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Fyn/Yes and non-canonical Wnt signalling converge on RhoA in vertebrate gastrulation cell movements

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Convergent extension (CE) cell movements during gastrulation mediate extension of the anterior–posterior body axis of vertebrate embryos. Non-canonical Wnt5 and Wnt11 signalling is essential for normal CE movements in vertebrate gastrulation. Here, we show that morpholino (MO)-mediated double knockdown of the Fyn and Yes tyrosine kinases in zebrafish embryos impaired normal CE cell movements, resembling the *silberblick* and *pipetail* mutants, caused by mutations in *wnt11* and *wnt5*, respectively. Co-injection of Fyn/Yes- and Wnt11- or Wnt5-MO was synergistic, but *wnt11* or *wnt5* RNA did not rescue the Fyn/Yes knockdown or *vice versa*. Remarkably, active RhoA rescued the Fyn/Yes knockdown as well as the Wnt11 knockdown, indicating that Fyn/Yes and Wnt11 signalling converged on RhoA. Our results show that Fyn and Yes act together with non-canonical Wnt signalling via RhoA in CE cell movements during gastrulation.

Keywords: Fyn; Yes; Wnt; convergence and extension; signalling
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INTRODUCTION

Vertebrate gastrulation is driven by morphogenetic cell movements of the three germ layers. Convergent extension (CE) cell movements within the mesoderm, as a result of mesodermal cell polarization, cause cells to become distributed along the antero-posterior (A-P) axis (Warga & Kimmel, 1990; Keller *et al*, 1992). In vertebrates, this process is regulated by non-canonical Wnt signalling, which is similar to *Drosophila* planar cell polarity (PCP) signalling (Solnica-Krezel & Eaton, 2003; Veeman *et al*, 2003). The zebrafish mutants *silberblick* (*slb*) and *pipetail* (*ppt*) have mutations in Wnt11 (Heisenberg *et al*, 2000) and Wnt5 (Kilian *et al*, 2003), respectively. Non-canonical Wnt11 signalling induces CE cell movements predominantly in the anterior regions of the embryo (Heisenberg *et al*, 1996, 2000), whereas Wnt5 signalling is essential for CE cell movements in the posterior regions (Hammerschmidt *et al*, 1996; Kilian *et al*, 2003). *Slb/ppt* double mutants show severe CE cell-movement defects, and *wnt5* RNA partially rescues the *slb* mutant phenotype (Kilian *et al*, 2003), indicating that Wnt11 and Wnt5 are at least in part functionally redundant. Non-canonical Wnt signalling is initiated by the binding of Wnt5 or Wnt11 to their receptor, Frizzled. In *Xenopus*, this induces formation of a Dishevelled (Dvl)–Daam1–RhoA complex, leading to activation of RhoA and Rac (Habas *et al*, 2001, 2003). Marlow *et al* (2002) showed that, in zebrafish, RhoA activates Rho kinase 2 (Rok2), which mediates cytoskeletal remodelling and CE cell movements. Active Rac activates the Jun kinase pathway in *Xenopus*, which results in transcriptional activation (Habas *et al*, 2003).

Although the role of Src family kinases (SFKs) in cell signalling is well established (Thomas & Brugge, 1997), relatively little is known about their function in embryonic development. All eight SFK genes have been disrupted in the mouse, resulting in relatively mild phenotypes in four cases and no apparent phenotype in the others (Lowell & Soriano, 1996). Src, Fyn and Yes are broadly expressed during mouse development, and double knockout mice either die perinatally (Src/Fyn and Src/Yes) or undergo degenerative renal changes (Fyn/Yes; Stein *et al*, 1994). Dominant-negative mutants of the Src family members, Src, Fyn and Yes, disrupt gastrulation movements in *Xenopus laevis*, resulting in the inability to close the blastopore (Denoyelle *et al*, 2001). We set out to elucidate the role of Src, Fyn and Yes in zebrafish development.

