Regulation of neurogenesis by Fgf8a requires Cdc42 signaling and a novel Cdc42 effector protein

Alissa M. Hulstrand and Douglas W. Houston*
Department of Biology and Interdisciplinary Graduate Program in Genetics, University of Iowa, Iowa City, IA 52242, USA.

Abstract

Fibroblast Growth Factor (FGF) signaling is required for numerous aspects of neural development, including neural induction, CNS patterning and neurogenesis. The ability of FGFs to activate Ras/MAPK signaling is thought to be critical for these functions. However, it is unlikely that MAPK signaling can fully explain the diversity of responses to FGFs. We have characterized a Cdc42-dependent signaling pathway operating downstream of the Fgf8a splice isoform. We show that a Cdc42 effector 4-like protein (Cdc42ep4-l or Cep4l) has robust neuronal-inducing activity in Xenopus embryos. Furthermore, we find that Cep4l and Cdc42 itself are necessary and sufficient for sensory neurogenesis in vivo. Furthermore, both proteins are involved in Fgf8a-induced neuronal induction, and Cdc42/Cep4l association is promoted specifically by the Fgf8a isoform of Fgf8, but not by Fgf8b, which lacks neuronal inducing activity. Overall, these data suggest a novel role for Cdc42 in an Fgf8a-specific signaling pathway essential for vertebrate neuronal development.

Keywords
neurogenesis; Fgf8; Cdc42; Cdc42 effector protein; Xenopus

INTRODUCTION

Neuronal differentiation within the neural plate is controlled by the inhibition of Notch signaling (Chitnis et al., 1995), resulting in the expression of neurogenic bHLH transcription factors including neurog2 and neuroD (Lee et al., 1995; Turner and Weintraub, 1994). The specific cell fates of primary neurons (i.e., larval-specific) are regulated differently along the mediolateral axis of the embryo, which is later transposed into dorsoventral patterning of the neural tube following neurulation. In amphibians, primary neurons appear in three stripes on either side of the dorsal midline within the neural plate, corresponding to lateral sensory neurons, intermediate interneurons and medial motorneurons. Primary neuronal differentiation takes place mainly in the deep (sensorial) layer of the neural plate, the cells of which are not polarized, and is excluded from the apically polarized cells of the superficial epithelium (SE; Hartenstein, 1989; Chalmers et al., 2002). Cell cycle exit and neuronal differentiation occur in a spatiotemporal wave from the lateral to medial neural plate...
The lateral primary sensory (Rohon-Beard) neurons are the first to terminally differentiate, with birthdating experiments suggesting this occurs mostly during the mid-gastrula stages in both frogs and fish (Lamborghini 1980; Metcalfe et al., 1990). Different neuronal subtypes are specified by graded amounts of specific molecular signals. Sonic hedgehog signaling from the notochord and floor plate is largely responsible for the specification of the ventral motorneurons and interneurons (Briscoe and Ericson, 2001). By contrast, the dorsal primary sensory neurons are specified independently from Shh and are regulated by the same signals that also induce non-neuronal fates in cells derived from the neural plate border region. These cell fates include hatching gland, preplacode, and neural crest, all of which also derive from the deep layer of the ectoderm (Park et al., 2012; Rossi et al., 2008; Rossi et al., 2009). Dorsal neuron and neural plate border inducing signals include BMPs and Wnts from the non-neural ectoderm as well as FGFs. In particular, graded Fgf8a induces preplacode and neural crest fates at low doses, whereas high doses induce sensory neurons and inhibit neural plate border fates (Fletcher et al., 2006; Hardcastle et al., 2000; Park et al., 2012).

In Xenopus, FGF signaling through Fgf4 and multiple Fgf8 splice isoforms is critical for many aspects of both mesoderm and neural development. Interestingly, Fgf8 splice isoforms, Fgf8a and Fgf8b, differ by 11 amino acids but elicit different responses in vitro and in vivo (Fletcher et al., 2006; Lee et al., 1997; Liu et al., 1999; MacArthur et al., 1995; Olsen et al., 2006; Sato et al., 2001; Sato and Nakamura, 2004). In Xenopus, Fgf8a induces ectopic neurogenesis throughout the epidermis whereas Fgf8b induces mesoderm (Fletcher et al., 2006; Hardcastle et al., 2000). The basis for these differences is not known, but may result from weaker binding of Fgf8a to the set of Fgf8-binding receptors (Olsen et al., 2006). In the chick, Fgf8a is thought to act as a weak Fgf8 ligand, since electroporation of low levels of Fgf8b resembled the Fgf8a effect (Sato and Nakamura, 2004). However, in frog embryos, high amounts of fgf8a did not mimic fgf8b, suggesting a qualitative difference (Fletcher et al., 2006). Prior to the work presented here, specific signaling pathways downstream of Fgf8a have not been identified.

Activation of FGFR signaling and tyrosine phosphorylation generates a host of well-characterized signal transduction responses (Dailey et al., 2005). The Ras/MAPK kinase cascade is a commonly studied outcome of FGFR signaling, but Rho GTPases (Rho, Rac Cdc24, et al.) can also be activated (Dailey et al., 2005). The specific functions of Rho GTPases are mediated by the binding of different effector proteins. Individual Guanine nucleotide exchange factors (GEFs) are thought to help recruit specific effectors to the monomeric GTPases they activate, thereby assembling compartmentalized signaling complexes (Bos et al., 2007; Sinha and Yang, 2008).

We have identified a role for one such effector protein in neuronal development, a member of the Cdc42 effector protein family (a.k.a., Borgs or CEPs; (Hirsch et al., 2001; Joberty et al., 1999)), Cdc42ep4-like/Cep4l. Cep4l and Cdc42ep4 proteins have well-characterized N-terminal CRIB (Cdc42/Rac Interactive Binding) domain that mediates binding to activated Cdc42 and Rac, but notto Rho (Burbelo et al., 1995). Cdc42ep family proteins have been characterized using in vitro, cell line and yeast-two hybrid assays, and bind only to activated Cdc42 and TC10/RhoQ, but not to activated Rac or Rho (Hirsch et al., 2001; Joberty et al., 1999). Cdc42ep4 has been shown to induce pseudopodia formation when transfected into fibroblasts (Hirsch et al., 2001), but the overall functions of the Cdc42ep family are unknown.

In this study, we show for the first time that a Cdc42 and Cdc42 effector-regulated signaling pathway is necessary and sufficient for neurogenesis in Xenopus. We show that Cep4l and...
Cdc42 overexpression in *Xenopus* embryos induces primary sensory neurons throughout the deep layer of the epidermis, and antisense knockdown of these molecules reduces this neuronal population. These effects resembled the induction of neurons in the epidermis by the Fgf8a splice isoform and we further show that Fgf8a stimulates Cep4l association with Cdc42. We also find that these proteins are required for robust neuronal induction by Fgf8a. Overall, these data identify a specific Cdc42-dependent molecular mechanism for neuronal specification likely acting downstream of Fgf8a isoform signaling.

**MATERIALS AND METHODS**

**Xenopus Embryos and Explants**

*Xenopus* adult females were induced to lay eggs by injection of human chorionic gonadotropin (hCG). Eggs were fertilized *in vitro* using a sperm suspension, cultured in 0.1X MMR (1X MMR: 0.1 M NaCl, 1.8 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 15.0 mM HEPES, pH 7.6) and dejellied in cysteine (2% in 0.1X MMR). Animal caps were dissected at stage 9 (Nieuwkoop and Faber, 1994) and cultured in 1X MMR to the indicated stage. For dp-ERK assays, caps were cultured for 60 minutes to avoid wound-induced ERK activation.

**Plasmids and mRNA Synthesis**

Full-length cDNAs for *cep4l* and *cdc42* in pCMV-SPORT6 were obtained commercially (Open Biosystems). The *cep4l* and *cdc42* coding regions (CDS), or *cep4l* CDS along with the 5'UTR, were amplified by PCR and cloned into pCR8/GW/TOPO (Invitrogen). Clones were sequenced and inserted into pCS2+/GW, pCS2-HA/GW, or pCS2-FLAG/GW custom Gateway converted vectors via LR recombination (Invitrogen). For pCS2+/GW, reading frame A cassette (Invitrogen vector conversion kit) was inserted into the *Stu*I site of pCS2+. For the HA-tag vector, reading frame B cassette was cloned into *Cla*I digested/Klenow filled pCS2+HA to generate a 2x HA fusion C-terminally. For the FLAG-tag vector, reading frame A was cloned into the *Stu*I site of pCS2+FLAG to generate a 2x FLAG fusion N-terminally. The cassettes were chosen so that constructs with complete triplet codons cloned into pCR8/GW/TOPO and recombined into the vectors will be in frame with the epitope tags. The parent vectors were gifts from J. Yang. A CRIB domain mutant (*cep4lH31A,H34A-pCS2*) was produced using site-directed mutagenesis (Quickchange II kit; Stratagene). Template DNA was linearized for sense transcription of mRNAs from *cep4l*-pCS2, *cep4lH31A,H34A-pCS2, CDC42EP4-pCS2, *cep4l*DCS-pCS2-HA, *cep4l*UTR-pCS2-HA, *cdc42*-pCS2, *cdc42*42G12V-pCS2, and *cdc42*-pCS2-FLAG with NotI. *Fgf8a-a-pCS107* (a gift from R. Harland) was linearized with *Asc*I. *Beta-galactosidase-pING4* (*β*-gal) was linearized with *Bam*HI. Capped mRNAs were synthesized using the T3 (*cdc42*-GFP) or SP6 mMESSAGE mMACHINE kit (Ambion). PCR primers for cloning and mutagenesis are shown in Supplementary Information (Table S1).

