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Lrig3 regulates neural crest formation in *Xenopus* by modulating Fgf and Wnt signaling pathways

Hui Zhao, Kosuke Tanegashima, Hyunju Ro, and Igor B. Dawid*

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

Abstract

Leucine-rich repeats and immunoglobulin-like domains 3 (*Lrig3*) was identified by microarray analysis among genes that show differential expression during gastrulation in *Xenopus laevis*. *Lrig3* was expressed in the neural plate and neural crest (NC) at neurula stages, and in NC derivatives and other dorsal structures during tailbud stages. A prominent consequence of the morpholino-induced inhibition of *Lrig3* expression was impaired NC formation, as revealed by the suppression of marker genes, including *Slug*, *Sox9* and *Foxd3*. In the NC induction assay involving *Chordin* plus *Wnt3a*-injected animal caps, *Lrig3* morpholino inhibited expression of *Slug*, *Sox9* and *Foxd3*, but not of *Pax3* and *Zic1*. In line with this, *Lrig3* knockdown prevented NC marker induction by *Pax3* and *Zic1*, suggesting that *Lrig3* acts downstream of these two genes in NC formation. Injection of *Lrig3* and *Wnt3a* led to low-level induction of NC markers and enhanced induction of *Fgf3*, *Fgf4* and *Fgf8* in animal caps, suggesting a positive role for *Lrig3* in Wnt signaling. *Lrig3* could attenuate Fgf signaling in animal caps, did interact with Fgf receptor 1 in cultured cells and, according to context, decreased or increased the induction of NC markers by Fgf. We suggest that *Lrig3* functions in NC formation in *Xenopus* by modulating the Wnt and Fgf signaling pathways.

Keywords

Fgf8; MAPK; Neural crest; Slug; Wnt3a; *Xenopus laevis*; Leucine-rich repeats protein; Animal cap; DNA microarray

INTRODUCTION

The neural crest (NC) arises at the border between the neural plate and the epidermis, and represents multipotent cells that migrate to various parts of the embryo and differentiate into multiple cell types. Induction of the NC requires precisely balanced combinations of signals generated by the BMP, Wnt, Fgf, retinoic acid and Notch/Delta pathways (Cornell and Eisen, 2005; Huang and Saint-Jeannet, 2004; Steventon et al., 2005; Yanfeng et al., 2003). These signals lead to the activation of multiple transcription factors such as *Msx1*, *Pax3* (Monsoro-Burq et al., 2005), *Zic1* (Sato et al., 2005), *Foxd3* (Kos et al., 2001; Pohl and Knochel, 2001; Sato et al., 2005), *Sox9* (Spokony et al., 2002), *Slug* (Mayor et al., 1995), *Snail* (Essex et al., 1993; Mayor et al., 1993), *Myc* (Bellmeyer et al., 2003) and *Ap2* (Luo et al., 2003). Gain- and loss-of-function experiments suggested tentative regulatory networks containing many of these factors (Monsoro-Burq et al., 2005; Saint-Jeannet et al., 1997; Sato et al., 2005).

*Author for correspondence (idawid@nih.gov).

Supplementary material

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In addition to NC formation, Fgf family members have crucial roles in at least three steps in embryogenesis, neural specification (Curran and Grainger, 2000; Hongo et al., 1999; Ribisi, Jr et al., 2000; Streit et al., 2000), posterior mesoderm formation (Amaya et al., 1993; Umbhauer et al., 1995) and cell migration (Chung et al., 2007; Yokota et al., 2003). Fgf signaling involves binding to one of four Fgf receptors (FGFRs), inducing activation of at least three downstream cascades: the PI3K-Akt, Raf-MEK1/2-ERK1/2 and PLC γ pathways (Eswarakumar et al., 2005; Schlessinger, 2004; Tsang and Dawid, 2004), the first two having been linked to mesoderm formation (Carballada et al., 2001; Umbhauer et al., 1995). Inhibition of the Fgf signaling by dominant negative *Fgfr1* (Amaya et al., 1993), *Sef* (Tsang et al., 2002) or *Mkp3* (Tsang et al., 2004) results in defects of axis formation. Knockdown of *Fgf8* by antisense morpholino (MO) impairs mesoderm and NC formation (Fletcher et al., 2006; Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005). It is notable that in NC formation, Fgf signaling is essential, but an excess of Fgf signaling is inhibitory (Hong and Saint-Jeannet, 2007).