RESULTS AND DISCUSSION

We identified zebrafish Src, Fyn and Yes on the basis of high overall protein sequence identity with their human counterparts (81%, 92% and 85%, respectively; cf. supplementary information online). *In situ* hybridization experiments showed that *src*, *fyn* and *yes* are ubiquitously expressed in zebrafish embryos (supplementary information online and data not shown). Their role in zebrafish development was assessed by injection of morpholinos (MOs) at the one-cell stage, inducing specific knockdown of protein expression (Nasevicius & Ekker, 2000). To focus on the redundant functions of SFKs and to ensure that the MOs did not induce inadvertent nonspecific defects, we titrated the MOs to amounts that did not induce a phenotype on their own, and we co-injected the MOs in pairs to knock down two SFKs at the same time. It is noteworthy that Tsai *et al* (2005) recently found that injection of high amounts of Yes-MO (15 ng) by itself induced

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epiboly defects in zebrafish. Micro-injection of low levels of Fyn-MO or Yes-MO (5 ng each) by itself did not affect early development, whereas co-injection of Fyn-MO and Yes-MO (5 ng each) induced specific defects (Fig 1), characterized by reduced extension of the embryonic axis at the one- and 14-somite stages (Fig 1A–F). At 24 h post fertilization (hpf), the forebrain structures did not extend anteriorly to the eyes (Fig 1G–I) and the eyes were partially or completely fused at 3 days post fertilization (dpf; Fig 1J–L). The Fyn/Yes-MO-induced defects strikingly resembled the *slb* phenotype in the anterior region of the embryo (Heisenberg *et al*, 2000; Ulrich *et al*, 2003). We phenocopied the *slb* mutant using Wnt11-MO (8 ng), as described previously (Lele *et al*, 2001), and compared the Fyn/Yes-MO and Wnt11-MO knockdowns. Indeed, they are highly similar (Fig 1), although the most severe *slb* phenotype, characterized by complete cyclopia, was not observed in the Fyn/Yes knockdown.

We used molecular markers to compare the Fyn/Yes and Wnt11 knockdown phenotypes. In the Fyn/Yes knockdown embryos, the neural plate, expressing *dlx3* at the edges, was wider at 10 hpf and the hatching gland, stained with the *hgg* marker, was located more posteriorly, similar to the Wnt11-MO knockdowns (Fig 1P–R). Moreover, the *sonic hedgehog* (*shh*) expression domain in the midline did not extend as far anteriorly as in the noninjected control, resulting in a gap between *dlx3* and *shh* expression in the Fyn/Yes and Wnt11 knockdown embryos (Fig 1S–U). Knockdown of Wnt11, on the one hand, and Fyn/Yes, on the other, induced highly similar defects, as assessed by analysis of morphology and molecular markers (Fig 1). This indicated that CE movements are impaired after knockdown of Wnt11 and Fyn and Yes.

To assess whether cell migration was impaired as a result of Fyn/Yes knockdown, we performed cell tracing experiments using caged fluorescein dextran. At 6 hpf, the fluorophore was uncaged by a short, localized pulse of ultraviolet light in the dorsal shield to assess extension of axial mesoderm cells (Fig 2A–F,M), and in lateral marginal cells 90° from the dorsal shield to evaluate dorsal migration (convergence; Fig 2G–L,N). Comparison of cell movements in wild-type (WT) and Fyn/Yes knockdown embryos showed that both convergence and extension cell movements during gastrulation were affected in Fyn/Yes-MO-injected embryos. Quantification of the cell movements in ten embryos indicated significant reductions in the anterior extension of axial mesoderm cells (Fig 2M) and even more pronounced effects on the dorsal migration of the lateral marginal cells (Fig 2N). The morphology of cells in 10 hpf embryos in which the fluorophore was uncaged in the dorsal shield at 6 hpf is consistent with a defect in polarization and medio-lateral intercalation of cells in Fyn/Yes knockdown embryos (Fig 2O,P). These results indicate that the defects in Fyn/Yes knockdown embryos are due to defects in CE cell movements.

The defects were specific for Fyn and Yes knockdown, because co-injection of Fyn-MO or Yes-MO with Src-MO (5 ng each) did not induce CE phenotypes (data not shown). To establish definitively that the MO-induced defects were specific, we co-injected synthetic RNA, encoding the corresponding gene(s). Four silent point mutations were introduced in the synthetic *fyn* and *yes* RNAs to avoid quenching of the MOs by the co-injected RNAs. Synthetic *fyn* and *yes* RNA largely rescued the Fyn/Yes-MO-induced phenotype (Fig 3A–F). *yes* RNA by itself also rescued the

