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Swap70b is required for convergent and extension cell movement during zebrafish gastrulation linking Wnt11 signalling and RhoA effector function ☆

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ABSTRACT

Swap70 functions as a guanine nucleotide exchange factor for Rac and RhoA regulating F-actin cytoskeletal rearrangements and playing a crucial role in mammalian cell activation, migration, adhesion and invasion. Here we show that the zebrafish orthologue, Swap70b, is required for convergent and extension cell movement during gastrulation. Swap70b morphants exhibited broader and shorter body axis but cell fate specification appeared normal. While ectopic Swap70b expression robustly rescued Wnt11 morphants, RhoA overexpression was sufficient to rescue Swap70b morphants, establishing Swap70b as a novel member of the non-canonical Wnt/PCP pathway downstream of Wnt11 and upstream of RhoA. This is distinct from the related Def6a protein that acts downstream of Wnt5b. Def6a/Swap70b morphants resemble Ppt/Slb double mutant embryos suggesting that Swap70b and Def6a delineate Wnt11 and Wnt5b signalling pathways.

Introduction

Mammalian switch-associated protein-70 (Swap70) was originally identified in a B cell-specific DNA recombination complex (Borggrefe et al., 1998) and Swap70-deficient mice exhibited an impaired IgE response (Borggrefe et al., 2001). However, Swap70 is a multi-domain protein containing two EF-hand motifs at the N-terminus potentially binding Ca^{2+}, a domain (DSH) highly conserved between Swap70 and Def6 (the only homologue of Swap70 exhibiting the same conserved domain structure; (Hotfilder et al., 1999; Mavrakis et al., 2004)), a pleckstrin homology (PH) domain that binds phosphatidylinositol-3,4,5-trisphosphate (PtdIns (3,4,5) P3) and a coiled-coil domain at the C-terminus that has similarities with, and functions as, a Dbl homology domain (DH) of guanine nucleotide exchange factors (GEFs) for Rho GTPases (Shinohara et al., 2002). Upon growth factor stimulation, Swap70 binds PtdIns (3,4,5) P3 and translocates to the cell membrane activating Rac and regulating cell motility through F-actin rearrangements (Shinohara et al., 2002; Ihara et al., 2006; Wakamatsu et al., 2006; Fukui et al., 2007). These functional properties of Swap70 have been shown to play a role in cell activation, migration, adhesion and invasion (Sivalenka and Jessberger, 2004). In particular, Swap70$^{-/-}$ B cells showed defective polarisation and impaired uropod and lamellipodia formation essential for B cell entry into lymph nodes (Pearce et al., 2006). B cell activation results in Swap70 phosphorylation at tyrosine 517 by the kinase Syk inhibiting F-actin binding (Pearce et al., 2011) indicating that post-translational modifications alter Swap70 function as it was described for Def6 (Gupta et al., 2003; Bécart et al., 2008; Hey et al., 2012). In dendritic cells, it was shown that Swap70 binds activated Rac and RhoA and it was demonstrated that Swap70 is required for RhoA-dependent motility and endocytosis of dentritic cells (Ocana-Morgner et al., 2011).

Here we show that Swap70 (Swap70b in zebrafish see below) is required for convergent and extension (CE) cell movements during gastrulation in zebrafish. During vertebrate gastrulation several morphogenetic cell movements including CE movements take place that shape the embryo proper. Mesodermal and neuroectodermal cells move towards the dorsal midline and intercalate, leading to the medio-lateral narrowing (convergence) and anterio-posterior lengthening (extension) of the body axis (Warga and

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Kimmel, 1990; Concha and Adams, 1998; Keller, 2002; Wallingford et al., 2002). A mutagenic screen in zebrafish revealed that embryos showing broadened and shortened body axis at the end of gastrulation without altering cell fate specification, indicative of CE movement defects, carried mutation in protein members of the non-canonical Wnt/PCP pathway (Hammerschmidt et al., 1996; Heisenberg et al., 2000; Mlodzik, 2002; Kilian et al., 2003; Solnica-Krezel, 2003). Two of these mutants, *silberblick* (*slb*) and *pipetail* (*ppt*), were identified as alleles of Wnt11 and Wnt5b ((Rauch et al., 1997) Heisenberg et al., 2000; Stoick-Cooper et al., 2007), respectively. Although Wnt11 and Wnt5b have partially redundant and overlapping functions in the non-canonical Wnt/PCP pathway, Wnt11 is predominantly required anteriorly while Wnt5b is essential in the posterior parts of the embryo (Tada and Kai, 2009). In *Xenopus*, Wnt11 signalling through frizzled receptors results in complex formation of Dishevelled (Dsh), formin-homology protein Daam1, RhoA and the GEF WGEF activating RhoA (Habas et al., 2001; Tanegashima et al., 2008). In zebrafish Wnt11 signalling also results in RhoA activation with its downstream effector Rok2 regulating actin cytoskeleton rearrangements and CE cell movements (Marlow et al., 2002) but it is yet unknown which GEF is required for RhoA activation.

We have recently shown that the Swap70 relative Def6 (Def6a in zebrafish) is required for CE cell movements during zebrafish gastrulation downstream of Wnt5b signalling (Goudevenou et al., 2011). Although morpholino-mediated knockdown of Def6a and Wnt11 resulted in synergism, Def6a overexpression was insufficient to rescue Wnt11 morphants suggesting an overlapping but distinct pathway for Wnt11 and Wnt5b (Goudevenou et al., 2011). We now present evidence that Swap70b mediates Wnt11 signalling through RhoA. Swap70b morphants displayed a phenotype similar to non-canonical Wnt/PCP pathway mutants. While the body axis of Swap70b morphants was wider and shorter compared to wild type embryos, cell fate specification appeared normal. Wnt11 morphants were partially rescued through overexpression of Swap70b and ectopic expression of RhoA was sufficient to rescue Swap70b morphants suggesting that Swap70b acts downstream of Wnt11 and upstream of RhoA. Given the similarity in amino acid sequence and domain structure, Def6a and Swap70b could partially rescue each other. However, compared to single morphants, Def6a/Swap70b double morphants exhibited an enhanced CE phenotype resembling Ppt/Slb double mutants suggesting that these two novel GEFs delineate the non-canonical Wnt/PCP pathway in zebrafish.

Materials and methods

Ethics statement

All animal work was approved by the ethics review committee of the University of Nottingham and carried out under United Kingdom Home Office project licence no. 40/2893.

Cloning of zebrafish Swap70b and RhoA

CDNA sequences encoding full-length zebrafish *swap70b* (BC115308 and NM_001044986) and *rhoA* (BC056556) were amplified by PCR and then subcloned into pβUT3+EGFP vector as described by Goudevenou et al. (2011) to establish GFP/Swap70 and/or GFP/RhoA fusions. *Swap70b* was also subcloned into pBluescript (Stratagene) to generate sense and antisense probes for *in situ* hybridisation. *GFP*-tagged *def6a* mRNA was derived as described by Goudevenou et al. (2011).

Zebrafish husbandry, whole-mount in situ hybridisation and RT-PCR

Zebrafish were maintained and embryos were collected and raised according to standard laboratory conditions (Westerfield, 2000). Whole-mount *in situ* hybridisation was performed essentially as previously reported (Thisse and Thisse, 2008) with probes transcribed from the following zebrafish cDNAs: *swap70b*, *dlx3b*, *hgg1* (kindly provided by Steve Wilson), *ntl*, *shh, cdx4*, and *pax2* (kindly provided by Martin Gering), *myoD* (kindly provided by Simon Hughes), *chd*, *gsc*, *bmp2b* (kindly provided by Paul Scotting).

Total RNA was extracted from zebrafish embryos of different developmental stages using Buffer RLT Plus and the RNAeasy Plus Mini Kit (QIAGEN) according to the procedure provided by the manufacturers. DNA contamination was removed by DNase I (Roche) treatment and first strand cDNA was synthesised utilising the SuperScript II Reverse Transcriptase (Invitrogen). Reverse transcription was carried out at 42 °C for 50 min, followed by 15 min at 70 °C to stop the reaction, and 1 μl of cDNA was used for PCR. The following primers were used for amplification.

swap70a: exon 11 FP 5′-CCTGCTTAAACTCGTTCAACC-3′ and exon 12 RP 5′-TGCCACATCTTCTCCAGTTC-3′.
swap70b: exon 6 FP 5′-AGCTTTGAAATCAGCGCC-3′ and exon 8 RP 5′-CTCTTTCTCCATTTCCAGCC-3′.
def6a: exon 10 FP 5′-GCAAGCACCAATGTTAAACAC-3′ and exon 11 RP 5′-CACACCCTCCTCTACTTTCC-3′.
def6b: exon 1 FP 5′-AGTGTCCAAATCACAACTCAAG-3′ and exon 3 RP 5′-TTTAACAAACGTGCCCTCC-3′.
def6c: exon 4 FP 5′-TCTGCCTGTTCAACCTACTC-3′ and exon 5 RP 5′-TCAAATGCTCCAAAAACTCCC-3′.
ef1α: FP 5′-GAGAAGGAAGCCGCTGAGAT-3′ and RP 5′-GCATCAAGGGCATCAAGAAG-3′.

