Mechanisms of Development 124 (2007) 129–136

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Essential role for Csk upstream of Fyn and Yes in zebrafish gastrulation

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Received 22 June 2006; received in revised form 12 October 2006; accepted 21 October 2006
Available online 28 October 2006

Abstract

Morphogenetic cell movements during gastrulation shape the vertebrate embryo bodyplan. Non-canonical Wnt signaling has been established to regulate convergence and extension cell movements that mediate anterior-posterior axis elongation. In recent years, many other factors have been implicated in the process by modulation of non-canonical Wnt signaling or by different, unknown mechanisms. We have found that the Src family kinases, Fyn and Yes, are required for normal convergence and extension cell movements in zebrafish embryonic development and they signal in parallel to non-canonical Wnts, eventually converging on a common downstream factor, RhoA. Here, we report that Csk, a negative regulator of Src family kinases has a role in gastrulation cell movements as well. Csk knock down induced a phenotype that was similar to the defects observed after knock down of Fyn and Yes, in that gastrulation cell movements were impaired, without affecting cell fate. The Csk knock down phenotype was rescued by simultaneous partial knock down of Fyn and Yes. We conclude that Csk acts upstream of Fyn and Yes to control vertebrate gastrulation cell movements.

Keywords: Gastrulation; Cell movements; Csk; Src family kinases; Zebrafish

1. Introduction

A series of morphogenetic cell movements during gastrulation results in the formation of the three germ layers, endoderm, mesoderm and ectoderm and in so doing creates the basic bodyplan of the developing embryo (Warga and Kimmel, 1990). Convergence and extension (CE) represents one of this series of movements during which cells converge towards the midline of the developing embryo, forming the medial/lateral axis, where they intercalate with one another and so extend around the yolk giving rise to the anterior/posterior axis (Keller et al., 1992). In vertebrates this process is governed primarily by the non-canonical Wnt pathway which is similar to the planar cell polarity (PCP) pathway identified in *Drosophila* (Solnica-Krezel and Eaton, 2003).

The vertebrate non-canonical Wnt pathway becomes activated when Wnt11 or Wnt5 bind to Frizzled receptors resulting in the translocation of Dishevelled to the plasma membrane where it forms a complex with Daam1, RhoA and Rac. RhoA and Rac subsequently become activated and propagate the signal to their respective downstream effectors, including Rok and JNK (Habas et al., 2001, 2003; Veeman et al., 2003). In *C. elegans*, this cascade will remodel the cell establishing polarity and allowing it to mount a proper chemotactic response (Goldstein et al., 2006). Non-canonical Wnt signaling induced cell polarization may be at the basis of vertebrate CE cell movements as well. In zebrafish, a number of mutants have been identified that harbor mutations in genes regulating this process (Heisenberg et al., 2000; Sepich et al., 2000; Topczewski et al., 2001; Kilian et al., 2003). The phenotype that all of these mutants have in common is that the embryos are shorter and broader as one might expect if CE has been disrupted. More recently a number of studies have come to light that show that CE is not solely governed by the non-canonical Wnt pathway. Other factors involved include Gα12/13 (Lin et al., 2005), Has2 (Bakkers et al., 2004), Cyclooxygenase-1 (Cha et al., 2005), Widerborst (Hannus et al., 2002), ERRα (Bardet et al., 2005),

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0925-4773/$ - see front matter
doi:10.1016/j.mod.2006.10.003

Scribble-1 (Wada et al., 2005), Fyn and Yes (Jopling and den Hertog, 2005), Ephrins (Oates et al., 1999), Slit (Yeo et al., 2001) and Stat3 (Yamashita et al., 2002). These serve to either modulate non-canonical Wnt signaling directly, highlighted by the recent finding that scribble-1 is a key CE regulator that genetically interacts with trilobite, a known component of non-canonical Wnt signaling (Wada et al., 2005). Alternatively, they function independently of it as shown with *widerborst*, which is not necessary for the activation of non-canonical Wnt signaling but is essential for the correct cellular localization of some of its components (Hannus et al., 2002). Recently, we have shown that signaling through the Src family kinases (SFK) Fyn and Yes converges with non-canonical Wnt signaling and serves to modulate the activity of the small GTPase RhoA during CE cell movements (Jopling and den Hertog, 2005). Furthermore, we have shown that the protein-tyrosine phosphatase (PTP) Shp2, an indirect activator of SFKs (Zhang et al., 2004), is also involved in regulating CE during gastrulation via Fyn/Yes and RhoA (CJ and JdH, unpublished data). Csk antagonizes Shp2 and inhibits SFKs by phosphorylation of a regulatory tyrosine in their COOH-terminus, rendering SFKs inactive (Nada et al., 1991). Therefore, we asked the question ''Is Csk involved in CE during vertebrate gastrulation?''.