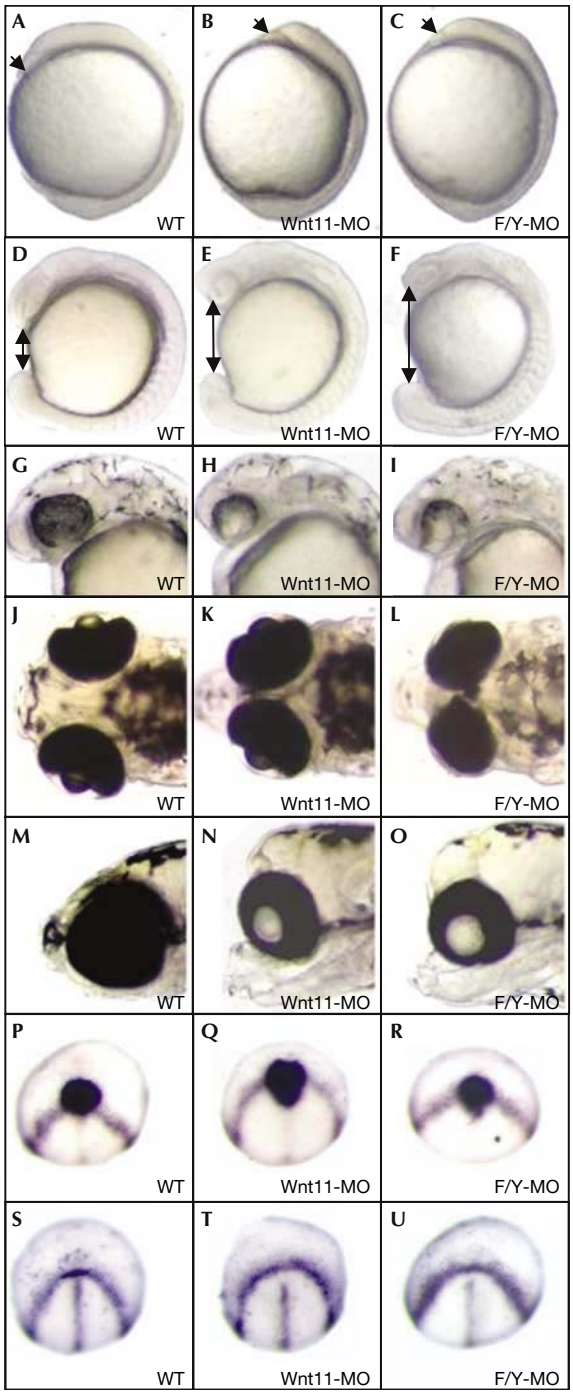

Fig 1 | Fyn/Yes knockdown-induced CE movement defects resemble those of Wnt11 knockdown. Wnt11-MO (8 ng) or Fyn- and Yes-MO (F/Y-MO; 5 ng each) were injected at the one-cell stage and the morphology was assessed at different stages: (**A–C**) one somite, the anterior-most structure is indicated with an arrow; (**D–F**) 14 somite, the gap between the anterior- and posterior-most structures at the 14-somite stage is indicated with a double-headed arrow; (**G–I**) 24 hpf; (**J–L**) 3 dpf; (**M–O**) 5 dpf. (**P–U**) *In situ* hybridizations of 10-hpf-old embryos with a *dlx3* probe (staining the edges of the neural plate) and an *hgg* probe (staining the hatching gland; **P–R**), or an *shh* probe (staining the midline; **S–U**). Dorsal views with the anterior to the top are depicted.