Morpholino and mRNAs injections

Swap70b MOs were designed and synthesised by GeneTools (Philomath, USA): AUG MOs: 5′-TGAGAAGCTCGTCCCTTAGTCC**CAT**-3′ (targeting the translation initiation site shown in bold); splice MO1: 5′-AGAGCAAAACGACAAACCTTCAGCT-3′ (targeting splice-donor site of exon 1); splice MO2: 5′-TGAATCATCTCTAACCTTGAATCCA-3′ (targeting splice-donor site of exon 6); and control MO 5′-CCTCTTACCTCAGTTACAATTTATA-3′. The *wnt11* and *def6a* MOs have been described previously (Lele et al., 2001; Goudevenou et al., 2011). Synthetic mRNA was prepared using mMessage mMachine (Ambion). MOs and mRNAs were injected into 2-cell stage zebrafish embryos and independent rounds of injections were repeated for at least three times.

Data quantification

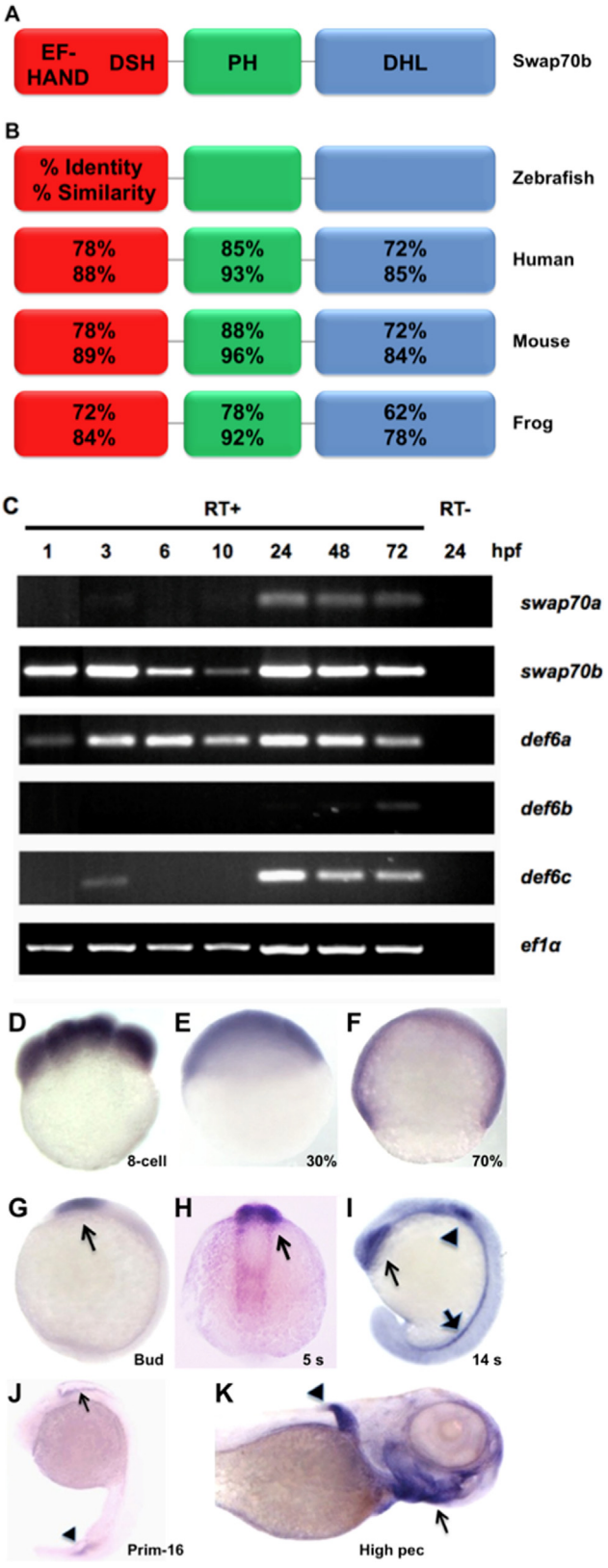
Images were captured using a Nikon-DS5M camera, a NIKON DS-1 control unit and Nikon ACT-2U 1.40 software. Measurements of angles and length were taken using the same magnification and ratios were calculated as described. Two-tailed Student's *t*-tests were used to determine statistical significance of quantitative data.

Results

Expression of swap70b during early zebrafish development

According to the Ensembl database (http://www.ensembl.org) five *swap70*/*def6* paralogues have been identified in zebrafish (protein family ID ENSFM00250000001889; Suppl. Table 1). Phylogenetic analysis revealed that *ENSDARG00000012247* gene

represents the zebrafish orthologue of human and mouse *def6* (referred to as *def6a*) whereas *ENSDARG00000044524* and *ENSDARG00000034717* are *def6* co-orthologues (referred to as *defb* and *def6c* respectively; see Suppl. Table 1) (Goudevenou et al., 2011). Similarly, *ENSDARG00000057286* gene (referred to as *swap70b*) is the orthologue closest to human and mouse *swap70* and *ENSDARG00000051819* is a *swap70* co-orthologue (referred to as *swap70a*) (Shuen et al., in preparation). Accordingly, the predicted amino acid sequence of Swap70b shares high sequence identity and



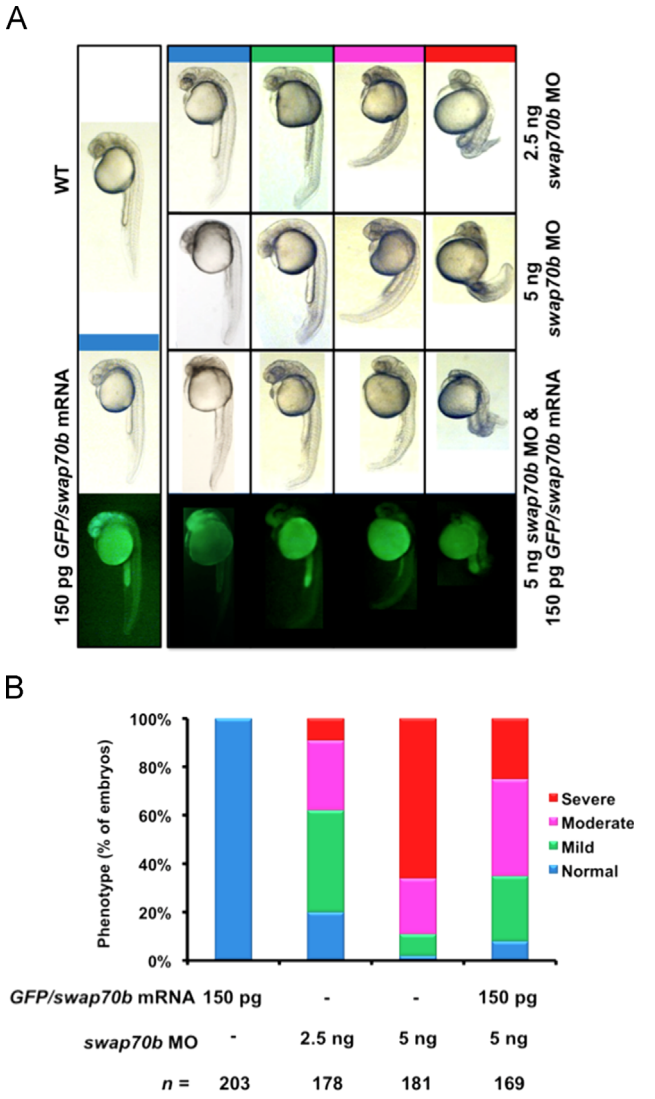


Fig. 2. Swap70 morphants exhibiting reduced length of the body axis can be rescued by ectopic expression of GFP/Swap70b fusion protein. (A) Zebrafish embryos were either un-injected (WT) or injected with *GFP/swap70b* mRNA (150 pg), *swap70b* MO1 (2.5 ng, 5 ng) or co-injected with *GFP/swap70b* mRNA (150 pg) and *swap70b* MO1 (5 ng). Swap70 morphants at 24 hpf were grouped according to the severity of their morphological phenotypes into normal (blue), mild (green), moderate (pink) and severe (red). Images of representative embryos are shown. (B) Quantification of the phenotypic analysis. *n*: total number of embryos derived from at least three independent experiments.

Fig. 1. Swap70b expression during zebrafish development. (A) Schematic representation of zebrafish Swap70b with putative Ca^{2+}-binding EF-hand motifs at the N-terminus followed by a Def6/Swap70 homology (DSH) domain, a pleckstrin-homology (PH) domain at the centre, and a Dbl-homology-like (DHL) domain that contains a coiled coil motif at the C-terminus. (B) The numbers refer to amino acid identities and similarities between the different orthologues. (C) Expression profiles of *swap70*/*def6* paralogues during zebrafish development. RT-PCR was performed to indicate the expression of five *swap70*/*def6* paralogues at various developmental stages. hpf: hours post-fertilisation. PCR reactions without reverse transcription (RT-) are shown for 24 hpf only and *ef1α* expression served as an internal control. (D–J) *In situ* hybridisation with a *swap70b*-specific antisense probe at different developmental stages: (D) 8-cell; (E) 30% epiboly (30%); (F) 70% epiboly (70%); (G) bud; (H) 5 somites (5 s); (I) 14 somites (14 s); (J) primordium 16 (prim-16) and (K) high pectoral (high pec). Sense control probes did not give rise to any signal (data not shown).

similarity with its human, mouse and frog counterparts (Fig. 1B, Suppl. Fig. 1) and exhibits N-terminal EF-hand motifs, a central PH domain and C-terminal dbl-homology-like (DHL) domain containing a coiled coil motif (Fig. 1A). The region between EF-hand motifs and PH domain is highly conserved between Def6 and Swap70 and is referred to as Def6-Swap70 Homology (DSH) domain (Mavrakis et al., 2004). Phylogenetic analysis confirms the close relationship between Def6a/Swap70b and demonstrates that this novel GEF family is clearly distinct from other Rho GEFs in zebrafish such as WGEF and Vav2 (Suppl. Fig. 2).