**Embryo Microinjections**

Fertilized embryos were injected with mRNAs or morpholino oligos (MO) into the animal hemisphere at the two-to-eight cell stages in Ficoll (2% Ficoll in 0.5X MMR). Antisense MOs complementary to *cep4l*, *fgf8a*, and *cdc42* were synthesized (Gene Tools) with the following sequences: *cep4l*UTR MO 5’ GGATCTTTTCACTTCCTTCCCAACG 3’ (new) against the 5’ UTR of *cep4l* (blocks translation); *fgf8a* MO 5’ CTCTGCTCCCTACATGCTGTGTAA 3’ (Fletcher et al., 2006) (disrupts splicing of the Fgf8a isoform); *cdc42* MO 5’ CTACACATTTAATTGTCTGCATGGC 3’ (Rana et al., 2006) (blocks translation; binds *X. laevis* and *X. tropicalis* mRNAs with no mismatches). The control MO was obtained from Gene Tools.
Recombinant human FGF8a, FGF8b and FGF17b were obtained commercially (R&D Systems) for protein injections. Proteins were dissolved in sterile 1X PBS to make stock solutions of 0.5 μg/μl (FGF8a, FGF17b) or 0.25 μg/μl (FGF8b). For injection, proteins were diluted to 0.5-1.5 ng per 40 nl in 1X PBS/0.1% BSA and microinjected (~40 nl/embryo) into the blastocoel at stage 9, as described (Pera et al., 2003). Following injection, embryos were cultured in 0.5X MMR to the desired stage.

**Whole Mount In situ Hybridization**

Whole mount in situ hybridization was performed as described (Kerr et al., 2008). Anti-digoxigenin-AP antibody (Roche Applied Science) was used at a concentration of 1:2500. Hybridization was detected through color reaction with BM Purple (Roche Applied Science), stopped with Bouin’s fixative, and washed multiple times with 70% EtOH/10 mM Tris, pH 8.0 before bleaching, as described (Sive et al., 2000). DNA template for digoxigenin-labeled RNA probes were linearized with restriction enzymes and in vitro transcribed using appropriate polymerases (Promega): cep4l-pCMV-SPORT6, SalI/T7; tubb2b-p24-10, NotI/T3; islet1-pExpressl, EcoRI/T7; runx1-pCMV-SPORT6, SalI/T7; pax2-pCMV-SPORT6, EcoRI/T7; pax6-pCS2, EcoRI/T3; sox2-pCS2, EcoRI/T7; sox9-pCMV-SPORT, EcoRI/T7; xk81a1-pBlueScript SK-, EcoRI/T7; foxi1-pBlueScript SK-, EcoRI/T7; crc-cpBlueScript SK-, EcoRI/T7. Probes were diluted to 1 μg/ml in hybridization buffer for use.

Beta-galactosidase staining was performed using Red-Gal (Research Organics) as described (Sive et al., 2000). Embryos sectioned following in situ hybridization were dehydrated in MeOH and embedded in PEDS wax (polyethylene glycol 400 diestearate, Polysciences) containing 10% cetyl alcohol. 10-12 μm sections were mounted on Superfrost Plus (Fisher) microscope slides with Citramount (Polysciences) for imaging.

Quantification of in situ hybridization stained neurons was performed by counting individual neurons in control and unilaterally-injected embryos. Significance was determined using two-tailed paired t-tests in Excel.

**Coimmunoprecipitation and immunoblotting**

In vitro translation was performed using TnT SP6 Coupled rabbit reticulocyte lysate kit (Promega). Capped mRNA synthesized as above was used as the template for protein translation according to manufacturer instructions. Half-volume reactions were incubated at 30°C for 90 minutes.

Coimmunoprecipitation was performed as described in Schneider et al., (2012) except that crosslinkers were not used. Coimmunoprecipitation of in vitro translated proteins with active His-tagged Cdc42 and Rac1 proteins (Cytoskeleton) was performed using nickel-chelated agarose beads (Pierce). Beads and protein pellets were resuspended in SDS-PAGE buffer, boiled and subjected to electrophoresis.

Immunoblotting was performed (Schneider et al., 2012) using peroxidase conjugated anti-FLAG (Sigma) or anti-HA (Roche) primary antibodies and non-conjugated primary antibodies, anti-beta-catenin (Sigma), anti-tubulin (beta) E7 (DSHB), anti-phospho-p44/42 MAPK (dp-Erk, Cell Signaling), and custom guinea pig anti-Cep4l (generated by Cocalico Biologicals) (all diluted 1:1000). Peroxidase conjugated anti-mouse IgG, anti-rabbit and anti-guinea pig secondary antibodies (Jackson ImmunoResearch) were diluted 1:20,000. Relative quantification of Western blot bands was carried out using ImageJ Gel Analysis.
**Immunostaining**

Embryos for immunostaining were fixed in MEMFA, washed in MeOH and bleached in 70% MeOH/10% H2O2. Following rehydration, embryos were incubated in PBT (1% Triton in 1X PBS) plus 10% goat serum three to four hours at room temperature and washed 30 minutes in PGT (PBT plus 1% goat serum). Mouse zn-12 hybridoma supernatant (DSHB) was diluted 1:5, and rabbit anti-diphospho-p44/42 MAPK (dp-Erk, Cell Signaling) was diluted 1:1000 in PGT. Embryos were incubated overnight at 4°C and washed five times for one hour in PGT. Peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch) were diluted 1:500 in PGT and incubated overnight at 4°C. Embryos were washed five times one hour in PGT and 30 minutes in PBS. DAB detection was performed using 0.3% DAB and 0.01% hydrogen peroxide diluted in PBS and stopped after 5-10 minutes by washing in PBS/0.1% Tween-20. TrueBlue substrate (KPL) detection was carried out for 15 minutes and reaction was stopped with water.

**Gene Expression Analysis by RT-PCR**

Total RNA isolation was performed as described (Houston and Wylie, 2005) using proteinase K and RNase-free DNase I. Negative controls lacked reverse transcriptase during cDNA synthesis to show absence of genomic DNA contamination. Both semi-quantitative and quantitative PCR using the Lightcycler480 (Roche) were carried out using ornithine decarboxylase (odc) as a loading control or normalization for quantitative PCR. PCR primers for RT-PCR are shown in Supplementary Information (Table S1).

**RESULTS**

**Cep4l is a vegetally localized RNA in Xenopus oocytes**

Cuykendall and Houston (2010) identified a number of novel mRNAs localized to the vegetal cortex. One of these mRNAs, cep4l, was verified by RT-PCR and showed an expression pattern during oogenesis similar to that of vg1 and vegt (Cuykendall and Houston, 2010). Preliminary overexpression of cep4l caused morphogenetic defects (see below), suggesting it has functions in early development. We therefore chose this cDNA for further characterization.

We obtained a full-length EST for Xenopus laevis cep4l from a commercial source. This EST encodes a predicted protein of 306 amino acids with a domain structure classifying the protein as a homologue of Cdc42 Effector Protein 4 (Cdc42ep4) (Fig. S1). Cdc42ep family proteins are conserved throughout the chordate lineage, with Cdc42ep4 showing independent duplications in Xenopus and in Teleost fish. We therefore designated this gene as Cdc42 effector protein 4-like (Cdc42ep4-like or Cep4l). Cdc42ep4 proteins contain three conserved domains, a Cdc42/Rac interactive binding (CRIB) domain and two other conserved regions, the CI and CII domains, located C-terminally to the CRIB domain (Fig. 1A). The CI/II regions and the acidic-rich C-termini of Cdc42ep4 homologues have no known function.

Cep4l mRNA is present maternally and expression continues throughout early development (Fig. 1B). Spatial analysis of RNA expression during oogenesis using in situ hybridization shows that cep4l mRNA is expressed uniformly in previtellogenic oocytes (data not shown). In full-grown stage VI oocytes, cep4l mRNA is localized in a broad expression domain in the cortex of the vegetal hemisphere (Fig. 1C). During embryogenesis, cep4l is expressed diffusely in the blastula and becomes enriched more in prospective dorsal mesoderm and ectoderm around the blastopore during gastrulation. In later gastrula stages, expression becomes enriched on the dorsal side of the blastopore (Fig. 1D-E). In early neurulae, cep4l is first expressed in patches adjacent to the anterior neural plate. This expression pattern persists, and by late neurulae cep4l is present in the migrating cranial neural crest (Fig. 1F-
In tailbud stage embryos, *cep4l* is found in the branchial arches populated by migrating cranial neural crest as well as in the somites at stage 25, and in migrating trunk neural crest at stage 31 (Fig. 1H, K-L).

**Overexpression of Cep4l induces ectopic neurogenesis**

To identify functions of Cep4l in development, we injected *cep4l* mRNA into 2-4 cell stage embryos. The effects of *cep4l* overexpression were first seen in blastula stage embryos as a disruption in the maternal animal cap pigment (Fig. 2A, D). *Cep4l*-injected embryos developed a perinuclear accumulation of pigment in affected blastomeres (data not shown), causing asymmetrical distribution of pigment as cell division proceeded. This pigment disruption was evident at stage 7, prior to the activation of zygotic transcription at MBT, indicating a direct effect of *cep4l* (data not shown). Other morphological effects of *cep4l* whole embryo overexpression include delayed closure of the blastopore, convergent extension defects and shortened anteroposterior axis, and defects in anterior structures, such as reduced forebrain and eyes (Fig. 2A-F).