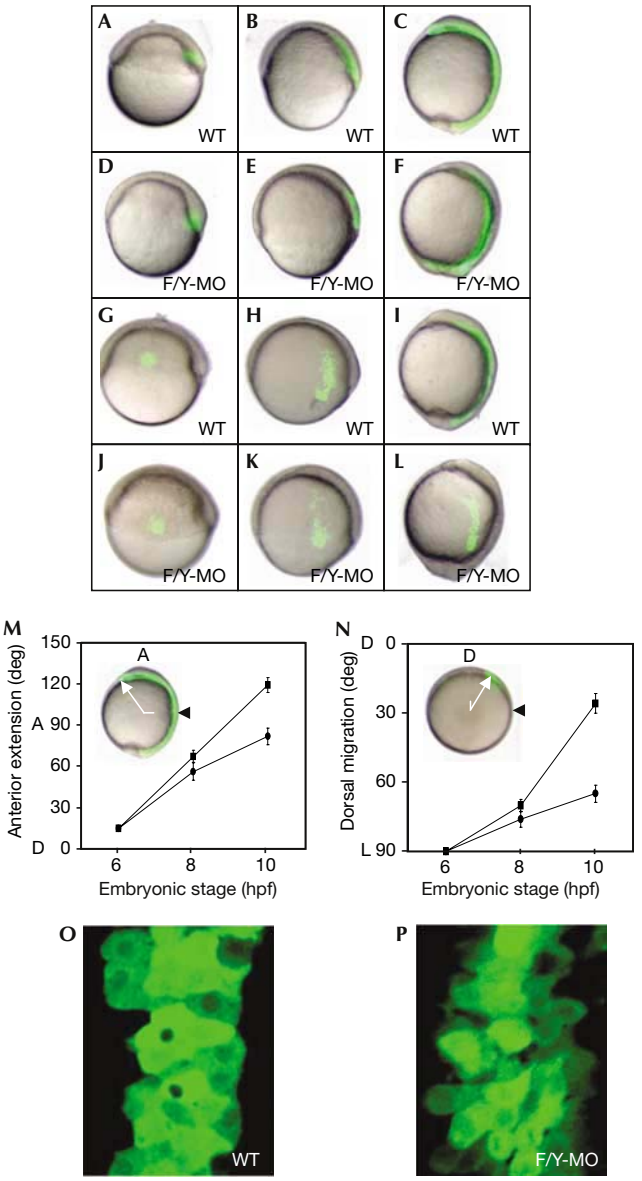

Fig 2 | Fyn and Yes are required for normal CE cell movements during gastrulation. Embryos were loaded with caged fluorescein dextran and the fluorophore was uncaged at the shield stage (6 hpf) dorsally to determine anterior extension (**A–F**) or laterally to determine dorsal migration (**G–L**). Cell labellings of the same embryos are depicted immediately after uncaging (**A,D,G,J**), at 80% epiboly, 8 hpf (**B,E,H,K**) and at the tailbud stage, 10–10.5 hpf (**C,F,I,L**). WT (**A–C,G–I**) and Fyn/Yes-MO-injected embryos (**D–F,J–L**) were assessed. (**M**) Anterior extension (white arrow) was quantified at the tailbud stage and is depicted as degrees of anterior movement (anterior, A, at the top in the inset) from the site of uncaging (black arrowhead). (**N**) Dorsal migration (white arrow in the inset) was quantified at the tailbud stage as degrees from dorsal (D), relative to the initial position at the shield stage. (**M,N**) Results are depicted for WT (filled rectangle) and Fyn/Yes-MO-injected (filled circle) embryos. Each experiment was performed ten times and the mean and standard deviation are shown. Dorsal view of a typical WT (**O**) and Fyn/Yes-MO (**P**) 10 hpf embryo in which the fluorophore was uncaged at 6 hpf in the dorsal shield.