RT-PCR analysis showed that all five genes are differentially expressed during early zebrafish development (Fig. 1C). While *swap70a* and *def6c* were not or weakly expressed early in development, expression of both was readily detectable at 24, 48 and 72 hpf. Expression of *def6b* was barely detectable until 72 hpf. In contrast, *def6a* and *swap70b* were both expressed from 1 hpf to 72 hpf indicating that both genes are maternally expressed (albeit at different levels; see 1 hpf) and zygotic expression of *def6a* and *swap70b* is apparent during gastrulation and segmentation (Fig. 1C; Goudevenou et al., 2011).

Whole mount *in situ* hybridisation using a *swap70b* antisense probe confirmed maternal expression of *swap70b* (Fig. 1D) and indicated ubiquitous expression of *swap70b* at blastula and gastrula stages (Fig. 1E and F). At tail-bud and 5 somites stage, *swap70b* expression was restricted to optic primordium (Fig. 1G and H) and at late segmentation *swap70b* expression was detectable in optic primordium, otic vesicle, and ventral mesoderm (Fig. 1I). At pharyngula stage, *swap70b* showed expression in caudal vein (Fig. 1J) and at hatching stage, *swap70b* transcripts were mainly observed in pectoral fins as well as pharyngeal arches (Fig. 1K). A *swap70b* sense probe did not give rise to any signal (data not shown).

Swap70b knockdown causes aberrant development

Our previous analysis of Def6a function revealed that that Def6a is required for convergent and extension cell movements during zebrafish gastrulation downstream of Wnt5b (Goudevenou et al., 2011). To address Swap70b function during zebrafish development, two different splice morpholinos (splice MO1 and 2) and an AUG morpholino were used to target *swap70b* mRNA splicing or translation (see M & M for details). Splice MO1 targeted the splice donor site of exon 1 resulting in the formation of mRNA containing intron 1. Splice MO2 targeted exon 6–intron 6 boundary resulting in mRNA lacking exon 6 as confirmed by RT-PCR (Suppl. Fig. 3). In both cases premature stop codons either in intron 1 or

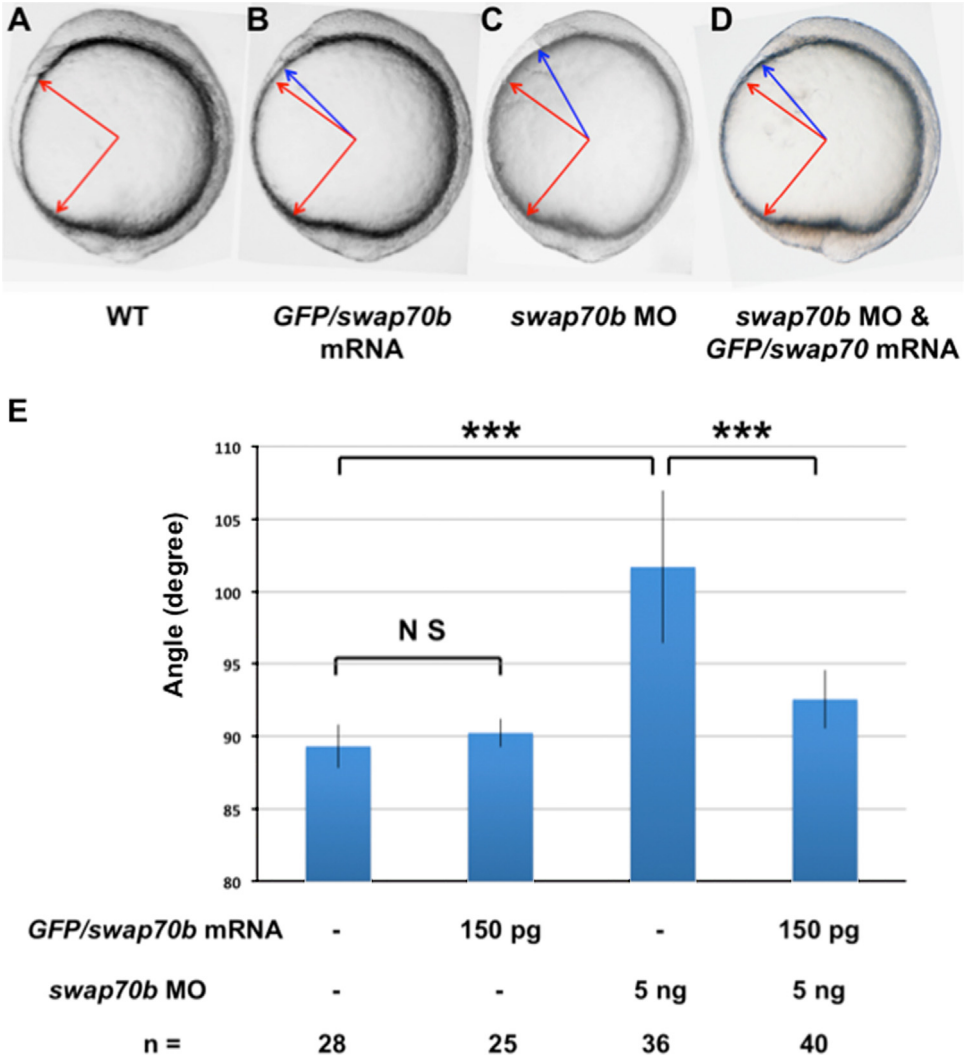


Fig. 3. Swap70b morphants exhibit a shorter body axis. Zebrafish embryos either un-injected (WT; A) or injected with *GFP/swap70b* mRNA (150 pg) (B), with *swap70b* MO1 (5 ng) alone (C), or co-injected with *GFP/swap70b* mRNA (150 pg) and *swap70b* MO1 (5 ng) (D) are shown at 10 1/3 hpf. (E) To assess the length of the body axis the angle between anterior and posterior ends was measured in wild type (WT; red arrows) and compared to the angle in morphant embryos (blue arrows). Standard deviation (error bars) is shown. Two-tailed Student's *t*-test was performed to establish significance. ***: $P < 0.001$. N.S.: not significantly different.

exon 7 (that is out-of-frame with exon 5) predicted a truncation of the Swap70b protein. The AUG MO targeted the translational start codon and its adjacent 5′ sequence predicting a lack of Swap70b protein.

Initial experiments injecting different amounts (2.5, 5, 7.5 or 10 ng) of splice MO1 or MO2, respectively, revealed a similar range of phenotypes in Swap70b morphants at 24 hpf (Fig. 2 and Suppl. Figs. 4–6). Swap70b morphants exhibited morphological alterations in brain, eyes, yolk extension and tail. In addition, the body axes of Swap70b morphants appeared shorter compared to wild type embryos (Fig. 2 and Suppl. Figs. 4–6). With increasing amounts of MO injected the phenotype became more severe and embryonic lethality increased (Fig. 2 and Suppl. Figs. 4–6). However, severity including embryonic death was much higher when splice MO1 was injected compared to splice MO2 (Suppl. Figs. 4–6) suggesting that splice MO2 injection resulted in a truncated Swap70b protein with residual function and milder phenotype whereas injection of splice MO1 resulted in a non-functional truncated protein. This is in line with recent data by Takada and Appel (2011), showing that targeting splice donor site of exon 5 resulted in a mild phenotype affecting neural precursor proliferation and differentiation. Indeed, embryos injected with 10 ng AUG MO exhibited a similar phenotype as embryos injected with splice MO1 (data not shown) supporting the notion that lack of Swap70b function results in aberrant early embryonic development.

To further ensure specificity of the observed phenotypes, splice MO1 and 2 were co-injected in low amounts (1.5 ng each) to test for synergy. The resulting Swap70b morphants exhibited similar phenotypes at 24 hpf to embryos that had been injected with either 2.5 ng splice MO1 or 7.5 ng splice MO2 (Suppl. Figs. 4–6). In addition, co-injection of 150 pg *GFP/swap70b* mRNA with 5 ng splice MO1 partially rescued the phenotype of Swap70b morphants, while injection of 150 pg of GFP/*swap70b* mRNA on its own did not cause obvious defects to early zebrafish embryogenesis (Fig. 2).

Taken together, these results strongly suggest that the observed phenotype at 24 hpf is specific to Swap70b knockdown.