Csk knockout mice die prenatally with a complex range of phenotypes including neural tube defects all of which are consistent with defective cell movements during gastrulation (Nada et al., 1993). Mouse knockouts such as *looptail* and *scribble* also display neural tube defects (Murdoch et al., 2001; Murdoch et al., 2003) while their zebrafish homologs, *trilobite* and *scribble-1*, respectively, have been linked directly to the regulation of non-canonical Wnt signaling and show disrupted CE movements during gastrulation (Sepich et al., 2000; Wada et al., 2005). Moreover, loss of Van gogh-like 2 in zebrafish *trilobite* mutants induced neural tube defects as well (Ciruna et al., 2006). Cultured fibroblast cells deficient for *csk* fail to migrate properly in response to various stimuli such as the growth factors PDGF and EGF, a defect that can be rescued by the inhibition of SFKs (McGarrigle et al., 2006). These cells show defective actin cytoskeletal remodelling, which is also observed in *Drosophila* wing cells with defective non-canonical Wnt signaling (Shimada et al., 2001) and in tissue culture cells with impaired non-canonical Wnt signaling (Wechezak and Coan, 2005; Aspenstrom et al., 2006). In *Xenopus*, overexpression of *csk* mRNA results in defective gastrulation cell movements, mimicking the phenotype caused by expression of dominant negative SFKs (Denoyelle et al., 2001).

Here we show that morpholino mediated knockdown of Csk in zebrafish results in defective morphogenetic cell movements during gastrulation without affecting overall cell fate, similar to the phenotype observed when positive regulators such as Fyn and Yes are knocked down. We also show that Csk exerts its effects through the negative regulation of Fyn and Yes.

2. Results

Zebrafish *csk* was identified (EST clone IMAGp998P2017182Q1) based on protein sequence homology with its human and mouse counterparts (86% and 85.6% identical, respectively) (Fig. 1A). *In situ* hybridization experiments using a *csk*-specific antisense probe show that it was ubiquitously expressed throughout early

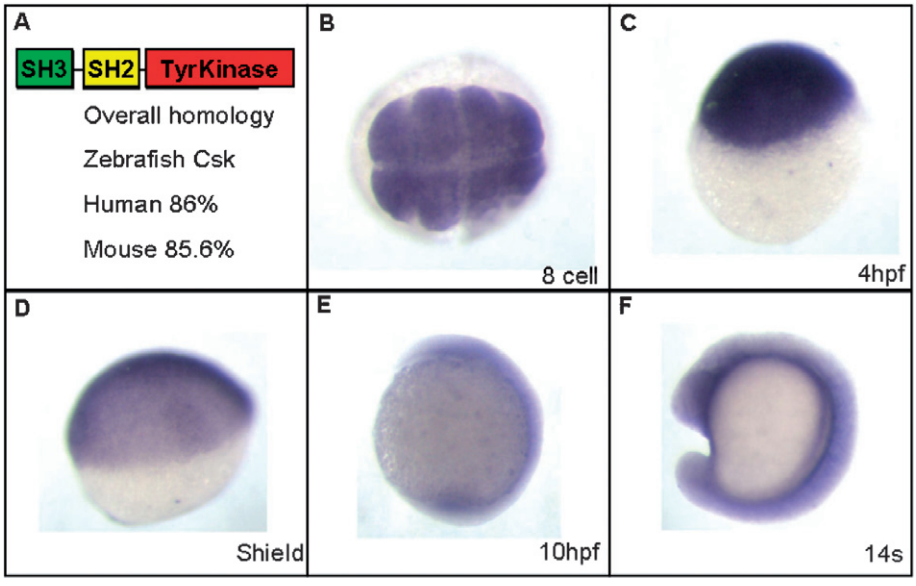

Fig. 1. Csk is ubiquitously expressed in early zebrafish development. (A) Schematic representation of zebrafish Csk with one Src homology 3 (SH3) domain and one Src homology 2 domain to the N-terminal side of the protein-tyrosine kinase domain. The overall sequence identity with human and mouse Csk is indicated. (B–F) *In situ* hybridization with a Csk-specific antisense probe at various stages of development: (B) 8 cell-stage; (C) 4 hpf; (D) shield stage; (E) 10 hpf and (F) 14 somite (14 s).