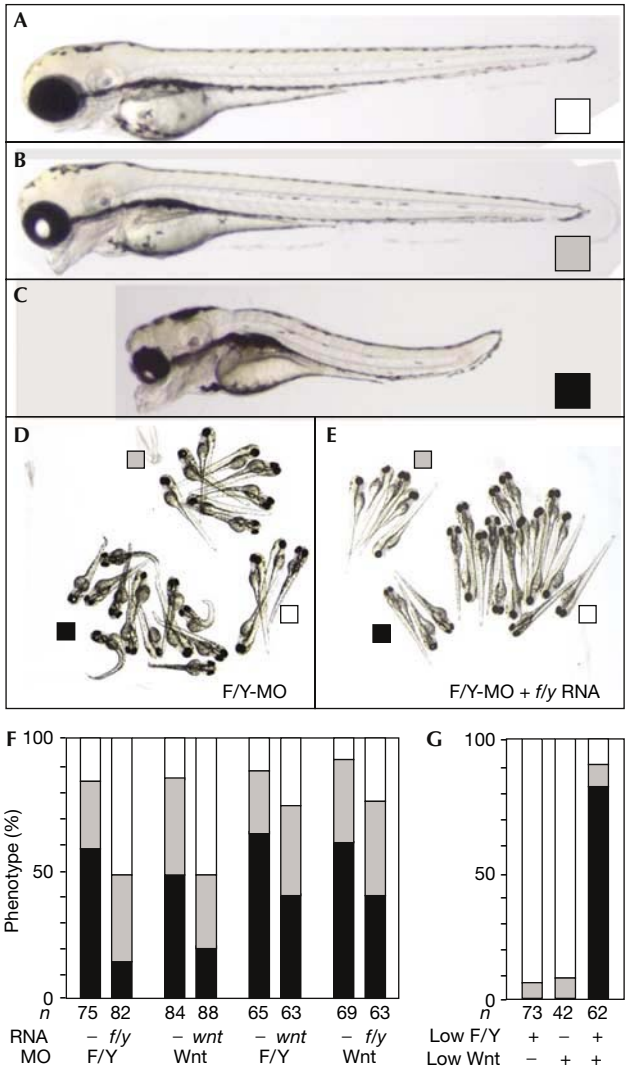

Fig 3 | Fyn/Yes- and Wnt11-MO-mediated knockdowns are specific and Fyn/Yes and Wnt11 do not seem to act in the same linear genetic pathway. Zebrafish embryos were co-injected with Fyn/Yes-MO (5 ng each) or Wnt11-MO (8 ng) and synthetic RNA encoding Fyn and Yes (1.0 pg each) or mouse Wnt11 (10 pg). At 3 dpf, the embryos were scored morphologically for CE movement defects into three categories: (**A**) WT (white box), (**B**) moderate (grey box), characterized by mild cyclopia and no forebrain structures anterior to the eyes, and (**C**) severe (black box), characterized by cyclopia and reduced body length. A typical rescue experiment is depicted in (**D**) Fyn/Yes-MO (F/Y-MO)-injected and (**E**) F/Y-MO + *f/y* RNA-injected embryos. (**F**) The phenotypes of the embryos from three independent experiments were scored and the percentages of WT (white bar), moderate (grey bar) and severe (black bar) are indicated. The injections with MO and with MO + RNA were performed in parallel on the same clutch of embryos, hence the small variations in percentages of phenotypes after injection of Fyn/Yes-MO (cf. bars 1,5). (**G**) Low levels of MO, 1.25 pg Fyn-MO and Yes-MO each and/or 2.0 pg Wnt11-MO, were (co-)injected and the phenotypes were scored as in (**F**). *n*, total number of embryos in three independent experiments.

Fyn/Yes-MO-induced phenotype, but nonrelated green fluorescent protein RNA did not (data not shown). Only low amounts of synthetic RNA (1.0 pg)—that did not induce phenotypes by itself—sufficed for these rescues. The MOs were injected in larger molar excess (more than four orders of magnitude greater) than the synthetic RNA. Therefore, the observed rescues cannot be due to quenching of the MOs. Synthetic mouse Wnt11 RNA rescued the Wnt11-MO-induced defect (Fig 3F). These results show that the MO-induced defects were specific.

Next, we investigated whether Fyn/Yes and Wnt11 acted in the same signalling pathway. Co-injection of all three MOs (5 ng Fyn-MO, 5 ng Yes-MO and 8 ng Wnt11-MO; cf. Fig 1) resulted in severe CE defects and none of the embryos survived past 48 hpf (data not shown). We tested decreasing concentrations of the MOs and found that co-injection of fourfold lower concentrations (1.25 ng Fyn-MO, 1.25 ng Yes-MO and 2.0 ng Wnt11-MO) induced severe CE movement defects (Fig 3G), whereas injection of Fyn/Yes- or Wnt11-MO at these concentrations by itself did not affect embryonic development. These results suggest that Fyn/Yes and Wnt11 act in either the same or parallel pathways.

We determined whether Fyn and Yes were genetically upstream or downstream of Wnt11 by attempting to rescue the Fyn/Yes-MO-induced phenotype with *wnt11* RNA and *vice versa*. We found that *wnt11* RNA (10 pg) did not significantly rescue the Fyn/Yes-MO-induced CE phenotype. Similarly, *fyn* and *yes* RNA did not significantly rescue the Wnt11-MO-induced phenotype (Fig 3F). The synthetic RNAs were functional in that they rescued their respective MO-induced defects (cf. *fyn/yes* RNA rescue of Fyn/Yes knockdown; Fig 3F). Taken together, these results indicate that the Fyn/Yes and Wnt11 pathways are partially overlapping and converge on a common downstream factor.

RhoA and Rac are downstream targets of the Wnt11 PCP pathway in *Xenopus* (Habas *et al*, 2001, 2003). We investigated whether RhoA and Rac are involved in the Fyn/Yes knockdown phenotype, by co-injection and assessment of morphological defects at 3 dpf. Co-injection of active RhoAV12 (0.5 pg RNA) in zebrafish embryos largely rescued the Fyn/Yes-MO-induced CE defects (Fig 4A). Consistent with previous work in *Xenopus* (Habas *et al*, 2003), RhoAV12 and, to a lesser extent, RacV12 (20 pg RNA) rescued the Wnt11-MO-induced phenotype in zebrafish as well. In contrast, RacV12 worsened the Fyn/Yes knockdown phenotype (Fig 4A). RhoAV12 and RacV12 alone did not affect zebrafish development at these concentrations (data not shown). We also assessed extension of the embryonic axis at the one-somite stage after co-injection of MOs and synthetic RNA, and measured the angle between the anterior and posterior ends of the embryos (Fig 4B–L). Consistent with the morphology at 3 dpf, we observed a significant increase in the angle after Fyn/Yes-mediated knockdown, which was significantly suppressed by co-injection of *fyn/yes* RNA as well as active *rhoA* RNA, whereas co-injection of active *rac* significantly reduced extension of the A-P axis, compared with Fyn/Yes knockdown alone (Fig 4B–F,K). The Wnt11 knockdown was suppressed significantly by *wnt11* and *rhoA*. Active *rac* did not rescue the Wnt11-MO-induced phenotype, but also did not worsen it (Fig 4G–J,L). Our results show that RhoA, but not Rac, is a common downstream signalling component in the Fyn/Yes and Wnt11 signalling pathway that mediates CE movements in zebrafish.