Swap70b is required for convergent and extension cell movement during gastrulation

Having established specificity of swap70b knockdown, we asked whether early zebrafish development is affected in Swap70b morphants. Using *swap70b* splice MO1, we analysed injected

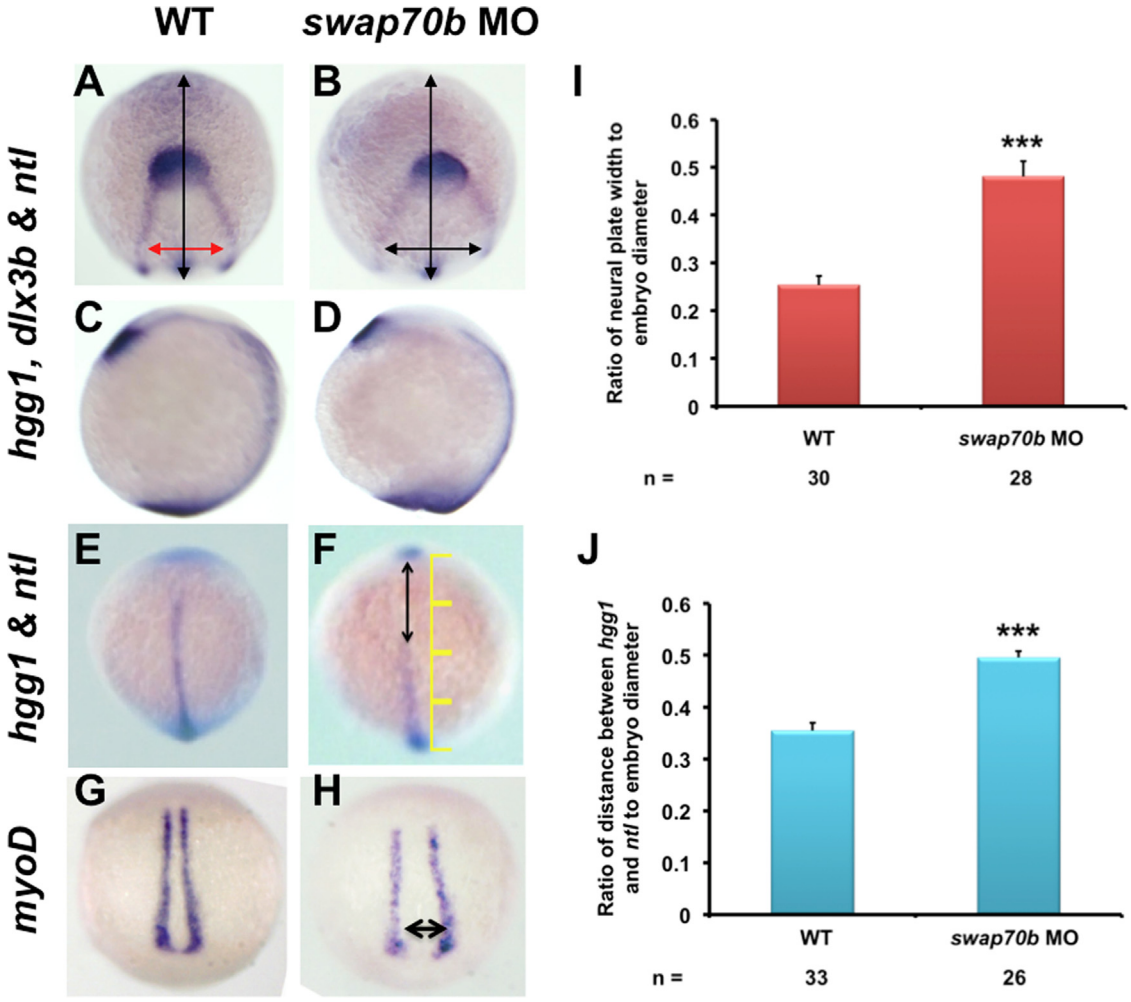


Fig. 4. CE cell movements are impaired in *swap70b* morphants. *In situ* hybridisation of un-injected embryos (A, C, E, G) and embryos injected with 5 ng *swap70b* MO1 (B, D, F, H) were performed at tail-bud stage with *hgg1* (marker of the polster/prechordal plate), *ntl* (marker of the notochord) and *dlx3b* (marker of the edges of the neural plate). Medio-lateral narrowing (convergence) was quantified by measuring the ratio (I) of width of the *dlx3b* staining (red/black double-headed arrow) at a constant distance (1/4 of the embryo diameter) from the *dlx3b* arc to the embryo width (vertical black arrow). The extent of anterior–posterior lengthening (extension) was quantified by measuring the ratio (J) of the distance between *hgg1* and *ntl* (double-headed arrow) to the embryo width (yellow scale). Two-tailed Student's *t*-tests were performed to determine statistical significance. ***: $P < 0.001$. *In situ* hybridisation with a *myoD* (G, H) revealed widening of the body axis (double-headed arrow) in Swab70b morphants.

embryos before (up to 50% epiboly) and after gastrulation at one somite stage. While Swap70b morphants were indistinguishable from wild type embryos before gastrulation (data not shown), they exhibited a significant shortening of their body axes after gastrulation indicated by a significant increase of the angle between the anterior (A) and posterior (P) ends of Swap70b morphants compared to wild type embryos (Fig. 3A, C and E). Co-injection of 150 pg of *GFP/swap70b* mRNA with 5 ng splice MO1 was sufficient to partially rescue the morphant phenotype (Fig. 3A, D and E) while embryos injected with 150 pg *GFP/swap70b* on its own were indistinguishable from wild type embryos (Fig. 3A, B and E).

Given that shortened body axis can be a result of impaired convergent and extension (CE) cell movements as observed for mutants in the non-canonical Wnt signalling pathway including Def6a (Goudevenou et al., 2011), we utilised marker gene expression to analyse CE movements in Swap70b morphants. Whole mount *in situ* hybridisation at tail-bud stage with *dlx3b* (distal-less homeobox gene 3b), which labels the borders of neural and non-neural ectoderm, *hgg1* (hatching gland 1) which marks the polster the anterior-most end of the prechordal plate, and no-tail (*ntl*) specific for the notochord, indicated that Swap70b morphants showed a significant increase in the ratio of neural plate width to embryo diameter (Fig. 4A, B and I). Similarly, the distance between the polster, marked with *hgg1*, and the notochord, marked with *ntl*, was significantly increased in Swap70b morphants (Fig. 4E, F and J). In addition, analysis of *myoD* expression at tail-bud stage revealed that the two stripes of adaxial cells of the paraxial mesoderm were medio-laterally expanded and also appeared shortened anterio-posteriorly in Swap70b morphants (Fig. 4G and H). At 24 hpf the morphant embryos exhibited a shortened body axis and curved tail, as described above, but the notochord did not exhibit undulation (data not shown) observed in some mutants with CE movement defects including Def6a (Goudevenou et al., 2011).

In order to clarify whether the observed phenotype was indeed caused by impaired CE movement or alternatively, through aberrant cell fate specification, we analysed Swap70b morphants by *in situ* hybridisation using a series of dorsal, ventral and mesodermal markers. As shown in Fig. 5, expression of the dorsal markers *chd* (chordin), *gsc* (goosecoid), ventral marker *bmp2b* (bone morphogenetic protein 2b) and non-axial mesodermal marker *cdx4* (caudal homeobox transcription factor 4) in Swap70b morphants was indistinguishable from wild type embryos (Fig. 5A–H). Similarly, *pax2* (paired box gene 2), marking the presumptive midbrain–hindbrain boundary was expressed in the Swap70b morphants but its expression domains were laterally expanded and posteriorly shifted, indicating a decreased convergence of the neural plate (Fig. 5I–L), consistent with the results described above (Fig. 4).

Taken together, these results strongly suggest that Swap70b has no obvious role in cell fate specification but is required for CE cell movement during gastrulation.

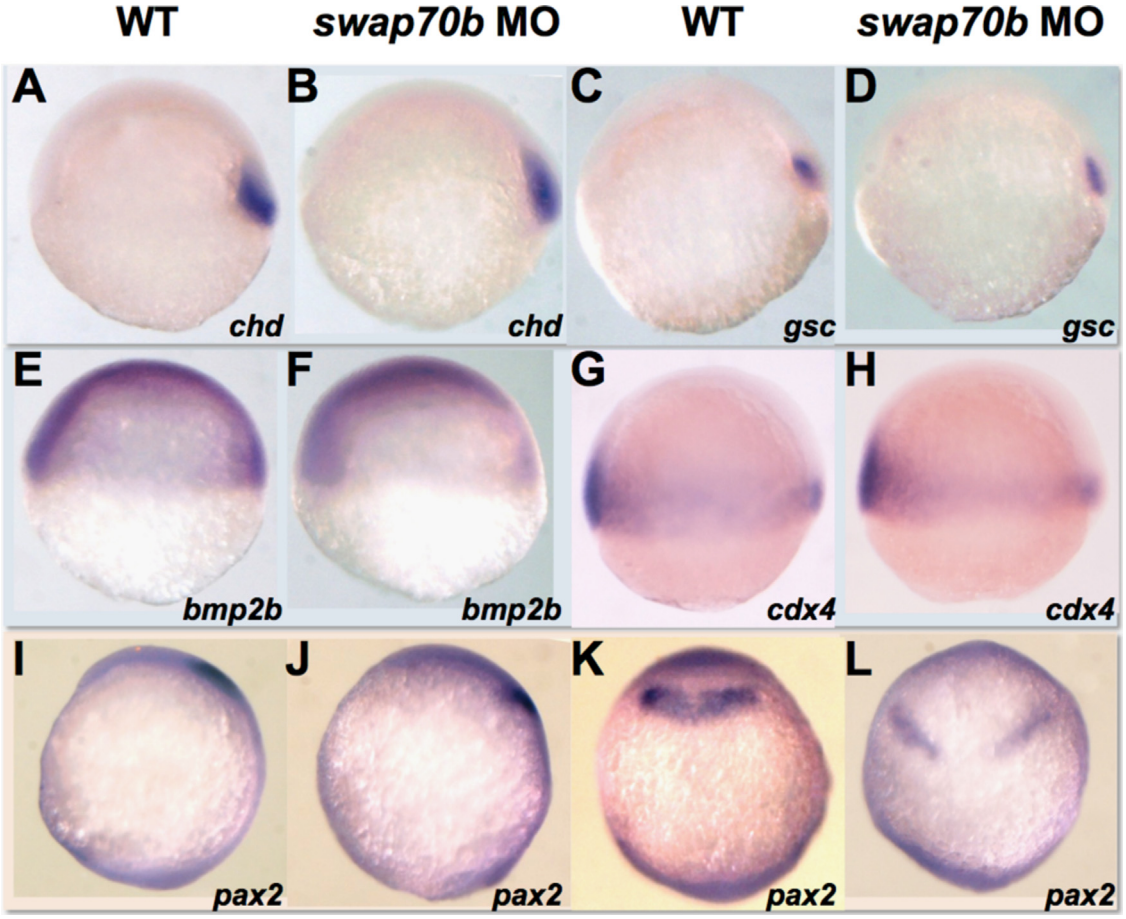


Fig. 5. Cell fate specification appears normal in swap70b morphants. Un-injected and 5 ng *swap70b* MO1-injected embryos at 6 hpf were hybridised with various probes: *Chordin*, *chd* (A, B); *goosecoid*, *gsc* (C, D); *bone morphogenetic protein 2b*, *bmp2b* (E, F); *caudal homeobox transcription factor 4*, *cdx4* (G, H). At tail-bud stage, the expression pattern of an anterior specific gene *pax2* shifted posteriorly and became broader in Swap70b morphants, but was still in the midbrain-hindbrain boundary (I–L). Lateral views (A–J) and dorsal views (K, L) with anterior to the top are shown.