zebrafish embryogenesis with a strong maternal contribution (Fig. 1B–F). The expression pattern of *csk* in zebrafish embryos is consistent with *csk* expression in the mouse (Imamoto and Soriano, 1993). To determine whether *csk* plays a role in early zebrafish development we employed a morpholino based strategy targeting the start codon of Csk. We injected the Csk-MO into single cell stage embryos which were subsequently monitored at specific intervals during embryogenesis. Injection of 5 ng Csk-MO reproducibly affected early zebrafish development producing a range of phenotypes consistent with defective gastrulation. No visible defects were detected as embryos progressed through epiboly. Only at 10 hpf it became apparent that the embryos had failed to extend properly around the yolk, similar to the phenotype seen in Fyn/Yes morphants (Fig. 2A–C). At 3 dpf, embryos were visibly shorter than un-injected controls (Fig. 2D). This was further confirmed by measuring the overall length of injected embryos from anterior to posterior at 3 dpf (Fig. 2E). The average length of wild-type embryos remained virtually invariant whereas morphant embryos were significantly shorter. Because morpholinos in general can produce non-specific side effects (Nasevicius and Ekker, 2000) we needed to establish that the observed defects associated with Csk knockdown were not artefactual. To this end, we (co-) injected varying amounts of RNA encoding human *csk* which is not recognized by the Csk-MO. We found that injection of 150 pg of human *csk* RNA by itself did not affect early zebrafish development morphologically (data not shown). However, co-injection of this amount of *csk* RNA with 5 ng Csk-MO restored normal body length (Fig. 2E) and rescued overall morphology (data not shown), indicating that the defects caused by Csk-MO injection were a direct result of specific Csk knockdown.

Next we investigated whether the observed defects were due to defective cell movement or incorrect cell specification, two very different processes which can give rise to similar phenotypes. To address this issue we performed cell tracing experiments using caged fluorescein. Embryos at the one cell stage were injected with either the caged fluorophore alone or in conjunction with the Csk-MO and then allowed to develop as normal. At 6 hpf a cluster of cells within the dorsal shield was labeled by uncaging the fluorescein with a short, localised pulse of UV light. The group of cells was then monitored every 2 h during gastrulation. The distance the cells migrated is directly proportional to embryonic extension. Repeating the process at 90° to the shield gives an effective measurement of how far the