In identifying potential effects of Cep4l expression on the nervous system, we examined *ntubulin/tubb2b* expression in primary neurons and noticed that *cep4l*-injected embryos displayed robust ectopic expression of *tubb2b* (Fig. 2G-H). *Xenopus* primary neurons are stereotypically organized in three mediolateral stripes. The medial and intermediate stripes (motor and interneurons) were unaffected in *cep4l*-injected embryos, whereas the lateral, sensory neurons were markedly increased and expanded throughout the lateral and ventral epidermis (102/145 embryos, 70%; over many experiments) (Fig. 2G, H). We also examined the function of a mammalian Cdc42ep4 homologue, which is a distinct paralogue of Cep4l. Interestingly, the neuronal-inducing activity was conserved in human *CDC42EP4* mRNA (14/29 embryos, 48%) (Fig. 2I). Sectioning confirmed that ectopic neurons induced by Cep4l formed in the deep layer of the ectoderm, as do endogenous primary neurons (Fig. 2J-K). To provide evidence that Cep4l was inducing differentiated neurons, we stained tailbud embryos using a monoclonal antibody against HNK-1 (mAb zn-12), a marker of branching neurons (Metcalfe et al., 1990). As with *tubb2b*, embryos overexpressing *cep4l* demonstrated ectopic neurons expressing HNK-1 well outside of the neural tube and in ventral regions (Fig. 2L, M).

We next injected *cep4l* unilaterally at the 2-cell stage and counted lateral domain neurons to determine whether the apparent increase in *tubb2b* was indeed owing to an increased number of neurons and not to a dispersal of existing neurons (Fig. 3). The average number of neurons on each side in control embryos was similar (Table 1; mean ~64 on each side), however *cep4l*-injected embryos showed a significant increase in neurons on the injected side (Table 1; >100 on injected side, Fig. 3A, E). Because the effect of *cep4l* overexpression appeared confined to the lateral neuronal cells (sensory), we confirmed their identity using more specific markers. Consistent with an induction of primary sensory neurons, cells expressing *runxl*, which is expressed by a subset of Rohon-Beard neurons (Park et al., 2012), were increased in *cep4l*-injected embryos (77/100 embryos, 77%; Fig. 3B, F). Quantification of neurons in unilaterally injected embryos showed a consistent statistically significant increase in mean numbers on the *cep4l*-injected side (Table 1; Fig. 3I).

Interneuron (*pax2*) and motor neuron (*islet1*) numbers were unaffected by *cep4l* (Figs. 3C, G; 3D, H). For motor neurons, similar numbers of *islet1* expressing cells along the midline of the neural tube were seen on the injected side of control and *cep4l*-expressing embryos (Table 1; Fig. 3I). However, lateral domain sensory neurons expressing *islet1* were increased in 20/35 embryos (57%), appearing in the lateral/ventral epidermis (Fig. 3G).
Counting islet-expressing cells in these embryos again indicated a significant increase in mean numbers of sensory neurons on the injected side, while control embryos showed no change (Table 1; Fig. 3I). For the interneuron population, counting of pax2 neurons showed no change in mean numbers of interneurons on the injected side compared to the uninjected side (Table 1; Fig. 3I).

Overall, these data show that misexpression of cep4l results in induction of primary neurons in the epidermal region of the embryo. Based on the expression of different neuronal markers (islet1, runx1), these ectopic neurons are likely to be subclasses of primary sensory neurons.

**Cep4l is required for primary sensory neurogenesis**

We next turned to loss-of-function experiments to determine the extent that Cep4l is required for endogenous neurogenesis. *Cep4l* is expressed maternally and maternal depletion of *cep4l* led to gastrulation lethality (data not shown), the etiology of which is under investigation. Additionally, we wished to specifically inhibit late Cep4l function in embryos, however the *cep4l* locus lacks introns (data not shown), precluding a splice-blocking oligonucleotide approach. We therefore designed a morpholino oligo (MO) that hybridizes to the 5’ UTR upstream of the starting methionine to block translation and production of the Cep4l protein (Fig. 4A). We tested the efficacy of the *cep4l* MO by immunoblotting against C-terminally HA-tagged constructs with or without the 5’ UTR MO target sequence. Addition of the HA tag did not hinder Cep4l function in overexpression assays (data not shown). Two-cell embryos were coinjected with *cep4lCDS-HA* or *cep4lUTR-HA* mRNAs and increasing doses of *cep4l* MO. Western blotting showed that Cep4lCDS-HA was expressed at similar levels in the presence or absence of the *cep4l* MO, whereas Cep4lUTR-HA was greatly reduced at all doses of MO (Fig. 4B).

To test the requirement for Cep4l in neurogenesis, we first injected both blastomeres at the two-cell stage with 40 ng of *cep4l* MO. Whole embryo depletion of Cep4l caused numerous morphological effects, including delay or failure of blastopore closure, shortened anteroposterior axis, and reduced anterior structures (Fig. 4C-F), possibly owing to interference with Cep4l function in the vegetal hemisphere. Since we were unable to assess any direct roles of Cep4l in these morphologically abnormal embryos, we more specifically tested the requirement of Cep4l by performing targeted blastomere injections. Eight-cell embryos were injected with 8 ng *cep4l* MO into either one dorsal animal (DA) or one ventral animal (VA) blastomere on the future right side of the embryo, and stained for neurons using *in situ* hybridization for tubb2b and islet1 (Fig. 4G-N). These injections are expected to target predominantly either the brain or the posterior CNS, respectively (Moody, 1989), and should avoid targeting maternally-derived vegetal *cep4l* transcripts. *Cep4l* MO injection into DA blastomeres caused a delay in blastopore and neural tube closure on the injected side, resulting in abnormal head morphogenesis. There was also a complete loss of tubb2b staining in the anterior neural tube and the trigeminal placode (16/20 embryos, 80%; Fig. 4K). Posterior tubb2b staining was unaffected. Unfortunately, we were unable to rescue this anterior morphogenetic defect using a wide range of *cep4l* mRNA doses (not shown), making interpretation of these effects problematic.

In contrast to dorsal animal (DA) injections, injection of *cep4l* MO into ventral animal (VA) derivatives strongly and reproducibly reduced spinal sensory neurons on the injected side and produced minimal morphogenetic defects. Expression of tubb2 and islet1 in the sensory neuron domain was reduced on the Cep4l-depleted side (44/56 embryos, 79%; Fig. 4L-N). Expression of these markers in the remainder of the neural tube, in particular the motor neuron expression of islet1 (Fig. 4N), was unaffected on the injected side. VA-targeting of
cep4l MO did not affect overall expression or patterning of a neural marker (pax6; Fig. S2A,C), suggesting that overall CNS formation is not dependent on Cep4l. Cep4l is also not required for neural crest fate specification, since a neural crest marker (snail/snail, Fig. S2B,D) was expressed normally. Spatially disrupted snail expression in later tailbud stages indicated that Cep4l could have an additional role in crest migration, although we did not pursue this further since Cep4l also affected gastrulation and neurulation, which could confound effects on the neural crest.

We confirmed the specificity of neuronal differentiation deficiencies in Cep4l-depleted embryos by performing rescue experiments (Fig. 4O-V). Embryos at the four-cell stage were injected on one side with low dose (125 pg) cep4l and β-gal as a lineage tracer (or β-gal alone), and subsequently injected with cep4l MO into a VA blastomere on same side at the eight-cell stage. Cep4l mRNA (lacking the MO-binding site) co-expression in VA blastomeres at this sub-phenotypic dose was able to restore sensory neurons on the injected side in the majority of cases in at least three separate experiments (25/35 embryos; 71%; Fisher's exact test p < 0.001, Fig. 4Q, U), indicating that the defects in neurogenesis were specifically the result of loss of Cep4l protein.

Taken together with our overexpression results, these data demonstrate that Cep4l activity is sufficient to induce primary sensory neurons in epidermal tissue, and is also necessary for endogenous sensory neuron formation in Xenopus.

**Cdc42 activity is required for primary neurogenesis**

Since Cep4l is a predicted Cdc42-effector protein, we further explored the functional relevance of its interaction with Cdc42. Prior studies on Cdc42ep proteins (Hirsch et al., 1999; Joberty et al., 1999) as well as high-throughput protein-protein interactome mapping (Rual et al., 2005), demonstrated that Cdc42ep4 binds specifically to activated Cdc42 and Rhoq, but not Rac or Rho proteins. We performed in vitro binding studies using commercially supplied active Cdc42 or Rac1 His-tagged proteins and in vitro translated Cep4l-HA. Consistent with these data, we found that purified active Cdc42 but not active Rac1 protein was able to interact with full-length Cep4l-HA (Fig. 5A). We did see a shorter product pulled down by active Rac1, which could represent use of an alternate downstream starting methionine. Altering the length can change the specificity of CRIB domains (Thompson et al., 1998), suggesting that a shorter Cep4l CRIB domain may weakly bind activated Rac1. We found no evidence that this shorter form is expressed from injected mRNA in vivo. Additionally, we confirmed that Cep4l-HA interacts with FLAG-Cdc42 in immunoprecipitations of embryo lysates (see below). Finally, we did not detect any expression of RhoQ in early embryos (data not shown). These data suggested that Cep4l would act primarily as an effector for activated Cdc42 during Xenopus embryogenesis.

Since members of the Cdc42ep family require interaction with Cdc42 for their function, we hypothesized that a Cep4l mutant CRIB domain deficient in Cdc42 binding would be inactive for neuronal inducing activity. We mutated well-characterized histidine residues in the Cep4l CRIB domain essential for binding to activated Cdc42 (Ash et al., 2003; Hirsch et al., 2001), cep4lH31A,H34A and compared expression of full-length and mutant proteins in embryo lysates. No differences in protein expression levels were found by immunoblotting against Cep4l using a guinea pig antiserum raised against Xenopus Cep4l (Fig. 5B). The mutant construct was inactive in neuronal induction (Fig. 5D-F), and in pigment disruption (data not shown), indicating a requirement for activated Cdc42 binding. We confirmed that Cep4l-H31A,H34A failed to interact with Cdc42 using co-immunoprecipitation of embryo lysates (Fig. 5C)
We next tested the idea that Cdc42 activation might be sufficient to induce neuronal differentiation. We first overexpressed wildtype Cdc42 (untagged) or a constitutively active mutant (Cdc42G12V) in embryos and assayed for ectopic tubb2b expression. Although ectopic neurons could be generated in over half the cases (29/45 embryos, 64%), associated blastopore or neural tube closure defects made it unclear whether these neurons were secondary to these other defects (data not shown). We therefore asked whether Cdc42 and Cep4l could synergize in neuronal induction. We co-injected sub-phenotypic doses of cep4l and either wildtype or constitutively active mutant Cdc42 constructs unilaterally into two-cell embryos. Injection of low doses (< 100 pg) of cdc42 or cdc42G12V did not affect tubb2b expression, nor did injection of the low dose of cep4l, alone or in combination with cdc42 (Fig. 5G-K). Coinjection of the cdc42G12V constitutively active mutant with cep4l, however, induced ectopic neurons and expanded the region of lateral neurons in the neural tube on the injected side (9/17 embryos, 53%; Fisher’s exact test p < 0.001; Fig. 5L).