Swap70b modulates CE cell movement downstream of Wnt11 and upstream of RhoA in the non-canonical Wnt/PCP pathway

We had previously shown that the Swap70b related protein Def6a was required for CE cell movements regulated through Wnt5b signalling, but that *def6a* overexpression failed to rescue Wnt11 morphants (Goudevenou et al., 2011). Therefore we tested whether Swap70b function was sufficient to compensate for Wnt11-deficiency. Co-injection of 5 ng *wnt11* MO with various amounts of *GFP/swap70b* mRNA revealed that 75 pg of *GFP/swap70b* mRNA was sufficient to partially rescue Wnt11 morphants. As shown in Fig. 6(A–D), the anterior–posterior body axes of co-injected embryos were significantly longer compared to embryos injected with *wnt11* MO alone (as indicated by reduced angle between the anterior and posterior ends of the embryos). In addition, *in situ* hybridisation with *hgg1*, *dlx3b* and *ntl* indicated

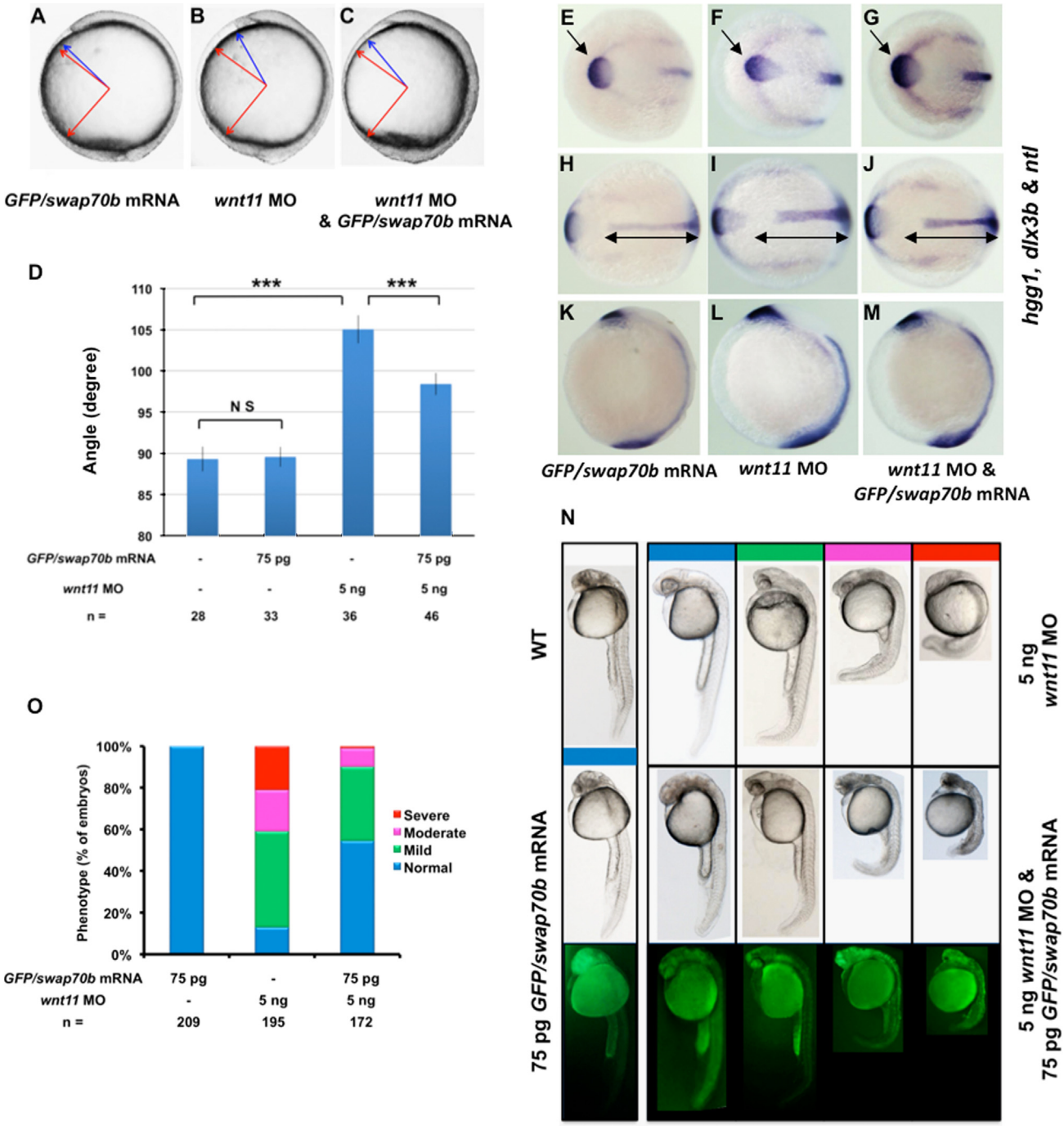


Fig. 6. Swap70b acts downstream of Wnt11. Zebrafish embryos at 2-cell stage were injected with *GFP/swap70b* mRNA (75 pg) (A), *wnt11* MO (5 ng) alone (B), or co-injected with *GFP/swap70b* mRNA (75 pg) and *wnt11* MO (5 ng) (C). (D) Quantification of A-P axis extension at $10\frac{1}{3}$ hpf. The angle between the most anterior and posterior embryonic structure was measured and quantified as described in Fig. 3. Two-tailed Student's *t*-tests were used to determine statistical significance. ***: $P < 0.001$. (E–M) *In situ* hybridisation with *hgg1*, *dlx3b* and *ntl* as indicated. Black double-headed arrows in H–J are given as reference to the WT. Arrows mark the *hgg1* expression domain indicating the altered position and shape of the polster/prechordal plate in Wnt11 morphants (F) that is essentially rescued by ectopic expression of GFP/Swap70b (G). (N) At 24 hpf stage, embryos were grouped into normal (blue), mild (green), moderate (pink) and severe (red) according to their morphological phenotype. Representative images of embryos are shown. Each panel of the bottom two rows depict identical embryos. Green fluorescence in yolk and yolk extension is due to auto-fluorescence. (O) Bar graphs show the percentage of embryos with normal, mild, moderate and severe phenotypes. *n*: total number of embryos derived from at least three independent experiments.

that ectopic Swap70b expression essentially reversed the characteristic phenotype (altered position and shape) of the polster/prechordal plate in Wnt11 morphants (Fig. 6E–M). Similarly, co-injected embryos exhibited a much less severe phenotype at 24 hpf compared to Wnt11 morphants (Fig. 6N and O) with more than 50% of embryos exhibiting a normal morphology. As expected, 75 pg (like 150 pg; see Fig. 2) of *GFP/swap70b* mRNA on its own did not give rise to any phenotype (Fig. 6D and O). However, when we co-injected 150 pg of *GFP/swap70b* mRNA with 5 ng of *wnt11* MO, the phenotype in Wnt11 morphants at 24 hpf appeared more severe (data not shown) suggesting that Wnt11 signalling is modulated through Swap70b and that a balanced Swap70b expression level is essential for appropriate downstream effects.