The Wnt/ β -catenin pathway has been implicated in NC formation by gain- and loss-of-function studies in vivo and in explants (Abu-Elmagd et al., 2006; Garcia-Castro et al., 2002; Monsoro-Burq et al., 2005; Saint-Jeannet et al., 1997; Sato et al., 2005). In NC formation, Wnt signaling cooperates with other signal cascades, notably an attenuated BMP signal, in the regulation of the specification and differentiation process (LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997).

In this paper, we report that *Lrig3*, a single-pass transmembrane protein, is involved in NC formation by modulating FGF and Wnt signaling. We identified *Lrig3* as a gene preferentially expressed in dorsal marginal zone explants from *Xenopus* gastrulae, using a DNA microarray approach. Among three human *Lrig* family members, *Lrig1* has been shown to function as an EGF signaling inhibitor by enhancing EGFR ubiquitination (Gur et al., 2004; Laederich et al., 2004), and has also been linked to HGF signaling as a negative regulator (Shattuck et al., 2007). However, little is known about the function of *Lrig* family proteins in embryonic development. In this study, we determined that *Lrig3* is required for NC formation in the *Xenopus* embryo.

MATERIALS AND METHODS

Embryo dissection and animal cap assay

Xenopus eggs were fertilized in vitro as described (Sive, 2000), embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Animal caps were dissected at stage 9 in 1 $\times$ MMR, and cultured in L-15 medium [67% L-15, 7.5 mM Tris-HCl (pH 7.5), 1 mg/ml BSA]. For microarray analysis, stage 10 *Xenopus* embryos were dissected into four explants. The dorsal marginal zone, composed mostly of endomesoderm, ventral marginal zone, animal cap and vegetal explant. To avoid possible cross contamination that could blur the microarray data, the junction regions between the explants were removed.

Microarray and data analysis

The dissected explants were homogenized in Stat 60 (TEL TEST), RNA was treated with DNase I and purified using RNeasy kit (Qiagen). Biotinylated probe was prepared from 100 ng total RNA using the OVATION RNA amplification system (Nugen Technologies). Probes were hybridized to *Xenopus* genome arrays (Affymetrix) according to the manufacture's instructions. Hybridized arrays were processed by the GeneChip Fluidics system (Affymetrix), and scanned in the GeneChip Scanner (Affymetrix). Gene expression profiles were analyzed by the GCOS software (Affymetrix).

DNA constructs

The ORF of *Lrig3* was cloned into pCS2+ (Turner and Weintraub, 1994) or into pCS2flag, pCS2myc and pCS2GFP. The tags were located c-terminal to *Lrig3*. Deletion mutants of *Lrig3* were generated by PCR and subcloned into pCS2myc.

Morpholino oligo

The splicing morpholino (Genetools) against *Lrig3* (L3MO) recognizes both pseudo-alleles is GGGTTTCTGAAAGATAAAAACAAGC, and the Control-MO is CCTCTTACCTCAGTTACAATTTATA.

lacZ staining, whole-mount in situ hybridization, Alcian Blue staining

lacZ staining was performed as described (Zhao et al., 2001). Whole-mount in situ hybridization was performed as described (Harland, 1991). The following probes were used: *Sox2* (Kishi et al., 2000), *Rx2a* (Yoshitake et al., 1999), *Krox20* (Bradley et al., 1993), *Slug* (*Snail2*) (Mayor et al., 1995), *Sox9* (Spokony et al., 2002), *Ap2a* (Luo et al., 2002), *Inca* (Luo et al., 2007), *Myc* (Bellmeyer et al., 2003), *Twist* (Hopwood et al., 1989) and *Traf4* (this laboratory) were examined. Alcian Blue cartilage staining was performed as described (Pasqualetti et al., 2000).