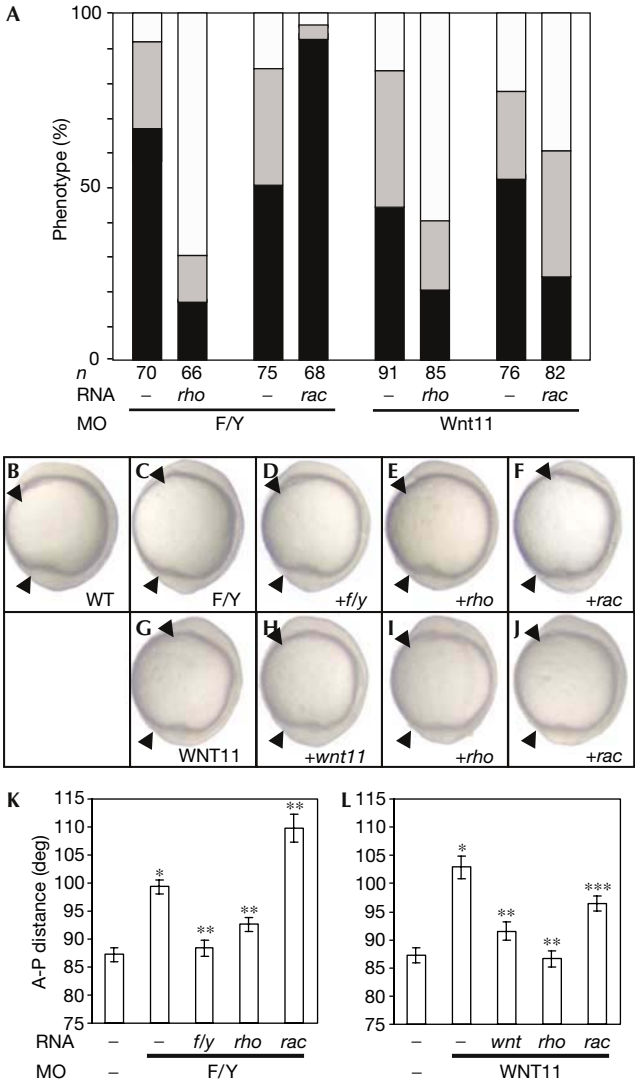

Fig 4 | Fyn/Yes and Wnt11 converge on RhoA. Zebrafish embryos were co-injected with Fyn/Yes-MO (5 ng each) or Wnt11-MO (8 ng) and synthetic RNA encoding active RhoAV12 (0.5 pg) or RacV12 (20 pg) and scored for morphological defects after 3 days (**A**), using the criteria described in the legend to Fig 3. *n*, total number of embryos in three independent experiments. (**B–L**) Embryos were co-injected with MOs and RNAs as in (**A**) and the embryonic axis extension (arrowheads) was assessed at the one-somite stage. (**C–F**) Fyn/Yes-MO-injected, co-injected with RNA encoding (**D**) *fyn* and *yes*, (**E**) active *rhoA* or (**F**) active *rac*. (**G–J**) Wnt11-MO-injected, co-injected with (**H**) mouse *wnt11*, (**I**) active *rhoA* or (**J**) active *rac*. (**K,L**) Quantification of A-P axis extension at the one-somite stage. The angle between the anterior and posterior ends was measured in 25–30 embryos that were (co-)injected as indicated. Averages are depicted; the error bars indicate standard errors of the mean. Two-tailed Student's *t*-tests indicate a significant increase in the angle after injection of the MOs alone ($P<0.001$, single asterisk) and a significant decrease in the angle after co-injection of *fyn/yes* or *wnt11* and *rhoA* ($P<0.001$, double asterisk). Co-injection of active *rac* with Fyn/Yes-MO induced a significant further increase in the angle ($P<0.001$, double asterisk), but *rac* co-injection did not significantly affect the Wnt11-MO-induced phenotype (triple asterisk).