Since these results suggested that Cep4l/Cdc42 interactions were sufficient to induce neurogenesis, we next determined the effect of Cdc42 loss-of-function on endogenous neuronal differentiation. We depleted Cdc42 in embryos using a translation-blocking morpholino that was used previously in X. tropicalis, and that recognizes the identical sequence in X. laevis (Rana et al., 2006). We confirmed that this oligo was effective in reducing expression of exogenous GFP-tagged Cdc42 (Fig. 6A). Injection of 60 ng of MO into one or two blastomeres of two-cell embryos did not visibly disrupt normal early development, although we did observe that tubb2b expressing neurons were reduced in a majority of cdc42 MO-injected embryos (44/57 embryos, 77%; Fig. 6B-F). In comparison, control morpholino injection at the same dose did produce a minor reduction in sensory neurons in a minority of embryos (22/65 embryos, 34%). The difference between the control and cdc42-MO groups remains statistically however (Fisher’s exact test, p < 0.01). These data suggest that Cdc42 plays a critical role in sensory neurogenesis, but also that these cells may be sensitive to high oligo doses. In unilateral injections, numbers of sensory domain tubb2b-positive neurons were moderately but significantly decreased on the Cdc42-depleted side compared to control uninjected embryos (Table 1, Fig. 6D-F). Overall, the synergy of neuronal induction in coinjection experiments, and the comparable effects of depletion of Cep4l and Cdc42, suggest that these proteins act together in endogenous sensory neuronal differentiation.

Cep4l is likely not a BMP antagonist or neural plate inducer

We next performed additional experiments to determine potential mechanisms by which Cdc42/Cep4l signaling might generate ectopic neurons. First, we treated cep4l-injected embryos with a combination of hydroxyurea and aphidicolin (HUA) to block the cell cycle in S phase (Harris and Hartenstein, 1991). Ectopic neurons were still induced by Cep4l (Fig. S3A-F), ruling out that ectopic neurons simply result from stimulation of cell division by Cep4l. Second, we found that ventral-animal overexpression of cep4l neither induced nor inhibited markers of neural plate (sox2), neural crest (sox9), or epidermis (sk8lal/epidermal keratin; Fig. 7A-E). Third, we analyzed Cep4l activity in tissue explants and found that Cep4l does not dorsoalize ventral marginal zone explants (data not shown) or induce neural fate in animal cap (Fig. 7F-I; Fig. S3G). These negative data suggest that Cep4l does not have strong pro- or anti-BMP activity or obvious pro- or anti-Wnt activity.

Next, we looked at possible effects on Notch signaling, which is known to inhibit neuronal differentiation (Chitnis et al., 1995), but can also inhibit multiciliated cell (MCC) and ionocyteWell (IC) fates (Stubbs et al., 2006). These cell types are derived from deep-layer ectoderm in the epidermal regions (Hayes et al., 2007). Surprisingly, however, overexpression of cep4l greatly suppressed MCC and IC fates (marked by ciliary-rootlet
coiled coil (crcc) and foxi1, respectively; Hayes et al., 2007) in a majority of cases (crcc 10/20, 50%; foxi1 24/34, 71%) while inducing neurons in those areas (Fig. 7N-O). Since Notch activation or Notch inhibition affects the differentiation of neurons, MCCs and ICs in the same manner, we conclude from these experiments that Cep4l is not directly involved in Notch signaling.

Finally, we compared the activity of Cep4l to Fgf8a, which induces primary sensory neurons throughout the lateral epidermis similarly to Cep4l (Hardcastle et al., 2000; Fletcher et al., 2006). We found that Fgf8a also suppressed MCC and IC fates while inducing neurons (Fig. 7P). The extent of the Fgf8a effect was greater in these assays owing to the fact that Fgf8a is secreted and easily diffusible and can act over a greater area. These roughly comparable effects of Fgf8a and Cep4l therefore suggested that Cep4l might functionally interact with Fgf8a signaling in regulating neuronal cell fate.

Cep4l is required downstream of Fgf8a in neuronal induction

In Xenopus, Fgf8a is required for both for posterior neural patterning and for neuronal differentiation (Fletcher et al., 2006). It is unclear however whether these activities involve similar signaling mechanisms, since posterior neural patterning requires Ras/MAPK signaling and neuronal differentiation does not (Ribisi et al., 2000). Since our initial experiments suggested that gain or loss of Cep4l function did not directly affect neural patterning, we more specifically examined the extent of Cep4l and Fgf8a functional interaction during neurogenesis using molecular epistasis experiments.

We first used minimally effective low doses of cep4l mRNA and human FGFR8a protein (injected into the blastocoe) either singly or in combination. Individual treatment alone caused minimal ectopic tubb2b expression, whereas co-injection of low-dose FGFR8a and cep4l resulted in many ectopic tubb2b-expressing cells throughout the epidermis (19/19 embryos; Fig. 8A-D). We additionally performed double-depletion experiments. Inhibition of Fgf8a protein expression using a splice-blocking MO specific for the Fgf8a isoform has been shown to reduce neuronal differentiation (Fletcher et al., 2006; Fig. S4A-F). We injected fgf8a MO and cep4l MO singly or in combination and assayed tubb2b expression. To minimize confounding morphological effects and provide an internal control, we injected fgf8a MO bilaterally into the ventral blastomeres at the four-cell stage and cep4l MO unilaterally into one ventral animal blastomere at the eight-cell stage. Consistent with Fgf8a/Cep4l interaction in overexpression experiments, the reduction of neuronal differentiation by Fgf8a knockdown was further enhanced on the side injected with cep4l MO (48/76 embryos, 63%; Fisher's exact test p <0.001; Fig. 8E-H). Fgf8a-depleted embryos had ~55 neurons on each side, and this was reduced to an average of six per side when both Cep4l and Fgf8a were depleted (Table 1).

Next, we analyzed the extent that Cep4l and Cdc42 were necessary for the induction of ectopic neurons by Fgf8a. We injected controls and Cep4l or Cdc42-depleted embryos (VA injection) with fgf8a mRNA (unilateral at two-cell) and examined tubb2b or runx1 expression. Fgf8a induction of neurons was robust on both sides of control embryos (91/91, 100%). Importantly, this activity was unilateral reduced on the sides of embryos by Fgf8a/Cep4l interaction in overexpression experiments, the reduction of neuronal differentiation by Fgf8a knockdown was further enhanced on the side injected with cep4l MO (48/76 embryos, 63%; Fisher's exact test p <0.001; Fig. 8E-H). Fgf8a-depleted embryos had ~55 neurons on each side, and this was reduced to an average of six per side when both Cep4l and Fgf8a were depleted (Table 1).

Lastly, to more directly address a possible role for Cep4l in posterior neural patterning by Fgf8a, we depleted Cep4l in the context of Fgf8a overexpression and examined the effects.
on anteroposterior neural pattern and neural crest formation. Unilateral injection fgf8a caused an expansion of sox2, krox20 and hoxb9 throughout the embryo and repressed snail expression on the injected side whilst inducing it on the contralateral side (Fig. S6). These observations indicate that Cep4l is not required for these effects of Fgf8a on the nervous system. Overall, these data demonstrate that primary sensory neurogenesis in Xenopus requires Fgf8a signaling involving activated Cdc42 and the Cep4l effector protein.

**Fgf8a signaling is independent of MAPK signaling and specifically promotes the association of Cep4l with Cdc42**

The molecular basis for the differential signaling activities of Fgf8 isoforms in chicken and frog embryos is not well understood. It has been suggested that Fgf8a/b isoforms differentially activate ERK during chicken midbrain-hindbrain development (Sato and Nakamura, 2004). We therefore tested the extent that this differential activation might occur in Xenopus. We injected embryos with equal doses of fgf8a or fgf8b mRNAs (500 pg) and dissected animal caps at the late blastula stage. These were cultured and assayed for the di-phosphorylated, active form of MAPK (dp-ERK) by immunoblotting (Fig. 9A). Caps expressing fgf8b showed robust activation of MAPK, whereas control caps and fgf8a injected caps lacked MAPK activation (Fig. 9A). Similar to fgf8a, cep4l (500 pg) also failed to induce MAPK in animal caps (Fig. 9A). Since Cep4l and Fgf8a do not induce neurogenesis in animal caps, but do so in whole embryos, we considered that caps might lack an additional component needed for MAPK signaling and neurogenesis. We therefore confirmed these results using whole-mount immunostaining against dp-ERK in intact gastrula stage embryos (Fig. 9B-E). These data are consistent with the results shown in the chick and suggest that in the frog, Cep4l and Fgf8a regulate neurogenesis independently of immediate MAPK pathway activation.

