency (net speed: along straight line between the start and endpoint) were impaired in mutants compared to WT controls, as was the persistence of migration (ratio of net : total speed) (Fig. 2G). Thus, Carmil3 is required for efficient migration of endodermal cells.

Aggregation of dorsal forerunner cells (DFC)

Static images from these time-lapse experiments with a *Tg[sox17:EGFP]* background also revealed the migration and cluster formation by dorsal forerunner cells (DFCs), which strongly expressed eGFP. DFCs in WT embryos usually formed a single large cluster (Fig. 2C). In mutant embryos, the DFC cluster was fragmented; we observed this in *MZcarmil3^{sa19830}* embryos (Fig. 2E) and in *MZcarmil3^{stl413}* mutant embryos (data not shown). In addition, we noted bright GFP-labeled cells that resided in an apparent gap between the DFC cluster and the leading edge of the migrating endodermal cells (Fig. 2E) in the mutant, but not WT embryos, which we suggest correspond to DFCs exhibiting abnormal migration.

We further examined DFC cluster morphology and DFC distribution during gastrulation using WISH probes for cells expressing *sox17*; these results also revealed the location of DFCs over time in larger populations of WT and mutant embryos. We examined embryos at 70-80% epiboly (midgastrulation; Fig. 3), and we observed variation in the size and coherence of the DFC cluster in the mutants compared to WT. To score the phenotype, we graded the morphology of the aggregated set of DFCs as “normal size, one cluster;” “normal size, split-mild,” “normal size, split-severe;” “small size, one cluster,” and “small size, split-mild.” Representative images are shown in Figure 3A-D, and graphs with quantification of these results, for control, *MZcarmil3^{sa19830}*, and *carmil3^{stl413}* mutant embryos, are presented in Fig. 3E. These results corroborate the observations from the time-lapse analyses, showing

that formation of a cohesive DFC cluster is impaired in *carmil3*-deficient gastrulae, which frequently displayed a fragmented and/or smaller DFC aggregate.

Figure 3. Patterns of dorsal forerunner cell (DFC) distribution in *MZcarmil3^{sa19830}* mutant embryos compared to heterozygous *+/+* *MZcarmil3^{sa19830}* embryos. Panels A to D illustrate the patterns observed, with results quantified in panel E. In most embryos, DFCs form a single tight cluster of normal size (light blue arrow in panel A and light blue bars in panel E). In some embryos, DFCs display three patterns of defects: B) normal size with mild splitting (i.e. two clusters); C) normal size with severe splitting (more than two clusters), and D) small size with mild splitting. (E) Percentages of embryos displaying the patterns of DFC defects in both *MZcarmil3^{sa19830}* and *MZcarmil3^{stl413}* mutants. The percentage of embryos in each group is indicated above each bar, and the total number of embryos in each group (N) is listed underneath the labels on the abscissa.

352 *Morphology of the Kupffer's Vesicle and Cilia*

353 The cluster of DFCs that forms during gastrulation, undergoes a morphogenetic
354 transition into an epithelial sac known as Kupffer's Vesicle (KV), the ciliated organ that
355 determines L/R asymmetry during zebrafish development (Gokey et al., 2016). Because we
356 observed a defect in DFC aggregate formation, we examined the morphology of the KV and
357 its cilia during segmentation (Fig. 4).

358 Imaging the KV in *MZcarmil3^{stl413}* mutant (*Tg[sox17:EGFP]*) embryos at the 6-somite
359 stage (Fig. 4A,B,B'), revealed that their KV during early segmentation was significantly
360 smaller than in control WT embryos (Fig. 4C). To visualize the epithelial KV morphology
361 during later development, we stained embryos fixed at the stage of 10-14 somites with
362 antibodies against the epithelial marker atypical PKC (aPKC) (Amack et al., 2007) and
363 acetylated tubulin (Ac-tubulin) (Piperno and Fuller, 1985), resolving the cilia as individual
364 structures, allowing us to count their number and measure their length. In these images, the
365 hollow empty center of the KV appears as a relatively dark unstained region in the aPKC
366 channel and as staining positive for cilia in the Ac-tubulin channel. In instances where the KV
367 did not "inflate" (*MZcarmil3^{sa19830}* in Fig. 4D, bottom panel), an unstained region was not
368 observed. As in earlier stages, the KV was often smaller or fragmented, resulting in a
369 decreased KV area for both mutants (Fig. 4C and 4E). The decrease in KV area was
370 statistically significant (Fig. 4E), and the magnitude of the decrease was sufficiently large that
371 the values were at or below the value for the area of the KV at this stage found to be
372 necessary for robust L/R patterning by Gokey and colleagues (Gokey et al., 2016), which is
373 drawn as a dotted line at 1300 μm^2 in Figure 4E.