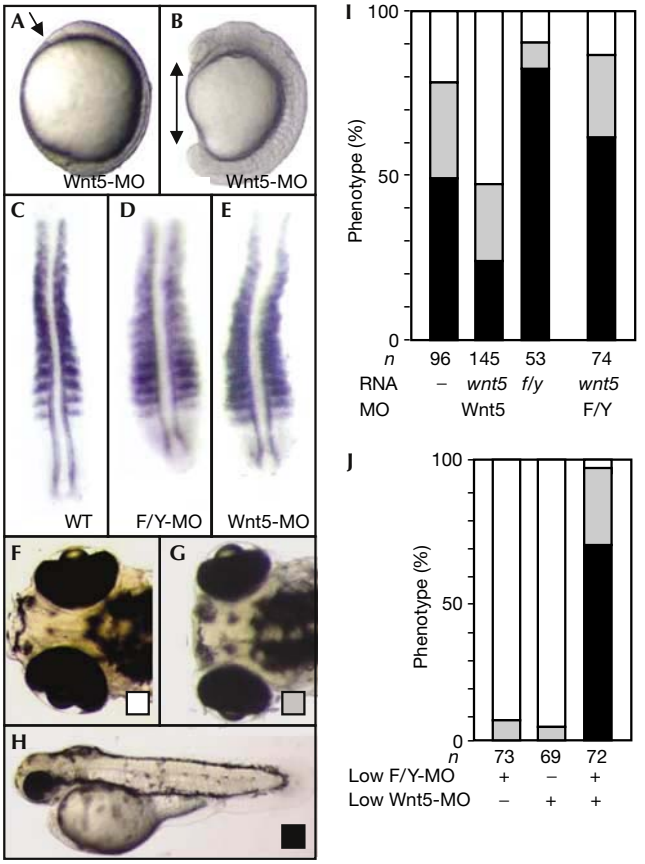

Fig 5 | Fyn/Yes and non-canonical Wnt5. Wnt5-MO (5 ng) was injected at the one-cell stage and phenotypes were scored morphologically at (**A**) 10 hpf (the arrow marks the anterior-most structure), and (**B**) 14-somite stage (the gap between the anterior- and posterior-most structures is indicated). (**C–E**) *In situ* hybridization with a *myoD* probe at the 14-somite stage: (**C**) WT, (**D**) Fyn/Yes-MO-injected and (**E**) Wnt5-MO-injected embryos. Knockdown embryos were scored morphologically at 3 dpf as (**F**) WT (white box), (**G**) moderate (grey box), characterized by abnormal development of the anterior-most structures, and (**H**) severe (black box), characterized by reduced body axis extension. (**I**) The Wnt5-MO (5 ng) was co-injected with mouse *wnt5* RNA (5 pg) or *fyn* and *yes* RNA (1 pg each) and the phenotypes were scored as depicted (**F,G**). (**J**) Low levels of MO (1.25 pg each) were (co-)injected and the phenotypes were scored as in (**F**). *n*, total number of embryos in three independent experiments.

Like Wnt11, non-canonical Wnt5 has a role in CE movements, although the effects are more pronounced in the posterior regions of the developing embryo, as illustrated by the *ppt* mutant phenotype (Hammerschmidt *et al*, 1996; Kilian *et al*, 2003). We phenocopied the *ppt* mutant by Wnt5-MO-mediated knockdown (Lele *et al*, 2001; Fig 5). At 10 hpf, extension of the anterior-most structures is reduced (Fig 5A) and at the 14-somite stage, extension of the A-P axis is reduced (Fig 5B). Fyn and Yes knockdown also resulted in reduced body length (cf. Fig 3C). *MyoD* staining showed that Fyn/Yes knockdown, like Wnt5 knockdown, resulted in much wider and shorter embryos (Fig 5C–E), suggesting that the role of Fyn and Yes in Wnt5 signalling may be similar to that in Wnt11 signalling. Fyn and Yes did not rescue the Wnt5 knockdown phenotype and *vice versa* (Fig 5F–I), whereas the *fyn* and *yes* RNAs readily rescued the Fyn/Yes-MO-induced phenotype (cf. Fig 3D–F), and mouse *wnt5* RNA largely rescued the Wnt5-MO-induced phenotype (Fig 5I). Co-injection of 1.25 ng each of the Fyn-, Yes- and Wnt5-MO (fourfold lower than normal) induced severe reductions in body axis extension (Fig 5J), whereas 1.25 ng of these MOs by themselves did not affect development. These results suggest that Fyn and Yes may be involved in Wnt5 signalling in a similar manner to Wnt11 signalling.