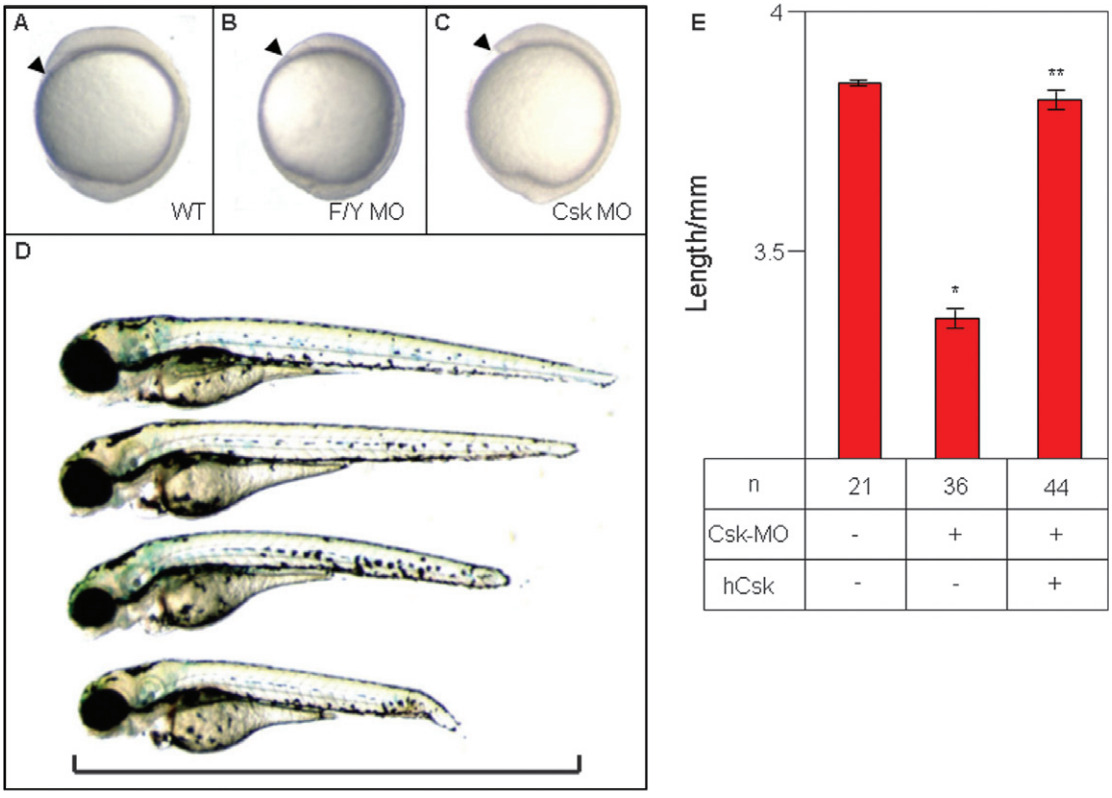

Fig. 2. Csk knockdown induced defects associated with impaired gastrulation. Zebrafish embryos were not injected (A) or microinjected with Fyn/Yes-MO (5 ng) (B) or Csk-MO (5 ng) (C) at the 1 cell stage and allowed to develop. (A–C) Morphology at 10 hpf shows reduced anterior extension of the Fyn/Yes-MO and Csk-MO injected embryos. (D) Csk knock down embryos at 3 dpf show a reduction in overall length (uninjected at the top). (E) Rescue of the Csk knock down phenotype by co-injection of synthetic human *csk* mRNA. Zebrafish embryos were injected with Csk-MO (5 ng) alone or in conjunction with human *csk* mRNA (150 pg). The length of the embryos was measured at 3 dpf and the average is shown here. Two tailed student *t*-tests indicate a significant decrease in length after injection of Csk-MO alone ($P < 0.001$, single asterisk) and a significant increase in the length after co-injection of *csk* mRNA ($P < 0.001$, double asterisk).

mesendodermal cells converge towards the dorsal midline. A comparison of wild-type and Csk-MO injected embryos revealed that Csk knockdown resulted in a significant reduction in the capability of cells to migrate both toward the dorsal midline (convergence) and anteriorly around the yolk (extension), indicating that Shp2 is required for correct CE during vertebrate gastrulation (Fig. 3).

To assess whether cell specification was affected by Csk knock down, we performed *in situ* hybridization with Csk-MO injected embryos using a panel of markers that are all known to be involved in cell specification. *Bone morphogenetic protein 2b* (*bmp2b*) specifies ventral cell fates but the expression pattern did not change significantly when Csk was knocked down (Fig. 4A and B). The expression of *Chordin* (*chd*), a dorsalising factor, was not significantly affected when compared to un-injected controls (Fig. 4C and D) which was also the case for *goosecoid* (*gsc*), another dorsal specific gene expressed in the zebrafish organiser (Fig. 4E and F). Finally, we also found that the expression of the mesendodermal marker *notail* (*ntl*) was similar in Csk morphants and wild-type embryos (Fig. 4G and H). These results clearly show that cell fate in zebrafish embryos was not affected by Csk knockdown, suggesting that the observed defects were due to morphogenetic cell movements that occurred during gastrulation.

The gastrulation defect we observed in Csk morphants may also have been caused by the mis-expression of known CE regulators such as Wnt11 and Wnt5 or Fyn and Yes. However, the expression of all these genes was similar in wild-type and Csk-MO injected embryos (Fig. 4I–P), suggesting that it is more likely that Csk is directly involved in the regulation of gastrulation cell movements.

Incorrect specification of the brain can result in embryos that lack certain brain structures. Obviously, this defect would make embryos appear to be shorter than wild-type embryos. For example, Six3 morphants have a severely reduced telencephalon when compared to uninjected controls and they appear to be shorter than uninjected controls (Ando et al., 2005). Because Csk morphants failed to extend properly around the yolk at 10 hpf (Fig. 2C) we wondered whether this was due to defective gastrulation or simply because they lack anterior structures. *Six3* is expressed in the developing forebrain of zebrafish embryos, *pax2* in the midbrain–hindbrain boundary and *krox20* labels rhombomeres 3 and 5. The expression of all of these genes was not affected in Csk morphants, indicating that these structures were present (Fig. 5A–F). However, the expression patterns of all 3 markers shifted posteriorly (Fig. 5A–F). When viewed from the dorsal side the expression patterns of *six3* and *pax2* were also broader than the control embryos (Fig. 5I–L). 8 hpf embryos express the axial mesendodermal marker *cyclops* (*cyc*) during gastrulation. The *cyc* expression pattern was clearly broader and shorter in Csk-MO injected embryos than in un-injected control embryos (Fig. 5M and N). These results demonstrate that the phenotype produced by Csk-MO was not due to the deletion of anterior structures but was in fact caused by the failure of cells to move to their correct positions during gastrulation.

Finally, we set out to determine whether Csk interacted with Fyn and Yes. To achieve this we co-injected MOs and measured the angle between the most anterior and posterior structures at 10 hpf to determine whether gastrulation had been adversely affected. Injection of the Csk-MO by itself led to an increase in this angle, a defect that was effectively rescued by co-injection of human *csk* mRNA (Fig. 6). Because Csk negatively regulates Fyn and Yes, we hypothesized that knockdown of Fyn and Yes might rescue the defects associated with Csk-MO. It is noteworthy that optimal knock down of Fyn and Yes induced severe gastrulation cell movement defects (Jopling and den Hertog, 2005), resulting in an increase in the angle