374 We analyzed the cilia in greater detail, and found that the number of cilia per KV was
375 decreased in both mutants (Fig. 4F). The decreases were substantial and significantly
376 different from WT embryos. In Figure 4F, the dotted line at 30 cilia per KV corresponds to the
377 value that was found to be necessary for robust L/R patterning by Sampaio and colleagues
378 (Sampaio et al., 2014). In both mutants, the number of cilia per KV was below this threshold
379 (Fig. 4F). In contrast, the length of the cilia did not differ in the mutant embryos compared to
380 WT embryos (Fig. 4G), suggesting that *Carmil3* is not required for cilia biogenesis per se, but
381 instead plays a role in the size and/or morphology of the KV.

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Figure 4. Morphology of Kupffer's vesicle (KV) and cilia in *carmil3* mutant embryos. Panels A through C are from one analysis, based on wide-field fluorescence images showing the morphology of sox17-EGFP labelling of the KV in control (A) and MZ*carmil3*^{stl413} mutant (B-B') embryos at the 6-somite stage. (C) Area of the KV in control and MZ*carmil3*^{stl413} mutant embryos. All the data points are shown. The red lines indicate the mean and one standard error of the mean. The results are statistically significant with a p value of < 0.0001. The number of embryos analyzed was 15 for control and 30 for mutant. In this set of experiments, the control was a heterozygous strain. Panels D through G are from a second experimental series with a different set of animals, based on confocal images of embryos at the 10-14 somite stage stained to visualize cilia and the KV, with antibodies to acetylated tubulin (Ac-tubulin, Green in Merge), and to atypical PKC (aPKC, Red in Merge) respectively. With anti-aPKC staining, the KV appears as a relatively dark area, owing to the absence of cells inside the vesicle. WT and two different *carmil3* mutant embryo lines are shown. The Merge panel shows examples of how the KV was outlined for calculation of area; the outline is based on both Ac-tubulin and aPKC images. Panels E through G are graphs of parameters quantified from the images, with the color scheme for WT and mutants as indicated. In each panel, the plotted values are the mean, and the error bars correspond to one standard deviation. E) Area of the KV. Horizontal dotted line corresponds to the value for area of KV found to be necessary for robust L/R patterning by Gokey and colleagues (Gokey et al., 2016). Values for the two mutants differ from the value for WT based on Student's t-test (p<0.005 (actual=0.0006 and 0.0005)). F) Number of cilia per KV. Horizontal dotted line corresponds to the value for number of functional cilia per KV (30) found to be necessary for robust left / right patterning by Sampaio and colleagues (Sampaio et al., 2014). Values for the two mutants differ from the value for wild type based on Student's t-test (p<0.005 (Actual=0.0001 and 0.0004)). G) Length of cilia. Values for the two mutant lines do not differ from the value for WT embryos based on Student's t-test (p=0.3277 and >0.9999). Values of N as in panel E. Values of N (embryos counted) as follows: WT, 9; MZ*carmil3*^{stl413}, 8; MZ*carmil3*^{sa19830}, 4 for panels to G.

413 Left-Right Patterning

414 Because we observed defects in formation of the KV, including its morphology, size
415 and the number of cilia; we asked whether further development revealed defects in L/R
416 asymmetry pathways and outcomes. Since the *carmil3* mutants displayed the KV area and
417 number of cilia below the thresholds shown to be required for robust L/R patterning by
418 previous studies (Gokey et al., 2016; Sampaio et al., 2014), we hypothesized that there
419 would be defects in L/R patterning in these mutants.