To further establish whether Swap70b acts downstream of Wnt11 in the non-canonical Wnt/PCP signalling pathway we asked whether Swap70b acts upstream of RhoA. RhoA is an essential component of the non-canonical Wnt signalling pathway downstream of Wnt11 and upstream of its effector Rok2 (Marlow et al., 2002; Jopling and Hertog, 2005; Zhu et al., 2006). In addition, direct interaction of Swap70 with RhoA has been shown in

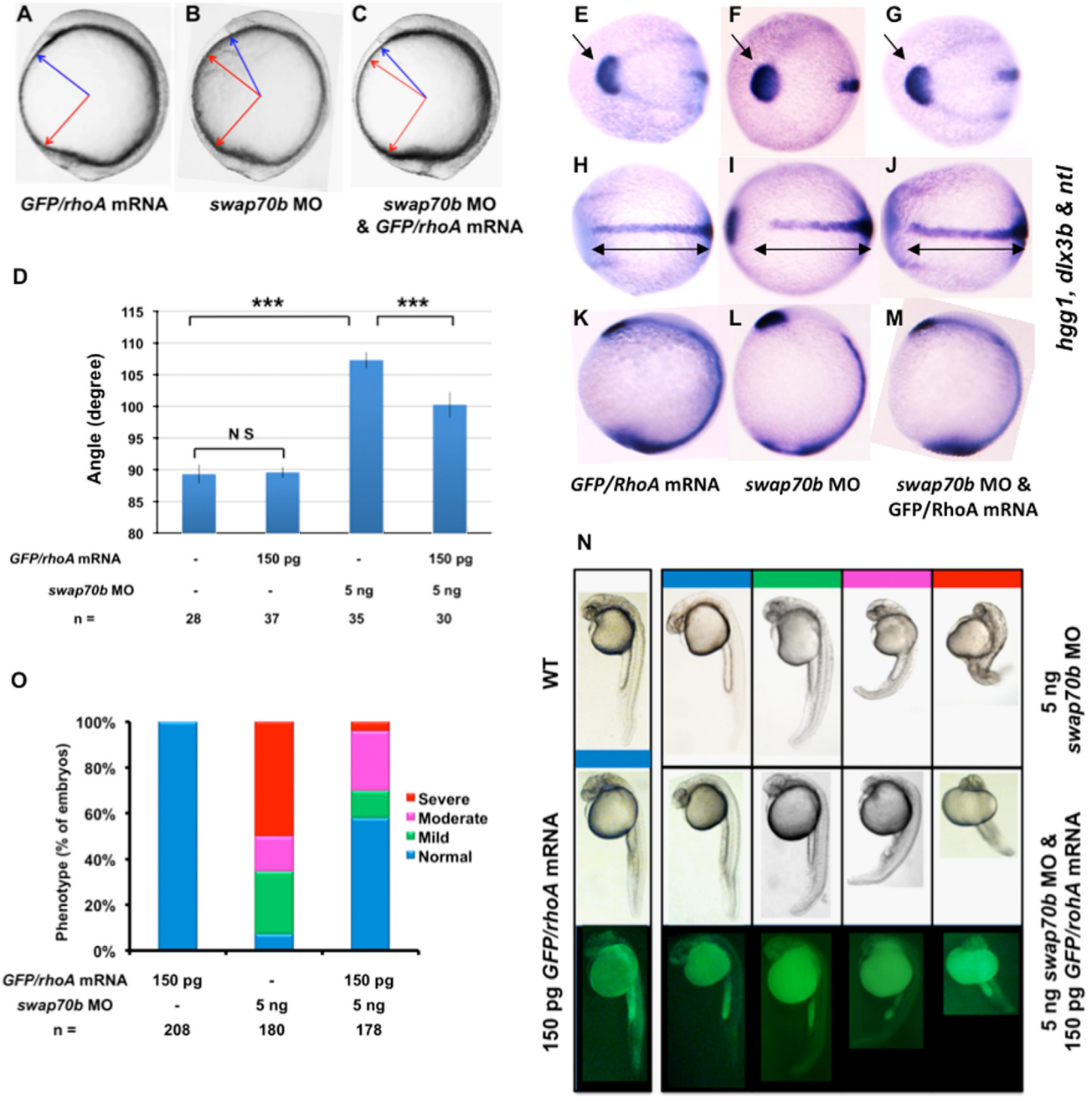


Fig. 7. Swap70b modulates CE cell movements via RhoA. Zebrafish embryos at 2-cell stage were injected with *GFP/rhoA* mRNA (150 pg) (A), *swap70b* MO (5 ng) alone (B), or co-injected with *GFP/rhoA* mRNA (150 pg) and *swap70b* (5 ng) (C). (D) Quantification of A-P axis extension at $10\frac{1}{3}$ hpf. The angle between the most anterior and posterior embryonic structure was measured and quantified as described in Fig. 3. Two-tailed Student's *t*-tests were used to determine statistical significance. ***: $P < 0.001$. (E–M) *In situ* hybridisation with *hgg1*, *dlx3b* and *ntl* as indicated. Black double-headed arrows in H–J are given as reference to the WT. Arrows mark the *hgg1* expression domain indicating the altered position and shape of the polster/prechordal plate in Swap70b morphants (F) that is essentially rescued by ectopic expression of GFP/RhoA (G). (N) At 24 hpf stage, embryos were grouped into normal (blue), mild (green), moderate (pink) and severe (red) according to their morphological phenotype. Representative images of embryos are shown. (O) Bar graphs show the percentage of embryos with normal, mild, moderate and severe phenotypes. *n*: total number of embryos derived from at least three independent experiments.

different cellular contexts (Ocana-Morgner et al., 2011). Co-injection of 150 pg of *GFP*-tagged *rhoA* mRNA with 5 ng *swap70b* splice MO1 resulted in a partial rescue of the Swap70b morphants. As shown in Fig. 7(A–D), the angle between the anterior and posterior ends of the co-injected embryos was significantly reduced compared to embryos injected with *swap70b* splice MO1 alone. In addition, *in situ* hybridisation with *hgg1*, *dlx3b* and *ntl* indicated that ectopic RhoA expression essentially reversed the altered position and shape of the polster/prechordal plate in Swap70b morphants (Fig. 7E–M). Similarly, the severity of the phenotype observed at 24 hpf was greatly reduced in co-injected embryos with almost 60% of the embryos exhibiting a normal morphology (Fig. 7N and O).

Taken together, these results strongly suggest that Swap70b is an essential component of the non-canonical Wnt/PCP signalling pathway linking Wnt11 signalling and RhoA effector function required for convergent and extension cell movements during gastrulation.

Swap70b and Def6a have distinct but overlapping function in the non-canonical Wnt signalling pathway

We had previously shown that Def6a overexpression was sufficient to rescue Wnt5b morphants but failed to rescue Wnt11 morphants (Goudevenou et al., 2011). Nevertheless, we also showed that co-injection of low amount of *def6a* and *wnt11* MOs resulted in a CE phenotype indicating synergism between Wnt11 signalling and Def6a function (Goudevenou et al., 2011). We therefore asked whether Def6a gain-of-function could compensate for Swap70b loss-of-function and vice versa, i.e. whether Swap70b overexpression could rescue Def6a morphants.

As shown in Fig. 8, Swap70b overexpression resulted in a robust rescue of Def6a morphants. Co-injection of 2.5 ng *def6a* MO with 150 pg *GFP*-tagged *swap70b* mRNA resulted in a significant reduction in the neural plate width at tail-bud stage compared to embryos injected with *def6a* MOs alone (Fig. 8A–D). As shown before (Fig. 2) injection of 150 pg *GFP*/*swap70b* mRNA did not give rise to any phenotype (Fig. 8A and D). At 24 hpf a robust rescue of the Def6a morphants through Swap70b overexpression was also apparent with 60% of embryos exhibiting a normal morphology compared to 10% of Def6a morphants (Fig. 8E and F). The reciprocal experiment, shown in Fig. 9, demonstrated that Def6a overexpression resulted in partial rescue of Swap70b morphants. Co-injection of 5 ng *swap70b* MO with 300 pg *GFP*-tagged *def6a* mRNA resulted in a significant reduction of neural plate width at tail-bud stage (as indicated by the expression domains of *hgg1* and *dlx3b*) compared to the greatly increased width in Swap70b morphants (Fig. 9A–D). Embryos injected with *GFP*/*def6a* mRNA alone did