The functional interaction data suggested that Fgf8a might stimulate the activation Cdc42 and increase recruitment of Cep4l. For these experiments, late blastula embryos expressing tagged Cep4l and Cdc42 constructs were treated with commercially prepared, purified FGF8 proteins injected into the blastocoel (Pera et al., 2003). We then performed immunoprecipitations 60 minutes later and analyzed the resulting immune complexes. In control embryos co-injected with FLAG-cdc42 and cep4l-HA, anti-FLAG (Cdc42) immunoprecipitation was able to bring down detectable amounts of Cep4l-HA (Fig. 9F). Interestingly, in the presence of exogenous human FGF8a protein injected into the blastocoel, the amount of Cep4l pulled down by FLAG-Cdc42 was increased (Fig. 9F). Whereas FGF8a significantly stimulated Cdc42/Cep4l complex formation, FGF8b did not, suggesting that regulation of Cdc42/Cep4l interaction is a specific outcome of Fgf8a signaling. The results from three independent experiments were quantitated, and indicated an average ~2 fold increase of Cep4l/Cdc42 interaction following Fgf8a treatment. Fgf8b treatment produced a small but reproducible reduction in Cep4l/Cdc42 interaction.

Fgf8a has been proposed to act as a weak Fgf8 ligand, since a one hundred-fold reduction in Fgf8b dose caused Fgf8a-like effects (Sato and Nakamura, 2004; Olsen et al., 2006). We therefore performed experiments using a fifty-fold dilution ofFGF8b. We found that this dose was similarly unable to induce neurons (Fig. S7). These results suggest that Fgf8 ligands exert primarily qualitative effects on downstream signaling mechanisms. To further test this, we also examined Fgf17 activity in Xenopus. Fgf17 (and Fgf18) is predicted to have a receptor affinity in between Fgf8a and Fgf8b and can produce an Fgf8a-like effect in chick neural tubes (Liu et al., 2003; Olsen et al., 2006). Consistent with this idea, we found that human FGF17b also greatly induced ectopic neurons in embryos (17/19 cases; 89%; three experiments; Fig. S7).
Since we observed a high level of Cdc42/Cep4l interaction in control embryos, we suspected this was the result of endogenous Fgf8a signaling. To determine whether this was the case, we performed immunoprecipitations to analyze Cep4l/Cdc42 interactions in embryos depleted of Fgf8a. Consistent with this hypothesis, the amount of Cep4l-HA immunoprecipitated by FLAG-Cdc42 was reduced in embryos co-injected with Fgf8a MO (Fig. 9H). Thus, Fgf8a is both sufficient and necessary to regulate Cep4l/Cdc42 interaction in the late blastula/early gastrula stages.

DISCUSSION

Cdc42 effector protein 4-like (Cep4l) regulates neurogenesis

As part of an ongoing effort to study the functions of maternally-localized transcripts in the Xenopus oocyte, we identified and characterized a Xenopus homologue of Cdc42 effector protein 4 (Cdc42ep4). Cep4l belongs to a little-studied class of Cdc42 effector proteins (Cdc42ep/Borg/CEP) implicated in actin filament regulation and cell shape (Hirsch et al., 2001). Through initial gain-of-function experiments in Xenopus embryos, we showed that Cep4l dramatically induced neurogenesis throughout the deep layer of the epidermis. The induced neurons were derived from the lateral domain of sensory neuron precursors present in the early neurula and express markers of primary sensory/Rohon-Beard neurons including pan-neuronal tubulin (tubb2b), islet1, runx1 and the zn-12 antigen. Markers of the motor and interneuronal subtypes were unaffected, suggesting a specific induction of the sensory population. Counting of neurons indicated that sensory neurons were increased in number in a cell/tissue autonomous fashion, suggesting that Cep4l does indeed induce neuronal fate as opposed to causing a dispersal of existing neuronal precursors. Interestingly, human CDC42EP4 had the same activity, indicating that this role in neuronal fate may be conserved. Nothing is known about the role of the Cdc42ep family in mammalian development however.

Loss-of-function experiments using antisense MOs support the conclusion that Cep4l is required for primary sensory neuron formation in vivo in Xenopus. Depletion of Cep4l in regions fated to form sensory neurons leads to a reduction in these neurons that can be rescued by MO-resistant cep4l mRNA. Importantly, this rescue could be achieved at doses that do not normally induce neurons and thus demonstrate the specificity of the MO injection. Primary spinal sensory/Rohon-Beard neurons become post-mitotic primarily during the late gastrula stages, with 80% being born at stage 11-12 (Lamborghini, 1980). Cep4l is expressed maternally in the vegetal hemisphere and is zygotically expressed around the blastopore and to a lesser extent throughout the animal cap by the midgastrula stage. Cep4l then becomes restricted to the dorsal half of the blastopore by late gastrula. Spinal cord and Rohon-Beard cells originate from the lateral blastoporal regions of the gastrula (Keller et al., 1992; Rossi et al., 2008), which would overlap with cep4l expression at these stages. Taken together with the gain-of-function data, we conclude that Cdc42ep4 proteins play a critical role in the specification and differentiation of primary sensory neurons in Xenopus.

Cep4l is likely to have other functions as well, since we saw effects on pigment dispersal as well as morphogenetic defects at high doses. Depletion of Cep4l in the whole embryo or in dorsal injections also results in gross morphogenetic defects, although we were unable to rescue this aspect. It is possible that precise dosage and temporal/spatial regulation of Cep4l expression would be needed to restore normal morphogenesis in depleted embryos. Cdc42ep proteins have been implicated in cytoskeletal regulation in cultured cells, however it is unclear to what extent Cep4l acts similarly in Xenopus. Cdc42ep4 and Cep4l are notable in their lack of an N-terminal extension of the conserved Cdc42ep CII motif, termed the Borg homology domain 3 (BD3), which has been characterized as a Septin-binding, cytoskeletal
regulatory motif (Joberty et al., 2001). Also, our preliminary experiments ruled out a direct role for Cep4l in actin assembly (data not shown), it is therefore possible that Cep4l overexpression affects morphogenesis indirectly by affecting intracellular transport. A more detailed analysis of Cep4l function at the cellular level would be instrumental in addressing these questions.

Role of Cdc42 in amphibian primary neurogenesis

One surprising finding from this work was the novel observation that Cdc42 is required for primary sensory neuron formation. The presence of a CRIB domain Cep4l and the characterization of related Cdc42ep proteins suggested that Cep4l would interact with activated Cdc42 and we verified this using *in vitro* and *in vivo* assays. Additionally, we made a CRIB domain mutant Cep4l that was unable to bind Cdc42 and correspondingly had no gain-of-function effect when expressed in embryos. This observation suggested that Cdc42 itself might function in sensory neuron specification. The ability of a constitutively active Cdc42 construct along with co-injected Cep4l to induce neurons supports this idea. Additionally, depletion of endogenous Cdc42 by MO injection greatly reduced the numbers of sensory neurons, demonstrating a requirement for Cdc42-mediated signaling in *Xenopus* neurogenesis. It was somewhat curious to note that injection of high doses of Cdc42 MO did not cause morphogenetic or other visible defects. Also, we did not observe the high rate of lethality seen when this MO was used in *X. tropicalis* (Rana et al., 2006). Cdc42 is likely to be an abundant protein and therefore MOs may be less effective than dominant-negative constructs which might act as sinks for a variety of Rho GTPase regulators.

It is unclear if Cep4l/Cdc42 signaling would have a conserved role in mammalian neurogenesis, since Cdc42ep4 gene targeted mice have not been reported. In mice Cdc42 is required to activate apical aPKC signaling to maintain ventricular zone neuronal progenitors and deletion of Cdc42 results in expansion of subventricular zone progenitors and expanded neurogenesis (Cappello et al., 2006). It is unlikely that Cdc42 is acting similarly at the level of maintaining apical identity in the context of primary neurogenesis in *Xenopus*. We find that gain of Cdc42 activity results in ectopic neurogenesis whereas deletion has this effect in mice (Capello et al., 2006). Additionally, primary neurogenesis occurs in unpolarized inner layer cells in the amphibian neural plate and is inhibited in the apically polarized superficial layer cells (Chalmers et al., 2002; Hartenstein, 1989), likely through aPKC activation (Ossipova et al., 2007; Sabherwal et al., 2009). These differences in the role of aPKC and Cdc42 could reflect bona fide species differences in function or could indicate differences in primary larval neurogenesis versus neurogenesis of adult neurons.

Regulation of Cep4l by Fgf8a isoform activity

A second major finding of this work is the involvement of Cep4l and Cdc42 in the regulation of neurogenesis by Fgf8a signaling. We show that Cep4l and Cdc42 are each needed for the neuron-inducing activity of Fgf8a. Furthermore, we find that Fgf8a is necessary and sufficient for the interaction of Cep4l with Cdc42. Fgf8a has been previously implicated in regulating neuronal fate, but the exact mechanisms and downstream signaling events have not been comprehensively identified. Many aspects of FGF signaling are mediated by MAPK activation, including anterior-posterior neural patterning, however the regulation of neurogenesis appears to occur independently of this pathway (Ribisi et al., 2000). We have shown that Fgf8a and Cep4l fail to activate MAPK in animal caps, whereas Fgf8b is strongly active in MAPK stimulation. These data are consistent with the ability of Fgf8b to activate brachyury (xbra) expression in *Xenopus* (Fletcher et al., 2006) and MAPK in the chick neural tube (Sato and Nakamura, 2004). Although previous Fgf8a gain-of-function experiments showed MAPK activation in a manner restricted to the diencephalon in the chick, this effect was likely indirect since it was enhanced rather than blocked by a
dominant-negative Ras construct (Sato and Nakamura, 2004). Fgf8a is required for anterior-posterior neural patterning in *Xenopus* (Fletcher et al., 2006) and may thus also regulate MAPK indirectly in neural tissue. Since Cep4l gain- or loss-of-function does not appear to specifically regulate neural patterning, this aspect of Fgf8a signaling is likely to be independent of Cep4l. Our data are the first to suggest a distinct, direct outcome of Fgf8a signaling in the context of neurogenesis.