420 First, we examined the pattern of expression of the lateral plate mesoderm marker
421 *southpaw* (*spaw*) in embryos at the 18-20 somite stage (Fig. 5) (Long et al., 2003). Whereas
422 WT embryos uniformly displayed *spaw* staining on the left, the two *carmil3* mutant lines
423 showed substantial percentages of embryos with bilateral or right-sided *spaw* staining.
424 Representative examples are shown in panel A of Figure 5, with quantitation in panel B and
425 Table I. Results for the two mutant lines were similar to each other and differed significantly
426 from results for WT embryos. These embryos were generated from the same set of mutant
427 and WT animals as those used in the experiments illustrated in panels D-G of Figure 4,
428 where the KV area and number of cilia per KV were below the critical threshold in the mutant.
429 When a different generation of mutant animals was used in a separate set of experiments to
430 generate MZ*carmil3*^{stl413} embryos, in which the KV area was not below the critical value
431 illustrated in panels A-C of Figure 4, little or no defects in *spaw* staining distribution were
432 observed (data not shown), consistent with the findings of Gokey and colleagues (Gokey et
433 al., 2016), and Sampaio and colleagues (Sampaio et al., 2014) noted above.

434

435 **Figure 5.** Patterns of *spaw* staining distribution at the 18-20 somite stage in *carmil3* mutant
436 embryos compared with WT embryos. A. Representative images illustrate observed patterns
437 of *spaw* staining, which is purple. B. Percentage of *spaw* staining patterns, comparing WT
438 embryos with embryos of two different *carmil3* mutant lines, MZ*carmil3*^{stl413} and
439 MZ*carmil3*^{sa19830}. The color scheme is indicated in panel A, below the images. In cases
440 scored as “absent,” no staining was observed. Values are listed in Table I.

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443 *spaw* regulates FGF signaling (Neugebauer and Yost, 2014), and *spaw* mutants are
 444 defective in L/R asymmetry, as revealed by the position of the heart (Ahmad et al., 2004;
 445 Long et al., 2003). Therefore, we asked whether mutant embryos had a phenotype related to
 446 heart position. Indeed, mutant embryos at ~30 hpf displayed a defect in the L/R positioning of
 447 the heart (Fig. 6). In a noticeable and significant number of embryos, the heart was in the
 448 middle or on the right side of the embryo, based on staining for cardiac myosin light chain
 449 (*myl7*), for which representative examples are shown in panel A of Figure 6. A combined
 450 count of embryos with altered heart position showed defects of 6 - 9% for each of the two
 451 mutants and a value less than 1% observed in WT embryos (Fig. 6, Panel B). The defects in
 452 the two mutant lines did not differ from each other by a statistically significant degree, and the
 453 differences between the two mutant lines and the WT line was highly significant in both
 454 cases.

455

Figure 6. Heart position in MZ*carmil3* mutant embryos compared to WT embryos at ~30 hpf, assessed by staining for transcripts of *myl7*, the gene encoding cardiac myosin light chain, in blue. A. Representative images illustrate observed patterns. B. Quantification of the sum of the two altered patterns, termed “heart jogging,” in two different MZ*carmil3* mutant embryo lines compared with WT embryos. WT embryos displayed the abnormal (to the right or center) heart jogging phenotype 0.25% of the time (N=784). This value was significantly higher in the mutant embryos: 8.6% for MZ*carmil3*^{stl413} (N=198) and 5.8% for MZ*carmil3*^{sa19830} (N=415). Error bars indicates standard error of proportion. P values were calculated from Fisher’s exact test calculated with GraphPad Prism. Asterisk indicate that for both mutants, compared with WT, p values were <0.0001.

As above for *spaw* staining, these data for heart position are from a set of animals and experiments where the KV area and number of cilia per KV in the mutant were below the critical threshold (Fig. 4 D-G). In a separate set of experiments, using MZ*carmil3*^{stl413} mutants, in which the KV area was not below the critical value (Fig. 4 A-C), little or no defects in heart position were observed (data not shown). Therefore, the penetrance and expressivity of the KV phenotype in MZ*carmil3*^{stl413} mutants correlated well with those of the *spaw* asymmetric staining defect during mid-segmentation stages and the L/R heart positioning defect at the end of embryogenesis, consistent with the findings of Gokey and colleagues (Gokey et al., 2016) and Sampaio and colleagues (Sampaio et al., 2014).