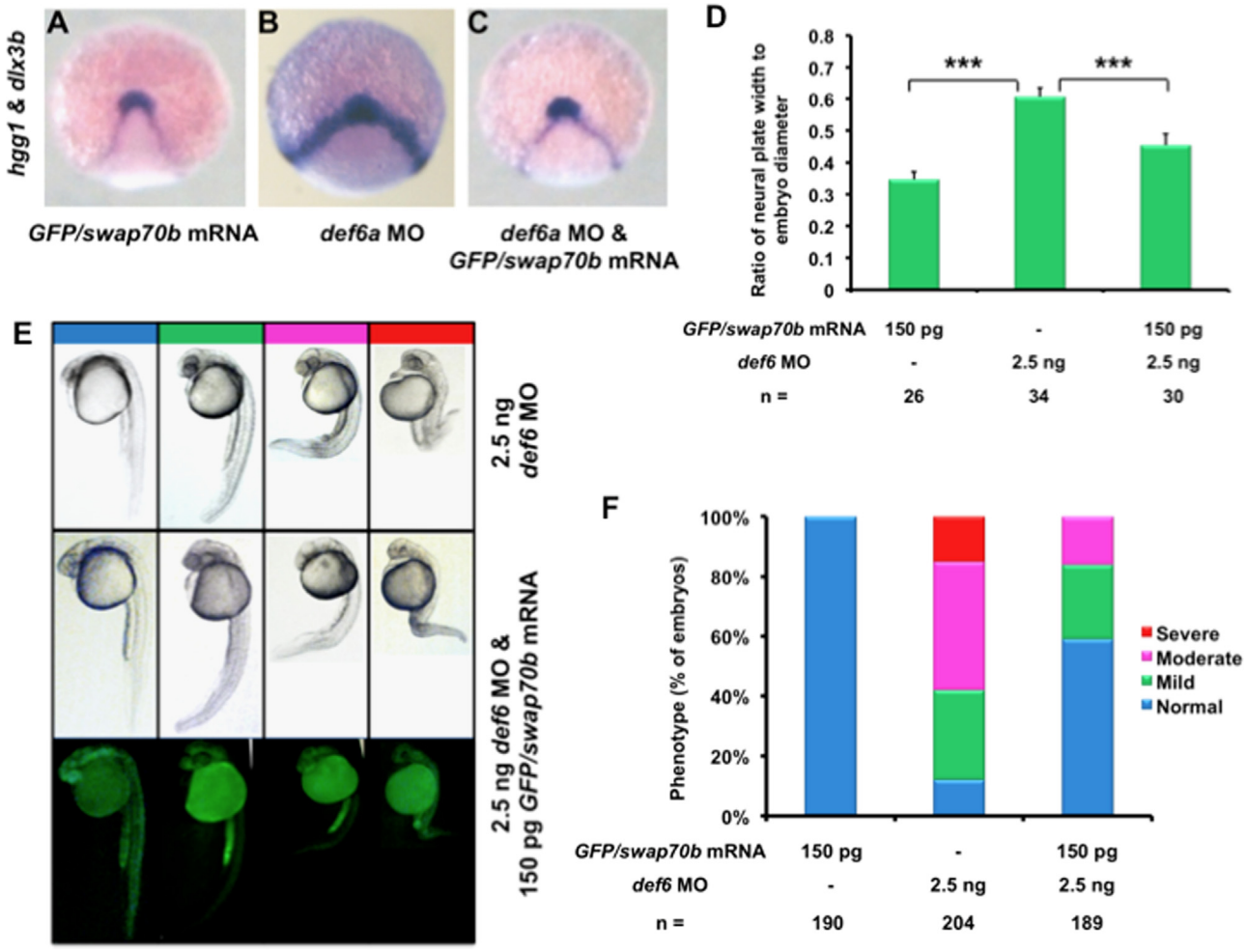


Fig. 8. Ectopic GFP/*Swap70b* expression rescued Def6a morphants. Embryos at 2-cell stage were injected with *GFP*/*swap70b* mRNA (150 pg) (A), *def6a* MO (2.5 ng) alone (B), or *GFP*/*swap70b* mRNA (150 pg) and *def6a* MO (2.5 ng) (C) and were stained at tail-bud stage with *hgg1* and *dlx3b* to evaluate CE using the same quantification method described above (Fig. 4I). (D) Two-tailed Student's *t*-tests were used to determine statistical significance. ***: $P < 0.001$. (E) Later at 24 hpf, embryos were grouped into normal (blue), mild (green), moderate (pink) and severe (red) according to their morphological phenotype. Representative images of embryos are shown. (F) Bar graphs show the percentage of embryos with normal, mild, moderate and severe phenotypes. Only 1/189 of the rescued embryos exhibited a severe phenotype as shown in E. *n*: total number of embryos derived from at least three independent experiments.

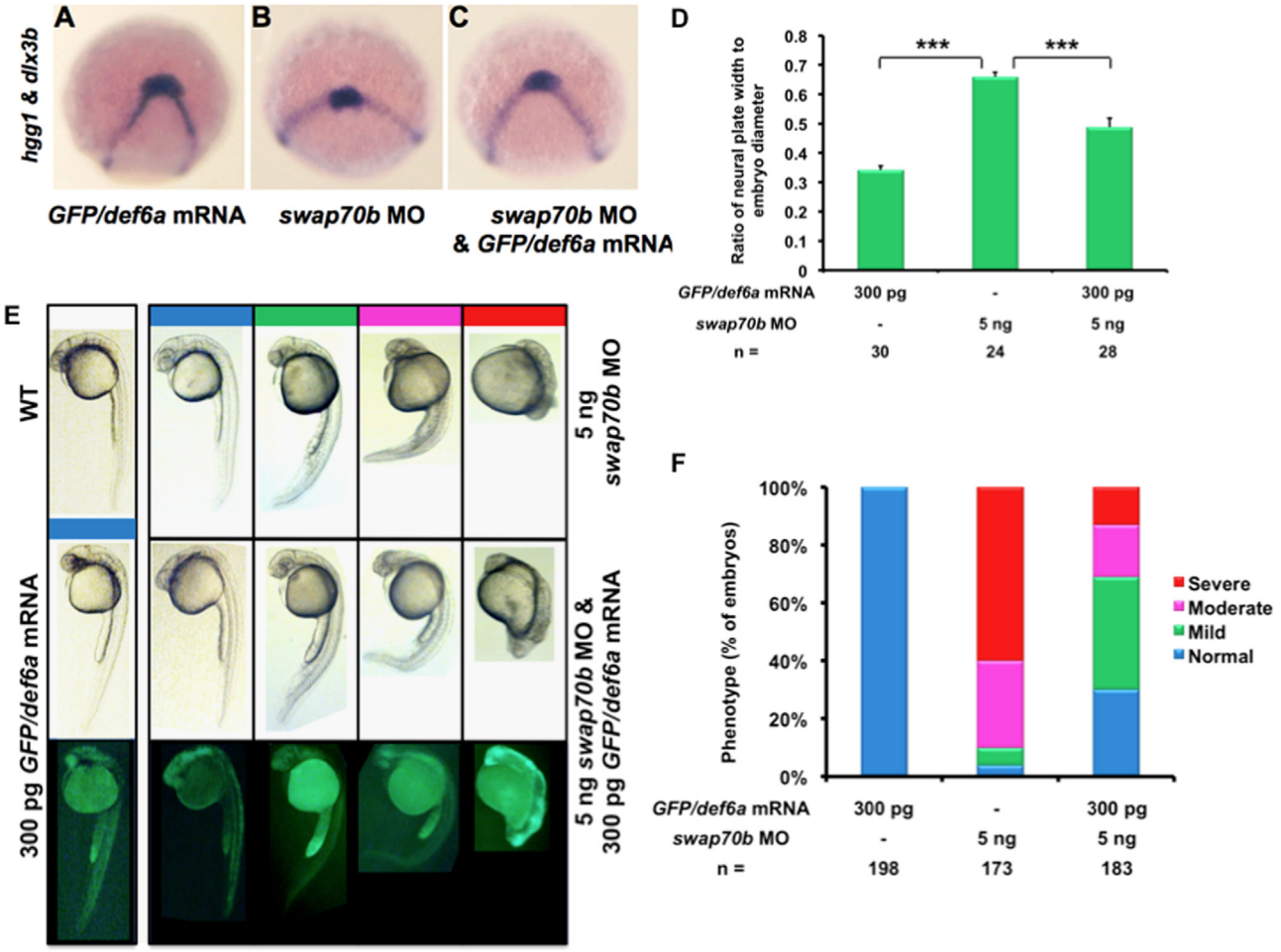


Fig. 9. Ectopic GFP/Def6a expression rescued Swap70b morphants. Embryos at 2-cell stage were injected with *GFP/def6a* mRNA (300 pg) (A), *swap70b* MO (5 ng) alone (B), or *GFP/def6a* mRNA (300 pg) and *swap70b* MO (5 ng) (C) and were stained at tail-bud stage with *hgg1* and *dlx3b* to evaluate CE using the same quantification method described above (Fig. 4I). (D) Two-tailed Student's *t*-tests were used to determine statistical significance. ***: $P < 0.001$. (E) Embryos at 24 hpf were grouped into normal (blue), mild (green), moderate (pink) and severe (red) according to their morphological phenotype. Representative images of embryos are shown. (F) Bar graphs show the percentage of embryos with normal, mild, moderate and severe phenotypes. *n*: total number of embryos derived from at least three independent experiments.

not show any phenotype (Fig. 9A). Similarly, the severity of morphological alterations was reduced in morphants co-injected with *swap70b* splice MO1 and *GFP/def6a* mRNA at 24 hpf compared to Swap70b morphants (Fig. 9E and F) indicating that Def6a can partially rescue Swap70b morphants. These data show that Swap70b and Def6a can partially rescue each other when expressed ectopically likely do to their similarity in structure and function. However, co-injection of *swap70b* and *def6a* MOs resulted in an additive effect (Fig. 10). Position and shape of the polster/prechordal plate (labelled by *hgg1*) that was altered in Def6a as well as Swap70b morphants (Fig. 10B and C compared to A) was more severely affected (Fig. 10D compared to B and C). To determine whether these differences were statistically significant, we established the ratio of prechordal plate length to embryo diameter (Fig. 10M). While the wild type embryos ($n=32$) had a ratio of 0.19 ± 0.02, Def6a morphants ($n=32$) exhibited a ratio of 0.31 ± 0.06 and Swap70b morphants ($n=32$) a ratio of 0.29 ± 0.05; both statistically different from wild type ($p < 0.001$). Double mutant morphants ($n=51$) showed a ratio of 0.35 ± 0.08 that was significantly different from Def6a ($p < 0.01$) and Swap70b ($p < 0.001$) morphants (Fig. 10M). In addition, the body axes of double mutant morphants was further reduced as indicated by the length of the notochord (Fig.10E and F). Similarly, co-injection of *wnt5b* and *swap70b* MOs resulted in an additive effect enhancing the CE phenotype (Suppl. Fig. 7). It seems therefore that Swap70b and Def6a have distinct functions during CE cell movements in zebrafish likely to delineate the Wnt11 and Wnt5b signalling pathways.

Discussion

Swap70b function in the non-canonical Wnt/PCP pathway

The results presented above demonstrate a novel function for Swap70b in regulating convergent and extension (CE) cell movements during zebrafish gastrulation. We show here that Swap70b acts downstream of Wnt11 signalling and upstream of RhoA effector function establishing a crucial role for Swap70b in the non-canonical Wnt/PCP signalling pathway.

The importance of the non-canonical Wnt/PCP pathway in CE cell movements during gastrulation in zebrafish was discovered through large-scale mutagenesis screens. Several mutants were identified that displayed a shorter and broader body axis at the end of gastrulation indicative of CE movement defects with normal cell fate specification (Driever et al., 1996; Haffter et al., 1996; Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996; Heisenberg and NussleinVolhard, 1997). Two of these mutants named *silberblick* (*slb*) and *pipetail* (*ppt*) were alleles of *wnt11* and *wnt5b* (previous name wnt5a, renamed after Stoick-Cooper et al. (2007)), respectively (Heisenberg et al., 2000; (Rauch et al., 1997).