It is interesting to speculate on whether Fgf8a/Cdc42ep signaling might function in other aspects of vertebrate development. *Fgf8* isoforms (of which *Fgf8b* is the major form) are co-expressed in many tissues in the embryo, including the gastrula, the mesoderm, the midbrain-hindbrain boundary and the limb bud apical ectodermal ridge (Crossley et al., 1995). Interestingly, elimination of the *Fgf8a*-containing spliceforms (a, c, e, g) in mouse, through gene targeted alteration of the relevant splice acceptor site, failed to cause embryonic defects in *Fgf8*-expressing tissues (Guo et al., 2010), in contrast to deletion of the b-containing forms or complete *Fgf8* nulls. Additionally, compound mutants of *Fgf8a* and *Fgf17*, which likely has Fgf8a-like activity, did not result in additional defects (Guo et al., 2010). Residual Fgf8a-like activity could be supplied by Fgf18 in these animals, but triple mutants have not been described. Interestingly, *Fgf17* and *Fgf18* orthologues and Fgf8 splice variants other than a/b are absent in *Xenopus* (Lea et al., 2009), which could explain the more severe effect of Fgf8a depletion on neural patterning in the frog compared to the mouse.

The mechanisms by which different Fgf8 family members and spliceforms could activate distinct signaling pathways are unclear. *In vitro*, Fgf8a and Fgf8b bind the same complement of FGFRs, namely the c-isoforms of FGFRs1-3 and FGFR4 with similar affinities, although Fgf8a binds them with about 10-fold weaker affinity (Olsen et al., 2006). Analysis of the Fgf8b-FGFR2c 3D structure suggested that Fgf8b binding creates a close apposition of FGFR tyrosine kinase domains, through binding-induced rotation of the linker region of the receptor (Olsen et al., 2006). It is possible that this tighter conformation is critical for MAPK activation, whereas weaker Fgf8a binding results in a looser arrangement allowing for activation of Cdc42 or other signaling cascades. Interestingly, we have shown that FGF17b regulates an Fgf8a-like response (Fig. S7), even though this isoform contains the same 11 amino acid region required for the high affinity interaction of Fgf8b, including a critical phenylalanine residue (F32, Olsen et al., 2006). It will thus be important to investigate additional determinants of signaling specificity of Fgf8 family ligands.

Following Cdc42 activation, binding of Cep4l or other effectors can occur. Cep4l and other Cdc42ep family members are hypothesized to be scaffold proteins (Hirsch et al., 2001), and thus Cep4l is likely to facilitate the formation of protein complexes to mediate neurogenic signaling. These signaling pathways remain unknown, but the identification of Cdc42/Cep4l-interacting proteins would likely provide clues to downstream pathways activated by Fgf8a. Surprisingly little is known about the specificity and usage of different Cdc42-effector proteins. The binding of activated Cdc42 to CRIB domains is considered rather promiscuous, and therefore compartmentalization of function is likely critical for the diversity of Cdc42 functions in the cell. The particular GEFs involved in initial activation are thought to provide much of the specificity in small GTPase effector recruitment (Bos et al., 2007; Sinha and Yang, 2008). We would therefore expect that specific Cdc42-activating GEFs activated by Fgf8a would recruit Cep4l and function in the context of sensory neuron fate in *Xenopus*.

**Developmental regulation of sensory neuron fate**

It is becoming increasingly appreciated that primary sensory neuron fates are regulated similarly to other neural plate border derivatives, including placode and neural crest derivatives (Hong et al., 2008; Park and Saint-Jeannet, 2008; Rossi et al., 2008; Cornell and...
Eisen, 2000). Notably, both neural and non-neural ectoderm can contribute to sensory neuron and neural crest lineages (Rossi et al., 2008; Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Graded amounts of Fgf8a and intermediate BMP levels are thought to specify different neural plate border lineages, with lower doses inducing preplacode and neural crest fates and higher doses inducing sensory neuron fates and inhibiting neural plate border fates (Hong et al., 2007; Hardcastle et al., 2000; Fletcher et al., 2006; Park et al., 2012). Our data show that Cep4l/Cdc42 signaling regulates sensory neurogenesis but not neural or neural plate border fates, suggesting that the Cep4l pathway is activated only at high levels of Fgf8a. Alternatively, Cep4l could allow integration with BMP and Wnt gradients, which are also involved in sensory neuron fate (Hong et al., 2008). Interestingly, we have found that Cep4l and Fgf8a overexpression induce sensory neurons but also inhibit the specification of intercalating deep layer epidermal cell types, multiciliated cells and ionocytes. Notch signaling is known to control the ultimate terminal differentiation of these cells, regardless of neural or epidermal fate. Fgf8a/Cep4l/Cdc42 signaling might therefore act to promote sensory neuron fate in multipotential deep layer ectodermal progenitors in both neural and non-neural ectoderm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

Ash J, Wu C, Larocque R, Jamal M, Stevens W, Osborne M, Thomas DY, Whiteway M. Genetic analysis of the interface between Cdc42p and the CRIB domain of Ste20p in Saccharomyces cerevisiae. Genetics. 2003; 163:9–20. [PubMed: 12586692]

Bos JL, Rehmann H, Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. Cell. 2007; 129:865–77. [PubMed: 17540168]

Briscoe J, Ericson J. Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol. 2001; 11:43–9. [PubMed: 11179871]

Burbelo PD, Drehsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J Biol Chem. 1995; 270:29071–4. [PubMed: 7493928]

Cappello S, Attardo A, Wu X, Iwasato T, Itohara S, Wilsch-Brauninger M, Itohara S, Eilken H, Rieger MA, Schroeder TT, Huttner WB, Brakebusch C, Gotz M. The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. Nat Neurosci. 2006; 9:1099–107. [PubMed: 16892058]

Chalmers AD, Welchman D, Papalopulu N. Intrinsic differences between the superficial and deep layers of the Xenopus ectoderm control primary neuronal differentiation. Dev Cell. 2002; 2:171–82. [PubMed: 11832243]

Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C. Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature. 1995; 375:761–6. [PubMed: 7596407]

Cornell RA, Eisen JS. Delta signaling mediates segregation of neural crest and spinal sensory neurons from zebrafish lateral neural plate. Development. 2000; 127:2873–82. [PubMed: 10851132]

Crossley PH, Martin GR. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development. 1995; 121:439–451. [PubMed: 7768185]
Cuykendall TN, Houston DW. Identification of germ plasm-associated transcripts by microarray analysis of *Xenopus* vegetal cortex RNA. Dev Dyn. 2010; 239:1838–48. [PubMed: 20503379]

Dailey L, Ambrosetti D, Mansukhani A, Basilico C. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev. 2005; 16:233–47. [PubMed: 15863038]

Fletcher RB, Baker JC, Harland RM. FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in *Xenopus*. Development. 2006; 133:1703–14. [PubMed: 16554360]

Guo Q, Li K, Sunmonu NA, Li JYH. Fgf8b-containing spliceforms, but not Fgf8a, are essential for Fgf8 function during development of the midbrain and cerebellum. Dev Bio. 2010; 1338:183–192. [PubMed: 19968985]

Hardcastle Z, Chalmers AD, Papalopulu N. FGF-8 stimulates neuronal differentiation through FGFR-4a and interferes with mesoderm induction in *Xenopus* embryos. Curr Biol. 2000; 10:6499–515. [PubMed: 1901716]

Hartenstein V. Neuronal determination without cell division in *Xenopus* embryos. Neuron. 1991; 6:399–411. [PubMed: 2642003]

Hartenstein V. Early neurogenesis in *Xenopus*: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. Neuron. 1989; 3:399–411. [PubMed: 16554360]

Hayes JM, Kim SK, Abitua PB, Park TJ, Herrington ER, Kitayama A, Grow MW, Ueno N, Wallingford JB. Identification of novel ciliogenesis factors using a new in vivo model for mucociliary epithelial development. Dev Biol. 2007; 312:115–30. [PubMed: 17961536]

Hirsch DS, Pirone DM, Burbelo PD. A new family of Cdc42 effector proteins, CEPs, function in fibroblast and epithelial cell shape changes. J Biol Chem. 2001; 276:875–83. [PubMed: 11035016]

Hong CS, Park BY, Saint-Jeannet JP. Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm. Development. 2008; 135:3903–10. [PubMed: 18997112]

Houston DW, Wylie C. Maternal *Xenopus* Zic2 negatively regulates Nodal-related gene expression during anteroposterior patterning. Development. 2005; 132:4845–55. [PubMed: 16207750]

Joberty G, Perlungher RR, Macara IG. The Borgs, a new family of Cdc42 and TC10 GTPase-interacting proteins. Mol Cell Biol. 1999; 19:6585–97. [PubMed: 10490598]

Joberty G, Perlungher RR, Sheffiel PJ, Kinoshita M, Noda M, Hayshead T, Macara IG. Borg proteins control septin organization and are negatively regulated by Cdc42. Nat Cell Biol. 2001; 3:861–6.