Discussion

We investigated the role of the actin assembly regulator, CARMIL3, in cell migration and morphogenesis during early zebrafish development. First, we confirmed that in actin polymerization assays, human CARMIL3 and zebrafish Carmil3 interact with and regulate the

488 activity of capping protein with respect to actin polymerization. More important, we discovered
 489 that *Carmil3* is required for normal migration of endodermal cells and the aggregation of
 490 dorsal forerunner cells (DFCs) during zebrafish gastrulation. Impaired aggregation of DFCs
 491 led to defects in the formation of the KV in terms of its shape and size. These KV defects
 492 were associated with decreased numbers of cilia present in mutant KVs; as a consequence,
 493 L/R asymmetry was impaired, manifested by malposition of the marker *spaw* during
 494 segmentation and later in the position of the zebrafish heart tube. These KV and L/R
 495 phenotypes displayed variable but correlated penetrance and expressivity.

496 The two *carmil3* mutant alleles we report here were generated by reverse genetic
 497 approaches: *carmil3*^{sa19830} by TILLING for ENU-induced nonsense mutations (Kettleborough
 498 et al., 2013) and *carmil3*^{stl413} by deploying TAL endonucleases (Boch et al., 2009; Moscou and
 499 Bogdanove, 2009). Both are premature stop codons predicted to produce truncated proteins
 500 and thus should represent strong or null alleles. Consistent with that view, the phenotypes
 501 are recessive. However, our study does not include an analysis of the protein products that
 502 would test this view definitively. The observation that only maternal zygotic but not zygotic
 503 *carmil3* mutants presented gastrulation phenotypes, is consistent with known significant
 504 maternal *carmil3* expression, which is likely to compensate for the lack of zygotic expression
 505 during gastrulation (Solnica-Krezel, 2020; Stark and Cooper, 2015). The lack of full
 506 penetrance for the later phenotypes indicates a substantial level of robustness in the
 507 processes of cell migration and morphogenesis, consistent with observations for many other
 508 genes regulating early embryogenesis (Chen et al., 2018; Kelly et al., 2000; Li-Villarreal et al.,
 509 2015; Solnica-Krezel and Driever, 2001). CARMILs are encoded by three conserved genes in
 510 vertebrates, including zebrafish, so redundant and overlapping functions contributed by
 511 CARMIL1 or CARMIL2 may also account for the variable level of penetrance seen for
 512 downstream phenotypes. In addition, as the two *carmil3* mutant alleles are nonsense
 513 mutations, the observed phenotypes could represent only partial loss-of-function and
 514 variability due to genetic compensation triggered by RNA degradation (El-Brolosy et al.,
 515 2019; Ma et al., 2019).

516 Our results provide new information about the function of CARMIL3, especially in the *in*
 517 *vivo* context of a whole vertebrate organism during the process of embryogenesis. Previous
 518 studies on CARMIL3 have used cell culture and mouse tumor models to uncover roles in
 519 neuronal synapse formation and cancer cell migration, based on actin assembly (Hsu et al.,
 520 2011; Lanier et al., 2016; Spence et al., 2019; Wang et al., 2020).

521 Previous studies, with human cultured cells that express both CARMIL1 and CARMIL2,
522 found overlapping but distinct functions for the two proteins, based on subcellular localization
523 and knockdown phenotypes (Liang et al., 2009; Stark et al., 2017). While the early
524 phenotypes observed here display strong penetrance, the later phenotypes are far less
525 penetrant, raising the question of compensatory and overlapping function among the
526 CARMIL-encoding genes. In support of the view, preliminary observations in our laboratories
527 have revealed stronger L/R asymmetry phenotypes in double mutant zebrafish embryos that
528 carry mutations in genes for both CARMIL2 and CARMIL3 (Stark, Solnica-Krezel and
529 Cooper, 2019, unpublished); these observations will merit further study in the future.

530 We discovered that Carmil3 is important for the migration of endodermal cells during
531 zebrafish gastrulation. Mutant endodermal cells had both reduced overall motility as well as
532 reduced persistence. Recent work demonstrated cultured cells lacking CARMIL3 had
533 reduced migration in the classical scratch assay and in trans-well migration. KO cells were
534 less polarized, had less polymerized actin and fewer focal adhesions (Wang et al., 2020).
535 Interestingly, the Rac-specific guanine nucleotide exchange factor, Prex1, was implicated in
536 regulation of Nodal-dependent actin dynamics and random endoderm cell motility during
537 gastrulation (Woo et al., 2012). Future experiments will determine if endodermal migration
538 defects in *MZcarmil3* mutants are associated with abnormal actin organization and/or focal
539 adhesion formation.