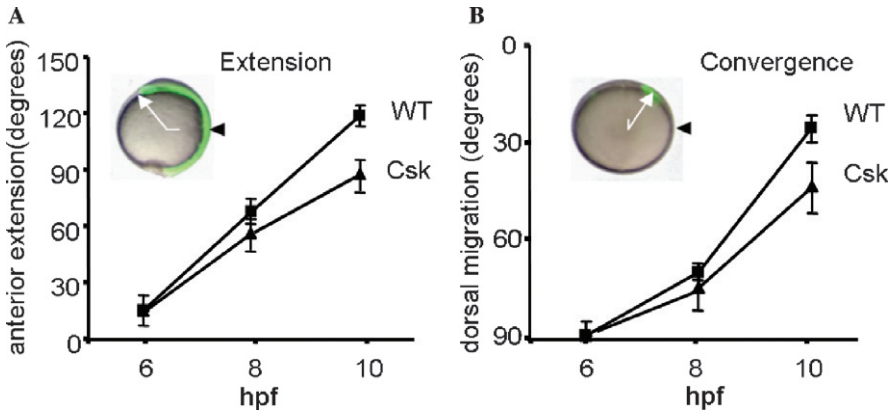

Fig. 3. CE cell movement defects in response to Csk knock down. (A) and (B) Embryos were loaded with caged fluorescein dextran and the fluorophore was uncaged at the shield stage (6 hpf) dorsally to determine anterior extension (A) or laterally to determine dorsal migration (B). Cell labelings of the same embryos were followed immediately after uncaging, at 80% epiboly, 8 hpf and at tailbud stage, 10–10.5 hpf. Wild-type and Csk-MO injected embryos were assessed. (A) Anterior extension (white arrow) from the site of uncaging (black arrowhead) was quantified at tailbud stage and is depicted as degrees anterior movement (inset is lateral view of a 10 hpf embryo, anterior at the top). (B) Dorsal migration (white arrow in inset) relative to the initial position at the shield stage (black arrowhead) was quantified at tailbud stage as degrees from dorsal (inset is a frontal view with dorsal to the top).