Keller R, Shih J, Sater A. The cellular basis of the convergence and extension of the *Xenopus* neural plate. Dev Dyn. 1992; 193:199–217. [PubMed: 1600240]

Kerr TC, Cuykendall TN, Luettofahn LC, Houston DW. Maternal Tgif1 regulates nodal gene expression in *Xenopus*. Dev Dyn. 2008; 237:2862–73. [PubMed: 18816846]

Lamborghini JE. Rohon-beard cells and other large neurons in *Xenopus* embryos originate during gastrulation. J Comp Neurol. 1980; 9:323–33. [PubMed: 7364967]

Lee JE, Hollenberg SM, Nidler L, Turner DL, Lipnick N, Weintraub H. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science. 1995; 268:836–44. [PubMed: 7754368]

Lee SM, Danielian PS, Fritzsch B, McMahon AP. Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. Development. 1997; 124:959–969. [PubMed: 9056772]

Liu A, Losos K, Joyner AL. FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development. 1999; 126:4827–4838. [PubMed: 10518499]

MacArthur CA, Lawshe A, Shankar DB, Heikinheimo M, Shackleford GM. FGF-8 isoforms diffeyp NIH3T3 cell transforming potential. Cell Growth Differ. 1995; 6:817–825. [PubMed: 7547503]

Mancilla A, Mayor R. Neural crest formation in *Xenopus* laevis: mechanisms of Xslug induction. Dev Biol. 1996; 177:580–9. [PubMed: 8806833]

Metcalfe WK, Myers PZ, Trevorrow B, Bass MB, Kimmel CB. Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. Development. 1990; 110:491–504. [PubMed: 1723944]

Moody SA. Quantitative lineage analysis of the origin of frog primary motor and sensory neurons from cleavage stage blastomeres. J Neurosci. 1989; 9:2919–30. [PubMed: 27693711]

Moury JD, Jacobson AG. The origins of neural crest cells in the axolotl. Dev Biol. 1990; 141:243–53. [PubMed: 2310034]
Nieuwkoop, PD.; Faber, J. Normal table of *Xenopus* laevis (Daudin) : a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Pub.; New York: 1994.

Olsen SK, Li JY, Bromleigh C, Eliseenkova AV, Ibrahim OA, Lao Z, Zhang F, Linhardt RJ, Joyner AL, Mohammadi M. Structural basis by which alternative splicing modulates the organizer activity of FGFR8 in the brain. Genes Dev. 2006; 20:185–98. [PubMed: 16384934]

Ossipova O, Tabler J, Green JB, Sokol SY. PAR1 specifies ciliated cells in vertebrate ectoderm downstream of aPKC. Development. 2007; 134:4297–306. [PubMed: 17993468]

Park BY, Hong CS, Weaver JR, Rosocha EM, Saint-Jeannet JP. Xaml1/Runx1 is required for the specification of Rohon-Beard sensory neurons in *Xenopus*. Dev Biol. 2012; 362:65–75. [PubMed: 22173066]

Park BY, Saint-Jeannet JP. Hindbrain-derived Wnt and Fgf signals cooperate to specify the otic placode in *Xenopus*. Dev Biol. 2008; 324:108–21. [PubMed: 18831968]

Pera EM, Ikeda A, Eivers E, De Robertis EM. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev. 2003; 17:3023–8. [PubMed: 14701872]

Rana AA, Collart C, Gilchrist MJ, Smith JC. Defining synphenotype groups in *Xenopus* tropicalis by use of antisense morpholino oligonucleotides. PLoS Genet. 2006; 2:e193. [PubMed: 17112317]

Ribisi S Jr, Mariani FV, Aamar E, Lamb TM, Frank D, Harland RM. Ras mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in *Xenopus* laevis. Dev Biol. 2000; 227:183–96. [PubMed: 11076686]

Rossi CC, Hernandez-Lagunas L, Zhang C, Choi IF, Kwok L, Klymkowsky M, Artinger KB. Rohon-Beard sensory neurons are induced by BMP4 expressing non-neural ectoderm in *Xenopus* laevis. Dev Biol. 2008; 314:351–61. [PubMed: 18191829]

Rossi CC, Kaji T, Artinger KB. Transcriptional control of Rohon-Beard sensory neuron development at the neural plate border. Dev Dyn. 2009; 238:931–43. [PubMed: 19301392]

Rual JF, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, Li N, Berriz GF, Gibbons FD, Dreze M, Ayivi-Guedehoussou N, Klitgord N, Simon C, Boxem M, Milstein S, Rosenberg J, Goldberg DS, Zhang LV, Wong SL, Franklin G, Li S, Albala JS, Lim J, Fraughton C, Llamosas E, Cevik S, Bex C, Lamesch P, Sikorski RS, Vandenhauwe J, Zoghbi HY, Smolyar A, Bosak S, Sequerra R, Doucette-Stamm L, Cusick ME, Hill DE, Roth FP, Vidal M. Towards a proteome-scale map of the human protein-protein interaction network. Nature. 2005; 437:1173–8. [PubMed: 16189514]

Sabherwal N, Tsutsui A, Hodge S, Wei J, Chalmers AD, Papalopulu N. The apicobasal polarity kinase aPKC functions as a nuclear determinant and regulates cell proliferation and fate during *Xenopus* primary neurogenesis. Development. 2009; 136:276–77.

Sato T, Araki I, Nakamura H. Inductive signal and tissue responsiveness defining the tectum and the cerebellum. Development. 2001; 128:2461–2469. [PubMed: 11493563]

Sato T, Nakamura H. The Fgf8 signal causes cerebellar differentiation by activating the Ras-ERK signaling pathway. Development. 2004; 131:4275–85. [PubMed: 15294862]

Schneider PN, Slusarski DC, Houston DW. Differential role of Axin RGS domain function in Wnt signaling during anteroposterior patterning and maternal axis formation. PLoS One. 2012; 7:e44096. [PubMed: 22957046]

Selleck MA, Bronner-Fraser M. Origins of the avian neural crest: the role of neural plate-epidermal interactions. Development. 1995; 121:525–38. [PubMed: 7768190]

Sinha S, Yang W. Cellular signaling for activation of Rho GTPase Cdc42. Cell Signal. 2008; 20:1927–34. [PubMed: 18558478]

Sive, HL.; Grainger, RM.; Harland, RM. Whole-mount *In situ* Hybridization.. Cold Spring Harbor Laboratory Press; 2000.

Stubbs JL, Davidson L, Keller R, Kintner C. Radial intercalation of ciliated cells during *Xenopus* skin development. Development. 2006; 133:2507–15. [PubMed: 16728476]

Thompson G, Owen D, Chalk PA, Lowe PN. Delineation of the Cdc42/Rac-binding domain of p21-activated kinase. Biochemistry. 1998; 37:7885–91. [PubMed: 9601050]

Turner DL, Weintrab H. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. Genes Dev. 1994; 8:1434–47. [PubMed: 7926743]
• We examine the role of a Cdc42 effector protein (Cep4l) in Xenopus development.
• Cep4l binds Cdc42 and induces primary sensory neurogenesis in epidermal regions.
• Fgf8a signaling is necessary for and specifically promotes Cdc42/Cep4l binding.
• Both Cep4l and Cdc42 are required for Fgf8a-induced primary neurogenesis.
Fig. 1. Cep4l is vegetally localized in oocytes and expressed in the developing mesoderm and neural crest during development

(A) Diagram of Cep4l protein domains. (B) RT-PCR showing expression of cep4l throughout development compared with the housekeeping gene odc. Stages as determined by Nieuwkoop and Faber. (C-H) Whole mount in situ hybridization of cep4l expression in oocytes and embryos. (C) Stage VI oocyte, lateral view. Early (D) and late (E) gastrulae, vegetal views. Early (F) and late (G) neurula. (H) Tailbud. Bp, blastopore. (I-L) Embryo sections following in situ hybridization. (I) Stage 15 neurula. (J-K) Stage 25 tailbud. (L) Stage 31 tailbud. All views transverse sections, dorsal to the top.
Fig. 2. Cep4l misexpression induces ectopic neurogenesis

(A-H) Cep4l overexpression phenotype. Two-cell embryos were injected with 500 pg cep4l mRNA and incubated to the indicated stage. (A,D) Stage 12, animal view. (B,E) Stage 17, dorsal view, anterior to the left. (C,F) Stage 26, lateral view, anterior to the left. (G-H) Cep4l and human CDC42EP4 overexpression induces ectopic neurons. Tubb2b expression in (G) uninjected (un), (H) cep4l-injected (500 pg), (I) CDC42EP4-injected (500 pg) embryos. (J-K) Tubb2b-stained stage 15 embryos were transverse sectioned, dorsal to the top left, ectopic neurons in the deep layer in (K) are indicated with arrows. (L, M) Zn-12 antibody-stained stage 24 embryos indicate ectopic neurons in cep4l-injected embryos.
(M, M’) compared with control (L, L’). Lateral views, dorsal to the top, (L’, M’) close-up images of (L, M) outlined by the boxed regions.
Fig. 3. Cep4l induces specifically primary sensory neurons

(A-H) Cep4l overexpression induces ectopic lateral neurons. (A-D) Unilateral β-gal alone, 50 pg (red). (E-H) β-gal + 250 pg cep4l. Neuronal markers are pan-neuronal tubb2b (A, E), Rohon-Beard sensory runx1 (B, F), sensory and motor islet1 (C, G), interneuron pax2 (D, H). Brackets in (E-G) indicate unilaterally expanded lateral neuron region in cep4l-injected embryos. (M) Quantification of the mean difference in marker positive neuron populations, Injected - Uninjected sides. Error bars indicate 95% CI.
Fig. 4. Cep4l is required for neurogenesis
(A) Cep4l morpholino design and cep4l constructs. (B) Cep4l MO blocks translation of cep4l mRNA. Immunoblotting against HA in embryo lysates with the indicated constructs and MO doses. -catenin was used as a loading control. (C-T) Cep4l depletion inhibits endogenous neurogenesis. (C-D) Phenotypes of controls and embryos injected with 40ng Cep4l MO. Lateral views, anterior to the left. (E-F) Tubb2b expression in Cep4l MO-injected embryos. Dorsal (top) and lateral (bottom) views, anterior to the left. (G-L) Dorsal and ventral Cep4l depletion differentially affects neurogenesis. (G) Diagram of lineage targeting strategy. Tubb2b (H, I, K-M) or islet1 (J, N) expression in controls (H, I, J), and
in embryos injected with 8 ng Cep4l MO into the right dorsal animal (DA) (K) or ventral animal (VA) (L, M, N) cells. Arrow in (K) marks depleted brain and placode neurons. Brackets in (M, N) mark depleted sensory neurons, small arrows mark unaffected areas. (O-V) cep4l rescue injections. Embryos were injected at two cells with 50pg -gal mRNA alone (O, S) or with a sub-phenotypic dose (125 pg) of cep4l (R, V), and subsequently at eight cells with 8 ng Cep4l MO into the right VA blastomere (P, T; Q, U). Bracket in (P, T) marks depleted sensory neurons. Panels (S-V) are closeup images of those above. Arrowheads in (Q, U) marks rescued sensory neurons. m.n., motoneurons, s.n., sensory neurons.
Fig. 5. Cep4l binds Cdc42 through the CRIB domain to induce neurons
(A) Co-immunoprecipitation of in vitro translated Cep4l-HA with active His-tagged Cdc42 and Rac1. (B-F) The Cdc42 binding (CRIB) domain is required for induction of ectopic neurons by Cep4l. (B) Immunoblot of embryos injected with cep4l or cep4l H31A,H34A CRIB domain mutant. (C) Co-immunoprecipitation of Cep4l-HA or mutant Cep4l H31A,H34A-HA with FLAG-Cdc42 in embryo lysates. Tubb2b expression at stage 18, uninjected control (D), cep4l (E), cep4l H31A,H34A (500 pg each) (F). (G-L) Activated Cdc42 functionally interacts with Cep4l. Tubb2b expression in embryos unilaterally injected with 125 pg cep4l (J-L) and 50 pg cdc42 (H,K) or cdc42-G12V (I,L). Arrow indicates ectopic neurons on the injected side in (L).
Fig. 6. Cdc42 is required for neurogenesis