Our data indicate that Fyn/Yes and non-canonical Wnts converge on RhoA in CE movements during vertebrate gastrulation. Active RhoA rescued the Fyn/Yes as well as the Wnt11 knockdown (Fig 4). Fyn/Yes and Wnt11 may act independently in parallel pathways, activating different pools of RhoA, hence the ability of dominant active RhoAV12 to rescue both the Fyn/Yes and the Wnt11 knockdowns. However, the combined knockdown of Fyn/Yes and Wnt11, inducing severe CE defects (Fig 3G), indicates that Fyn/Yes and Wnt11 signalling have common downstream components. Our results are consistent with data from *Caenorhabditis elegans*, in which Wnt and Src signalling were postulated to act in parallel, upstream of Rho, to specify the endoderm and to orient the cell division axis in the EMS cell (Bei *et al*, 2002). In *Xenopus*, Wnt11 signalling bifurcates downstream of the Dvl/ Daam1 complex, leading to activation of RhoA and Rac (Habas *et al*, 2003). The finding that RhoA, but not Rac, rescued the Fyn/Yes knockdown shows that Fyn and Yes act downstream of the Dvl/Daam1 complex and upstream of RhoA. Fyn/Yes-induced activation of RhoA in zebrafish may be due to inactivation of RhoGAP, similar to Src64-mediated inactivation of p190RhoGAP in *Drosophila* (Billuart *et al*, 2001), which is consistent with direct p190RhoGAP phosphorylation by SFKs (Brouns *et al*, 2001). Moreover, Fyn/Yes-induced RhoA activation may also be due to direct phosphorylation and activation of a guanosine 5′-triphosphate exchange factor for RhoA (Schuebel *et al*, 1998).

In conclusion, our results show that Fyn and Yes are required for normal CE cell movements during gastrulation, and provide a starting point to further unravel the interaction between SFKs and non-canonical Wnt signalling.

METHODS

Zebrafish and *in situ* hybridization. Zebrafish were kept and the embryos were staged as described previously (Westerfield, 1995). *In situ* hybridizations were performed as described (Thisse *et al*, 1993), using probes specific for *dlx3*, *shh*, *myoD* (gifts from Jeremy Wegner, Jean-Paul Concordet and Eric Weinberg, respectively) and *hgg1* (RZPD ID: IMAGp998O098963Q; RZPD, www.rzpd.de, Berlin, Germany).

Morpholinos, RNAs and injections. Antisense MOs were targeted close to the start ATG of the respective complementary DNAs and ordered from GeneTools (Philomath, OR, USA): Src, 5′-GCC TCGTCGAAAACCACACGAAATG; Fyn, 5′-TGTCCTTACATTGCA CACAG CCCAT; Yes, 5′-CCTCTTTACTCTTGACACAGCCCAT. The Wnt11- and Wnt5-MO have been described previously (Lele *et al*, 2001). Fyn and Yes cDNAs were amplified by PCR using oligonucleotides with four silent point mutations to avoid quenching of the MOs by the co-injected RNAs: mutant Fyn: 5′-ATGGGCTGCGTACAGTGCAAGGACAAAGAGGCA, mutant

Yes: 5′-ATGGGCTGCGTAAAAAGCAAAGAGGACAAGGGT. 5′-capped sense RNAs were synthesized using constructs encoding mutant Fyn and Yes, mouse Wnt11 and Wnt5 (gift from Andy McMahon) and active RhoAV12 and RacV12 (gift from Boudewijn Burgering) and the mMessage mMachine kit (Ambion, Austin, TX, USA). MO (0.1–10 ng) and synthetic RNA (0.5 pg–1.0 ng) were injected into embryos of the AB or TL strain at the one- to two-cell stage and phenotypes were assessed at the indicated stages.

Cell tracing. Embryos were (co-)injected at the one-cell stage with 0.25% 4,5-dimethoxy-2-nitrobenzyl (DMND)-caged fluorescein dextran (molecular mass 10,000; Molecular Probes, Leiden, The Netherlands). Uncaging was performed as described (Bakkers *et al*, 2004) at the shield stage (6 hpf) using an Axioplan microscope, equipped with an ultraviolet light source, adjustable pinhole and × 40 objective. Pictures were taken immediately after uncaging, at 80% epiboly (8 hpf) and the tailbud stage (10 hpf). The angles for dorsal convergence and anterior extension were determined using NIH imaging software.

Accession numbers. Sequences of zebrafish cDNAs reported here were deposited in the EMBL database: *src*, AJ620750; *fyn*, AJ620748; *yes*, AJ620749.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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