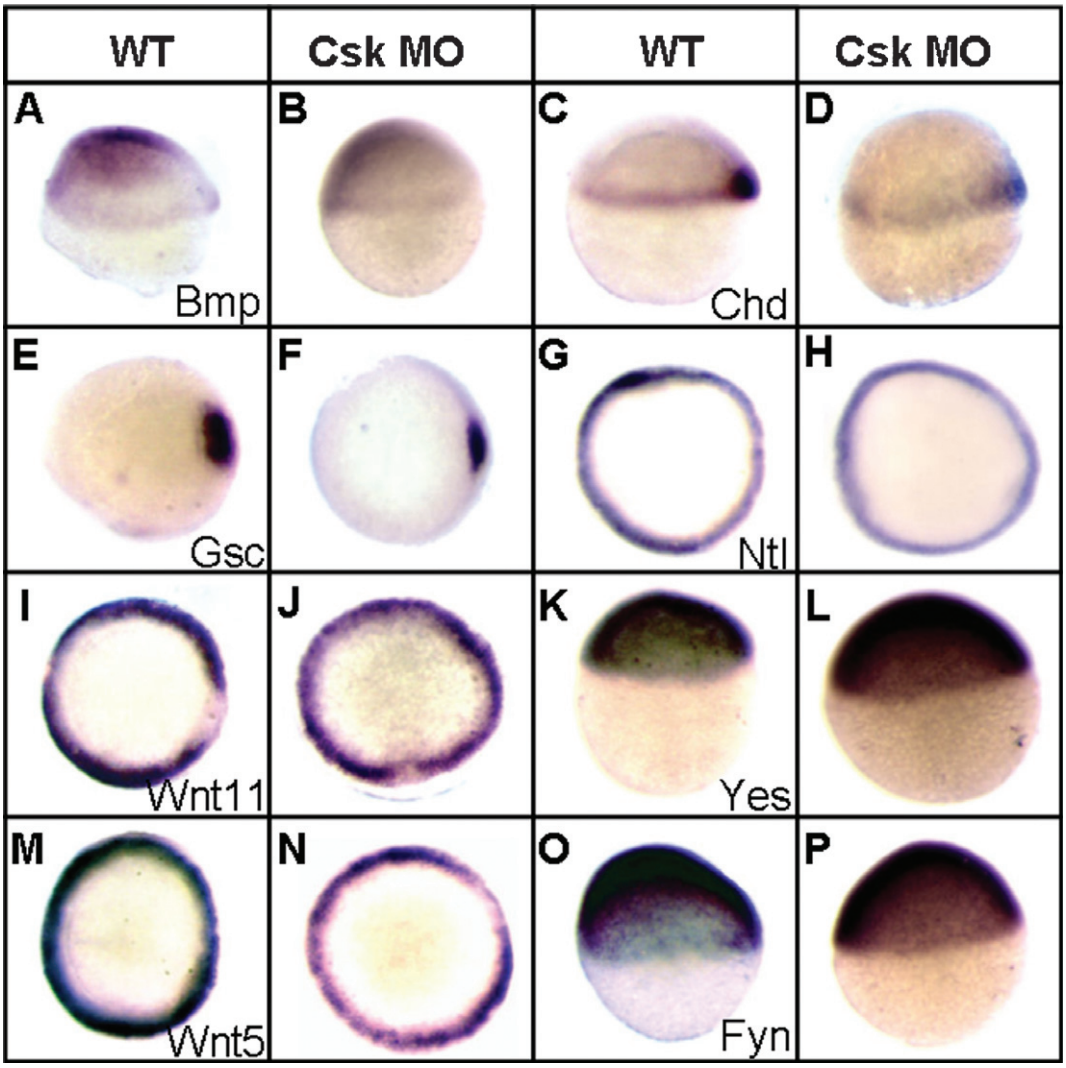

Fig. 4. Csk knock down did not affect cell specification, nor expression of known regulators of CE cell movements. Csk-MO injected embryos were fixed at 6 hpf and *in situ* hybridization was done with various probes: (A and B) *bone morphogenetic protein 2b, bmp*; (C and D) *chordin*, *chd*; (E and F) *goosecoid*, *gsc*; (G and H) *no tail*, *ntl*; (I and J) *wnt11*; (K and L) *yes*; (M and N) *wnt5*; (O and P) *fyn*. Either lateral views (A–D, K, L, O, and P) or animal pole views (E–H, I, J, M, and N) are depicted here.

between the anterior- and posterior-most embryonic structures at 10 hpf (Fig. 6). Co-injection of Csk-MO with optimal amounts of Fyn- and Yes-MOs (5 ng each) did not alter these defects (data not shown). However, we titrated the amount of co-injected Fyn/Yes-MO down and found that co-injection of 0.125 ng each of the Fyn/Yes MOs led to an effective rescue of the Csk-MO induced phenotype at 10 hpf (Fig. 6). Low Fyn/Yes-MO injections (0.125 ng each) did not induce phenotypes by themselves (data not shown). The Fyn/Yes-MO rescues of the Csk-MO persisted at later stages of development, because no morphological defects were detected anymore at 3 dpf (data not shown). These results are consistent with Csk knock down leading to hyperactivation of Fyn and Yes, causing the observed gastrulation defects. Partial knock down of Fyn and Yes in turn rescued the Csk knock down phenotype, placing Fyn and Yes downstream of Csk in zebrafish gastrulation cell movements.

3. Discussion

Here we provide evidence that Csk has an essential role in early zebrafish development. We show here that *csk* is expressed throughout early zebrafish development (Fig. 1). Knock down of Csk resulted in CE defects that were morphologically detectable at 10 hpf (Fig. 2A–C). Given the maternal expression of Csk (Fig. 1A), we cannot exclude the possibility that Csk also has an essential role earlier in embryogenesis. Nevertheless, the Csk knock down defects were reminiscent of previously documented morphants and mutants with compromised CE cell movements during gastrulation (Topczewski et al., 2001; Yamashita et al., 2002; Jopling and den Hertog, 2005). At later stages the embryos were visibly shorter than wild-type siblings, a common feature of CE mutants such as Pipetail (Wnt5) and Trilobite (Sepich et al., 2000; Kilian et al., 2003). We demonstrated using cell tracing