Wnt11 induces the accumulation of its receptor Fz7 and its intracellular mediator Dsh at distinct sites of cell contacts (Witzel et al., 2006). Direct binding of Wnt11 to Fz7 may be required to trigger this accumulation (Heisenberg et al., 2000; Djiane et al., 2000). In addition, relocation of Dsh from the cytoplasm to sites of

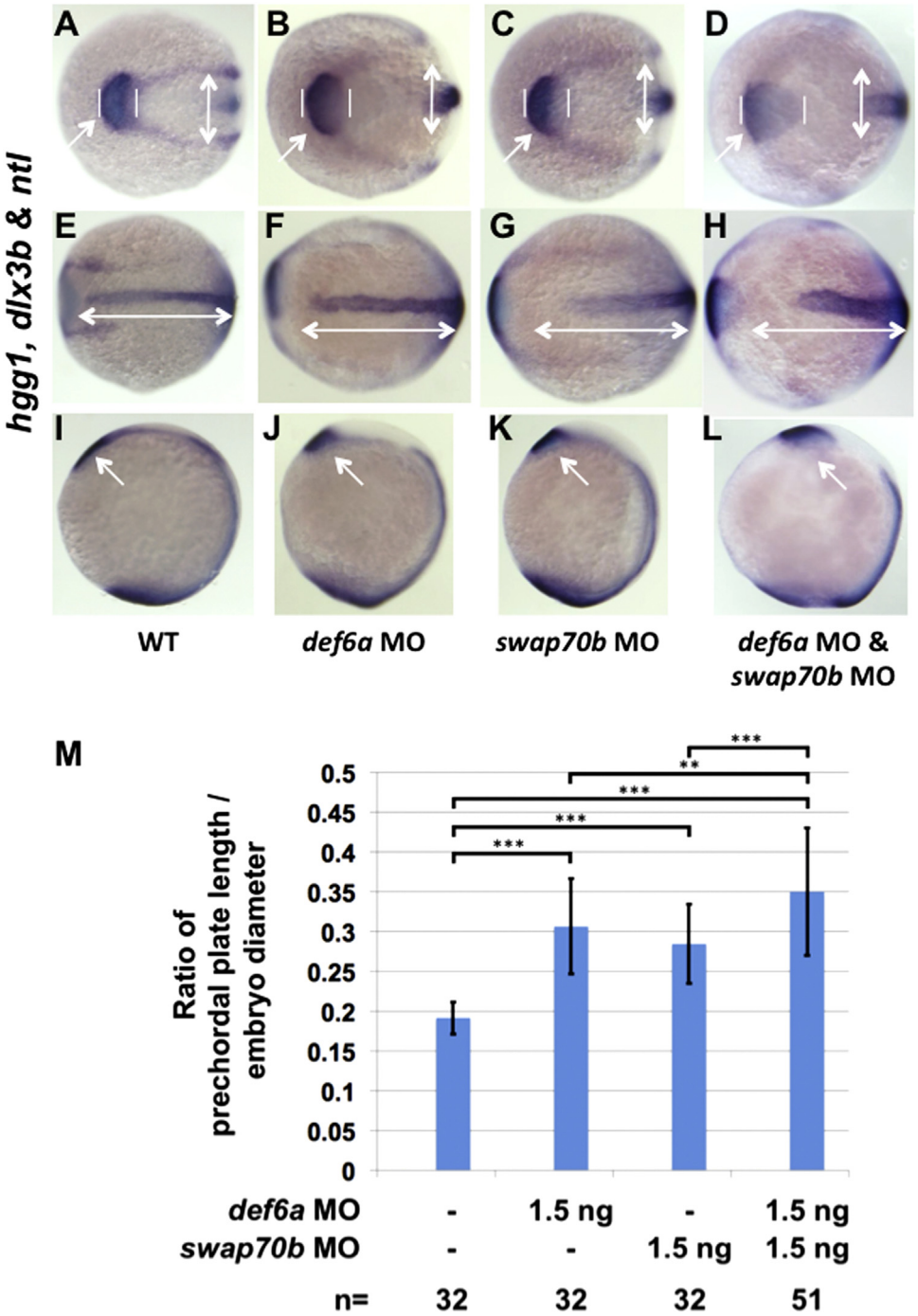


Fig. 10. Def6a and Swap70b have distinct functions in CE cell movement. *In situ* hybridisation with *hgg1*, *dlx3b* and *ntl* as indicated comparing WT embryos (A, E, I) with either Def6a (B, F, J), Swap70b (C, G, K) single morphants or Def6a/Swap70b double mutants (D, H, L). White double-headed arrows in E–H are given as reference to the WT. Arrows and vertical lines mark the *hgg1* expression domain indicating the altered position and shape of the polster/prechordal plate in Def6a (B, J), Swap70b (C, K) and Def6a/Swap70b (D, L) morphants compared to WT (A, I). (M) Quantification of the altered shape of the polster/prechordal plate. The ratio of prechordal plate length to embryo diameter is shown for wild type embryos (0.19 ± 0.02), Def6a morphants (0.31 ± 0.06), Swap70b morphants (0.29 ± 0.05) and double mutant morphants (0.35 ± 0.08). Two-tailed Student's *t*-tests were used to determine statistical significance. ***: $P < 0.001$. **: $P < 0.01$. Number of embryos (n) in each group analysed as indicated.

Fz7 accumulation appears to occur through direct binding of Dsh to Fz7 (Carreira-Barbosa et al., 2003). Studies in Xenopus embryos showed that Wnt-Fz signalling activates RhoA through activation of Dsh, and a direct interaction of Dsh with the adaptor protein Daam1 (Habas et al., 2001). In zebrafish overexpression of either RhoA or the RhoA effector Rok2 that regulates re-organisation of the actin cytoskeleton required for CE cell movements (Keller, 2002; Tada and Kai, 2009; Roszko et al., 2009; Gray et al., 2011), partially rescues the *wnt11*/*slb* mutant phenotype (Marlow et al., 2002; (Jopling and Hertog, 2005; Zhu et al., 2006). Given that Daam1 lacks GEF activity (Habas et al., 2001), a RhoA-family GEF was postulated and subsequently, it was shown that WGEF was required to activate RhoA in *Xenopus* (Tanegashima et al., 2008). Based on the data presented here showing that Swap70b is downstream of Wnt11 and upstream of RhoA, we propose that in zebrafish, Swap70b is the missing link providing the GEF

activity necessary to activate RhoA. Indeed it was shown that Swap70 regulates Sphingosine-1-phosphate-induced motility and endocytosis of mouse dentritic cells through RhoA activation (Ocana-Morgner et al., 2011). In addition, Wnt11-mediated migration of prechordal plate progenitor cells during zebrafish gastrulation is regulated through Sphingosine-1-phosphate receptors (Kai et al., 2008). Miles apart (Mil) was identified as a suppressor of defective anterior migration of the prospective prechordal plate in *slb/wnt11* mutant embryos (Kai et al., 2008).

Swap70b and Def6a exhibit an overlapping but distinct function in the non-canonical Wnt signalling pathway

Swap70 and Def6 are atypical GEFs for Rho GTPases sharing a unique domain structure comprising of a pleckstrin homology (PH) domain N-terminal to a dbl homology-like (DH-like) domain distinguishing them from all other known Rho GEFs that exhibit a DH domain N-terminal to a PH domain (Mavrakis et al., 2004; Tybulewicz and Henderson, 2009). We recently showed that Def6a morphants phenocopied CE cell movement defects during gastrulation observed in *ppt/wnt5b* mutants and discovered that Def6a can rescue Wnt5b morphants (Goudevenou et al., 2011). Wnt11 morphants on the other hand could not be rescued through overexpression of Def6a (Goudevenou et al., 2011) suggesting a specific requirement of Def6a downstream of Wnt5b signalling. Here we have shown that Swap70b function is required for Wnt11 signalling upstream of RhoA. Attempts to rescue Wnt5b morphants with overexpression of Swap70b failed and rather enhanced the CE cell movement phenotype characteristic for Wnt5b morphants (data not shown). Similarly, while RhoA overexpression partly rescued Swap70b morphants as shown here, RhoA could not rescue Def6a morphants (Goudevenou et al., 2011) suggesting that Def6a functions through other Rho GTPases than RhoA. Importantly, Swap70b and Def6a double mutant morphants exhibited a far more severe CE phenotype than each single mutant morphant. This result is reminiscent of the data by Kilian et al. (2003), showing that Ppt-Wnt5b/Slb-Wnt11 double mutants showed an additive effect exhibiting a more severe CE cell movement defect than single mutants. It seems therefore that the Swap70b and Def6a perform distinct functions in the non-canonical Wnt signalling pathway, delineating Wnt11/RhoA from Wnt5b signalling. However, a synergistic effect between Wnt11 and Def6a was observed; in particular, a quantity of wnt11 MO or def6 MO that individually resulted in little or no phenotype caused severe CE movement defects specific to wnt11 knockdown when injected in combination (Goudevenou et al., 2011) and as we have shown here, Swap70b overexpression robustly rescued Def6a morphants and Def6a overexpression partially rescued Swap70b morphants. It seems therefore that both proteins have distinct but overlapping functions perhaps explaining the finding that despite their distinct phenotypes, both Slb/Wnt11 and Ppt/Wnt5b act redundantly in the non-canonical Wnt signalling pathway to regulate morphogenetic movements during the course of gastrulation (Kilian et al., 2003).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.020.

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