540 We found that Carmil3 is important for the aggregation of DFCs during gastrulation.
541 DFCs typically migrate vegetalward ahead of the germ layers in one cluster of cells.
542 Occasionally a few cells may separate from the cluster. DFC coalescence and migration as a
543 cohesive cluster is strongly dependent on cell-cell adhesion. *cdh1*/E-cadherin
544 (*halfbaked/volcano*) mutants in which cell adhesion is reduced, exhibit delayed epiboly of all
545 germ layers and frequently fragmented DFC clusters (Shimizu et al., 2005; Solnica-Krezel et
546 al., 1996). A recent study showed loss of CARMIL3 in cultured cells inhibits cadherin based
547 adhesion through transcriptional downregulation of epidermal type gene expression (Wang et
548 al., 2020). Such a mechanism might account for the reduced adhesion of DFC *in vivo*. We
549 observed small and malformed KV, likely the direct result of the smaller DFC clusters.
550 However, we cannot exclude a more direct role of Carmil3 in KV morphogenesis.

551 CARMILs appear to regulate actin via direct biochemical interactions with and effects on
552 the actin-capping properties of CP (Edwards et al., 2013; Lanier et al., 2015; Stark et al.,

2017). Indeed, CP itself is known to have an important role in morphogenesis. Mutations in humans and in zebrafish of the gene encoding the beta subunit of CP, known as *capzb* in zebrafish, were found to cause craniofacial and muscle developmental defects, with effects on cell morphology, cell differentiation and neural crest migration (Mukherjee et al., 2016). In addition, the same study found that *capzb* overexpression produced embryonic lethality.

Among regulators of CP, CARMILs are only one family of proteins with CPI motifs. Among other CPI-motif protein families, which are unrelated to each other outside of their CPI motifs, several have been shown to have roles in actin-based processes of development. The CPI-motif protein CKIP-1 is important for myoblast fusion in mammalian and zebrafish systems (Baas et al., 2012). CapZIP, known as *duboraya/dub* in zebrafish, is important in zebrafish development for actin organization in cells lining the KV, cilia formation in the KV, and L/R asymmetry (Oishi et al., 2006). CD2AP, encoded by *cd2ap* in zebrafish, is important for the development and function of the kidney glomerulus, in mammals and zebrafish (Hentschel et al., 2007; Tossidou et al., 2019). Zebrafish CIN85, a homologue of CD2AP encoded by the gene *sh3kb1*, is also important for glomerular podocyte function (Teng et al., 2016), and it has a role in the formation and maintenance of the vascular lumen (Zhao and Lin, 2013). Finally, the twinfilin family of CPI-motif proteins, encode by four genes in zebrafish - *twf1a*, *twf1b*, *twf2a* and *twf2b*, has not been as well-studied, but may also affect actin assembly, as may the WASHCAP / Fam21 family of CPI-motif proteins, encoded by *washc2c* in zebrafish.

The *carmil3* mutant lines generated here provide a valuable tool for studying the regulation of actin dynamics during endoderm migration and KV morphogenesis in the context of a developing vertebrate embryo, and for testing potential functional interactions with CARMIL3 interacting proteins.

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753 Tables.

754 Table I. Pattern of expression of *spaw* at 18-20 somites, comparing *carmil3* maternal zygotic
755 (MZ) mutant embryos with WT embryos. The percentage of the total number of embryos with
756 different patterns are listed, along with 95% confidence intervals (CI) and the number of
757 embryos scored (N). Statistics, including confidence intervals, were calculated with GraphPad
758 Prism.

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	Phenotypic Pattern, Percent of Total (95% CI)				
Genotype	Left	Right	Bilateral	Absent	N
Wild type	98 (94–99)	1 (0–3)	2 (0–5)	0	172
MZ <i>carmil3</i> ^{stl413}	54 (46–62)	9 (5–15)	26 (20–34)	11 (7–17)	137
MZ <i>carmil3</i> ^{sa19830}	53 (43–64)	7 (3–14)	31 (22–41)	9 (5–17)	88

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