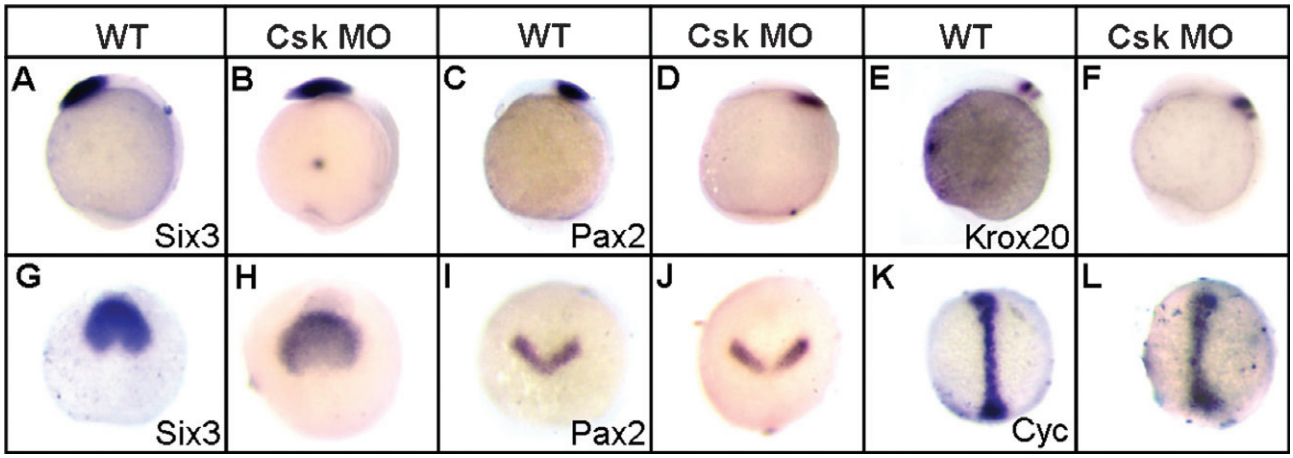

Fig. 5. Csk knock down induced a posterior shift. Molecular markers indicate there is no deletion of anterior structures only a posteriorwards shift in expression. Control and Csk-MO injected embryos were fixed at 10 hpf (A–J) or 8 hpf (K and L) and *in situ* hybridizations were done with the indicated probes: (A,B,G, H) *six3*; (C,D,I,J) *pax2*; (E and F) *krox20*; (K and L) *cyclops*, *cyc*. Lateral views (A–F) or dorsal views with anterior to the top (G–L) are depicted here.

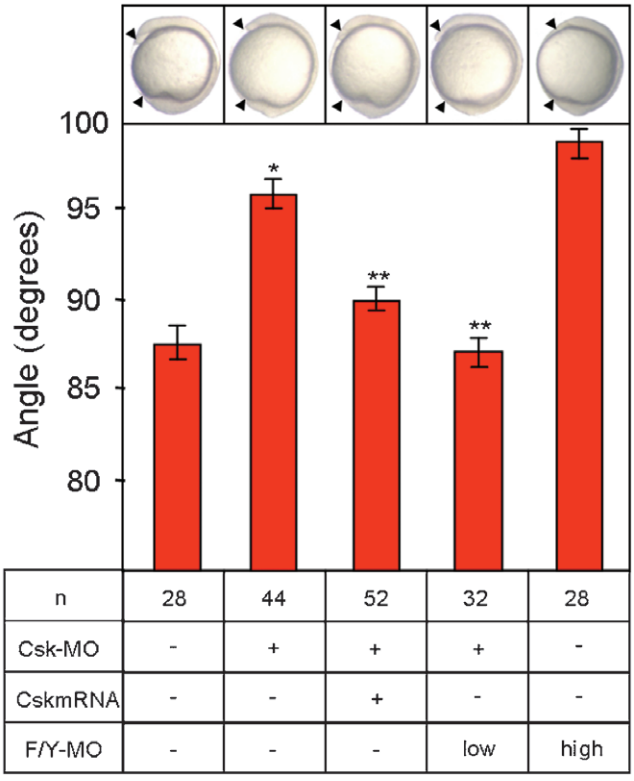

Fig. 6. Csk acts upstream of Fyn and Yes in gastrulation cell movements. The Csk-MO (5 ng) was injected alone or together with human *csk* mRNA (150 pg) or low levels of Fyn/Yes-MO (0.125 ng each, low). As a control, high levels of Fyn- and Yes-MOs that induce CE defects were injected (5 ng each, high). The angle between the most anterior and posterior embryonic structure was determined at the 1-somite stage in at least 20 embryos and the average angle is depicted here in degrees. Two tailed student *t*-tests indicate a significant increase in the angle after injection of Csk-MO alone ($P < 0.001$, single asterisk) and a significant decrease in the angle after co-injection of *csk* mRNA or Fyn/Yes-MO ($P < 0.001$, double asterisk).

experiments that during gastrulation both convergence and extension cell movements were impaired upon Csk knock down (Fig. 3). Csk phosphorylates and inhibits SFKs and is known to be involved in the regulation of a number of pathways which include growth factors and cell–cell signaling molecules (McGarrigle et al., 2006; Vidal et al., 2006). Some of these may regulate cell specification, which – when disrupted – can produce embryos with similar defects to those produced when CE is defective (Schulte-Merker et al., 1994; Schier et al., 1997; Rebagliati et al., 1998). However, the expression patterns of a panel of cell specification markers remained unaltered in Csk morphants (Fig. 4), indicating that the defects we observed were not caused by defective cell fate determination. Moreover, anterior patterning markers were still expressed, albeit there was a distinct posterior shift in expression (Fig. 5), similar to that observed in other CE defective morphants such as Stat3 (Yamashita et al., 2002). Our results indicate that Csk is essential for normal CE cell movements during gastrulation.