(A) Cdc42MO depletes Cdc42 protein. Immunoblot of GFP-tagged Cdc42 in embryos injected with 500 pg cdc42-GFP alone or with 60 ng cdc42 MO. (B-E) Cdc42 is required for neurogenesis. (B-C) Tubb2b expression in controls (B) and in embryos unilaterally injected with 60 ng cdc42 MO (C). (D-E) Quantification of tubb2b+ lateral neurons in control and cdc42 MO-injected embryos. (D) Mean number of neurons in uninjected and injected sides and (E) mean difference Injected - Uninjected sides. Error bars indicate 95% CI, ** = p-value < 0.001.
Fig. 7. Cep4l and Fgf8a expand neurons and inhibit non-neuronal deep cell fates

(A-E) Expression of neuronal tubb2b (A), neural plate sox2 (B), neural crest sox9 (C), and superficial epidermal xKs1al (D-E) in embryos injected with 200 pg β-gal and 250 pg cep4l. (F-I) Cep4l misexpression in animal caps. Tubb2b expression in controls (F-G) or cep4l-injected (H-I) embryos and explants. (K-P) Expression of epidermal deep layer multiciliated cell marker crcc (K-M) and ionocyte marker foxi1 (N-P) in controls (K, N), cep4l-injected (L, O), and fgf8a-injected embryos (M, P). Insets in N-P show expression of tubb2b.
Fig. 8. Cep4l interacts with and is required for Fgf8a in neuronal induction

(A-H) Cep4l functionally interacts with Fgf8a. Tubb2b expression in embryos overexpressing (A-D) or depleted of either Cep4l or Fgf8a (E-F). (A) BSA treated control, (B) cep4l-injected (125 pg), (C) FGF8a protein treated (0.5 ng), and (D) both cep4l and FGF8a-injected embryos. (E) Uninjected control, (F) cep4l MO-injected (8 ng VA), (G) fgf8a MO-injected (10 ng ventral blastomeres at 4 cell stage), and (H) both cep4l and fgf8a MOs. (I-L) Cep4l depletion blocks Fgf8a-induced ectopic neurons. Two-cell embryos injected unilaterally with 100 pg β-gal mRNA alone (I) or with 125 pg cep4l (J), 125 pg fgf8a (K-L) mRNA and subsequently with 8 ng cep4l MO in the right VA blastomere (I-J, L).
Fig. 9. Fgf8a acts independently of MAPK activation and regulates association of Cep4l with Cdc42

(A) Immunoblotting against diphospho-ERK in animal caps, isolated 60 minutes post dissection. Embryos were injected with 500 pg of the indicated RNAs. WE= whole embryo, stage 9; An= uninjected animal caps. (B-E) Immunostaining against dp-ERK in control BSA treated (B), cep4l-injected (500 pg) (C), FGF8a (1.5 ng) treated (D), FGF8b (1.5 ng) treated (E) gastrulae. Arrow indicates activated ERK staining. Animal views. Insets: vegetal views showing internal control staining of dp-ERK in the marginal zone. (F,H) Immunoblots of FLAG immune complexes from embryos co-injected at two cells with 500 pg cep4l-HA and FLAG-cdc42 RNA. (F) Co-immunoprecipitation of Cep4l-HA with FLAG-Cdc42 in embryos injected at stage 9 with BSA or 1 ng human FGF8a or Fgf8b protein into the blastocoel. (G) Relative quantification of HA pulldown normalized to FLAG pulldown and lysate levels of expression, using ImageJ. (H) Co-immunoprecipitation of Cep4l-HA with FLAG-Cdc42 in embryos co-injected at four cells with Fgf8a MO.
### Table 1

Cep4l signaling is sufficient and necessary for sensory neurogenesis.

| Experiment          | Marker       | N | Uninjected count (mean ± 95% CI) | Injected count (mean ± 95% CI) | Inj. - Un. Count (mean ± 95% CI) | p-value (paired t-test) |
|---------------------|--------------|---|----------------------------------|-------------------------------|----------------------------------|-------------------------|
| **Series 1 (≥2 experiments)** |              |   |                                  |                               |                                  |                         |
| βgal               | tubb2b      | 40 | 64 ± 4.11                        | 64 ± 3.44                     | −0.15 ± 3.32                     | n.s                     |
| βgal+cep4l        | tubb2b      | 38 | 68 ± 4.23                        | 102 ± 10.0                    | 33.5 ± 9.99                      | <0.001                  |
| βgal               | islet1 motor| 14 | 23 ± 1.22                        | 23 ± 1.22                     | 0.308 ± 1.55                     | n.s                     |
| βgal+cep4l        | islet1 motor| 30 | 14 ± 0.991                       | 14 ± 0.927                    | 0.516 ± 1.29                     | n.s                     |
| βgal               | islet1 sensory| 14 | 58 ± 3.22                        | 60 ± 3.24                     | 2.36 ± 4.84                      | n.s                     |
| βgal+cep4l        | islet1 sensory| 30 | 57 ± 2.42                        | 73 ± 3.48                     | 15.1 ± 6.17                      | <0.001                  |
| βgal               | pax2 inter  | 8  | 22 ± 2.59                        |                               | −0.25 ± 1.29                     | n.s                     |
| βgal+cep4l        | pax2 inter  | 36 | 18 ± 1.76                        | 19 ± 1.99                     | 0.833 ± 0.937                    | n.s                     |
| βgal               | ranx1 sensory| 49 | 39 ± 1.93                        | 40 ± 1.69                     | 0.592 ± 2.22                     | n.s                     |
| βgal+cep4l        | ranx1 sensory| 45 | 38 ± 2.12                        | 52 ± 3.89                     | 13.1 ± 3.86                      | <0.001                  |
| **Series 2 (4 experiments)** |              |   |                                  |                               |                                  |                         |
| Un                 | tubb2b      | 79 | 64 ± 2.33                        | 64 ± 1.25                     | 0.304 ± 1.69                     | n.s                     |
| Cdc42MO           | tubb2b      | 67 | 61 ± 3.70                        | 48 ± 3.82                     | −13.3 ± 2.49                     | <0.001                  |
| **Series 3 (1 experiment)** |              |   |                                  |                               |                                  |                         |
| Fgf8aMO           | tubb2b      | 14 | 53 ± 6.01                        | 58 ± 8.45                     | 4.77 ± 8.77                      | n.s                     |
| Fgf8aMO+Cep4lMO  | tubb2b      | 13 | 54 ± 11.1                        | 6 ± 4.89                      | −47.8 ± 12.4                     | <0.001                  |
| **Series 4 (1 experiment)** |              |   |                                  |                               |                                  |                         |
| βgal               | ranx1 sensory| 18 | 32 ± 2.86                        | 32 ± 3.14                     | 0 ± 2.44                         | n.s                     |
| fg8a               | ranx1 sensory| 25 | 130 ± 11.3                       | 127 ± 17.8                    | −3.32 ± 17.6                     | n.s.                   |
| Cep4lMO           | ranx1 sensory| 27 | 32 ± 2.63                        | 14 ± 2.99                     | −18.1 ± 4.77                     | <0.001                  |
| Cep4lMO+fg8a      | ranx1 sensory| 21 | 72 ± 14.2                        | 21 ± 12.3                     | −51.1 ± 20                       | <0.001                  |
| Cdc42MO           | ranx1 sensory| 21 | 36 ± 6.99                        | 28 ± 4.99                     | 10.9 ± 7.46                      | <0.01                  |
| Cdc42MO+fg8a     | ranx1 sensory| 19 | 95 ± 15.4                        | 27 ± 13.4                     | −67.6 ± 19.1                     | <0.001                  |

Unilaterally-injected embryos were scored for the following neuronal and neuronal subtype markers, and marker-positive neurons counted on each side in control and experimental embryos. Statistical significance was determined using a paired, two-tailed t-test in Excel, comparing the mean number of neurons on the injected versus uninjected side of each embryo. n.s. = not significant. **Series 1.** Cep4l overexpression induces sensory, but not motor or inter neurons. **Series 2.** Cep4l depletion reduces sensory neurons. **Series 3.** Low dose unilateral Cep4l depletion enhances reduction of sensory neurons in low dose bilateral Fgf8a depleted embryos. **Series 4.** Cep4l and Cdc42 depletion reduce sensory neurons and block Fgf8a neurogenesis.