Csk negatively regulates SFKs by phosphorylation of their COOH-terminal tyrosine. We demonstrate that Csk knockdown in zebrafish embryos was rescued by partial Fyn/Yes knock down (Fig. 6), indicating that Csk indeed is genetically upstream of Fyn and Yes in zebrafish development. Moreover, our results indicate that the Csk knock down phenotype is caused by hyperactivation of the SFKs, Fyn and Yes. Interestingly, Csk knock out fibroblast cells display a migratory defect that is rescued by a SFK inhibitor (McGarrigle et al., 2006), indicating that rescue of the loss of Csk by inhibition of SFK activity is not unprecedented.

Modulation of Csk expression in other species induces phenotypes that are consistent with gastrulation defects as well. For instance, Csk over-expression in *Xenopus* phenocopies the gastrulation defect caused by expressing dominant negative SFKs (Denoyelle et al., 2001). Homozygous

mouse knockouts with a targeted mutation in Csk lack functional Csk and harbor consitutively activated SFKs. The phenotype of these mice is compatible with defective gastrulation as well (Nada et al., 1993). We found that knock down of the SFKs, Fyn and Yes, induced gastrulation cell movement defects (Jopling and den Hertog, 2005), similar to the Csk knock down defects described here. The paradox that is emerging is that over-expression of a factor causes the same defects as knocking it out or down. The concept that ''too much or not enough'' can produce similar effects is not new and a number of studies have reported this finding in distinct biological processes, including gastrulation. For instance, overexpression of Rok2, a downstream effector of the non-canonical Wnt pathway, produces a similar phenotype as expression of a dominant negative form (Marlow et al., 2002). Similarly, Gα12 and 13 are involved in the control of CE movements and over-expression of WT Gα12/13 RNA produces a similar defect as Gα12/13 knock down (Lin et al., 2005). It appears that an activity window exists for these factors. If overall activity falls outside of this window (either positively or negatively) the resulting phenotypes are very similar. This model may explain why knockdown of Csk produces similar defects as Fyn/Yes knockdowns, despite their antagonistic roles in cell signaling.

In conclusion, we show here that Csk is required for normal vertebrate gastrulation to occur. The Csk knockdown phenotype is reminiscent of other CE defective phenotypes both morphologically and molecularly. Because we were able to rescue the Csk phenotype by knocking down Fyn and Yes, Csk is genetically directly upstream of Fyn and Yes in the regulation of cell movements during vertebrate gastrulation.

4. Materials and methods

4.1. Zebrafish and in situ hybridization

Zebrafish were kept and the embryos were staged as described before (Westerfield, 1995). *In situ* hybridizations were done essentially as described (Thisse et al., 1993) using probes specific for *bmp2b* (Martinez-Barbera et al., 1997), *chd* (Schulte-Merker et al., 1997), *cyc* (Rebagliati et al., 1998), *ntl* (Schulte-Merker et al., 1992), *gsc* (Schulte-Merker et al., 1994), *six3* (Kobayashi et al., 1998), *pax2* (Kelly and Moon, 1995), *krox20* (Oxtoby and Jowett, 1993) and *wnt5* (Rauch et al., 1997) (generous gifts from members of the zebrafish community) and *fyn, yes, wnt11* and *csk* (RZPD ID's: UCDMp611J0321Q114, MPMGp609A1681Q8, MPMGp637F0720Q2 and IMAGp998P2017182Q1, respectively, from www.rzpd.de, Berlin, Germany).

4.2. Morpholinos, RNA and injections

Antisense MOs were designed to include the start ATG of the respective cDNAs and ordered from GeneTools (Philomath, OR, USA): Csk, 5′-GCCAGGTCGCCTCAAAG GTAGACAT. The Fyn and Yes MOs were described before (Jopling and den Hertog, 2005). 5′ capped sense RNAs were synthesized using a construct encoding *csk* and the mMessage mMachine kit (Ambion, Austin, TX, USA). Ranges of MO (0.1–5 ng) were injected into embryos of the AB strain at the 1 cell stage and phenotypes were assessed at the indicated stages.

4.3. Cell tracing

Embryos were (co-)injected at the one cell stage with 0.25% 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged fluorescein dextran (10,000 MW; Molecular Probes, Leiden, the Netherlands). Uncaging was done as described (Jopling and den Hertog, 2005) at shield stage (6 hpf) using an Axioplan microscope, equipped with a UV light source, adjustable pinhole and 40X objective. Pictures were taken immediately following uncaging, at 80% epiboly (8 hpf) and tailbud stage (10 hpf). The angles for dorsal convergence and anterior extension were determined using NIH imaging software.

Acknowledgements

The authors thank all who have contributed probes for the *in situ* hybridizations described here. We also thank Gabby Krens for providing the caged fluorophore. This work was supported by a European Union (FP5) Research Training Network (HPRN-CT-2000-00085).

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