Cell culture and transfection

HEK293T and COS7 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's manual.

RT-PCR assay

Superscript III (Invitrogen) was used for cDNA synthesis. PCR primers are listed in Table 1.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer [137 mM NaCl, 5 mM EDTA, 1% TrionX-100, 10 mM Tris-HCl (pH 7.5)]. Immune complexes were captured by Fastflow protein G beads (GE Health), washed in lysis buffer five times, separated on NuPage Norvex 4-12% Bis-Tris gels (Invitrogen), and transferred to PVDF membranes (Invitrogen) for blotting.

ERK phosphorylation

Animal caps were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA and 5 mM sodium orthovanadate (Bottcher et al., 2004). The equivalent of 10 animal caps was blotted using monoclonal anti-diphospho-ERK antibody (clone MAPK-YT, Sigma) at 1:1000, and monoclonal anti-pan-ERK (BD) at 1:4000.

Immunofluorescence

Cells were plated on chambered microslides (Nunc). The cells were washed by cold 1× PBS, fixed by 2% PFA in 1× PBS, blocked with 20% serum in 1× PBS, and incubated with antibodies for 1 hour. Alexa Fluor 488- or 568-conjugated secondary antibodies were used. Slides were mounted in VECTASHIELD (Vector Laboratories) and viewed using a Zeiss LSM510 confocal microscope.

Luciferase assay

HEK293T cells were plated into 24-well plates for 1 day, a total of 0.8 µg/well of plasmid DNA including Topflash-luciferase (0.25 µg/well) and Renilla luciferase pRL-CMV (0.025 µg/well) were added, using pCS2+ DNA to adjust the total amount. After 1 day, mouse Wnt3a

(100 ng/ml) or LiCl (30 mM) was added. Luciferase activity was measured using the Dual luciferase system (Promega) after 24 hours.

RESULTS

Properties and expression of *Xenopus* and zebrafish *Lrig3*

To study genome-wide gene expression during embryogenesis, we used DNA microarrays to investigate spatial differences in gene expression. Stage 10 *Xenopus* embryos were dissected into four explants (see Materials and methods). Several known genes were used to test the dissection (Fig. 1A). The original results of these microarray experiments were deposited at GEO and will be discussed elsewhere. The Accession Numbers are GSM227883 (animal cap explant), GSM227884 (dorsal explant), GSM227963 (vegetal explant) and GSM227964 (ventral explant). Here, we focus on one gene identified in this study that encodes a protein most similar to human LRIG3; we named this gene *Xenopus* leucine-rich repeats and immunoglobulin-like domains 3 (*Lrig3*). *Lrig3* contains a signal peptide, 14 tandem leucine-rich repeats, N- and C-terminal specialized leucine-rich repeats, three immunoglobulin C2 domains, a single transmembrane domain and a cytoplasmic region with no identified domain structure (Fig. 1B). To determine whether a similar gene is expressed in different vertebrate embryos, we isolated zebrafish *lrig3* by 5' RACE (Fig. 1B). Accession numbers are EU126151 (*Xenopus*) and EU126152 (zebrafish). The phylogenetic tree in Fig. 1C presents the relationships within the vertebrate *Lrig* family, also including *Drosophila* Kekk as the closest homolog.

Lrig3 contains a putative signal peptide and transmembrane domain, and is thus predicted to be a transmembrane protein. To investigate the subcellular localization of *Lrig3*, we expressed epitope tagged protein in COS7 or CHO cells. A fraction of *Lrig3*-Flag was localized at the cell membrane, while a majority localized to the Golgi apparatus, as seen by colocalization with the *cis* Golgi marker GM130 (Fig. 1D). In addition, punctate distribution of *Lrig3*-Flag was observed in the cytosol. Only a small proportion of these puncta overlapped with the early endosome marker EEA1 (Fig. 1E). The nucleus was essentially devoid of *Lrig3*-Flag staining.

In the *Xenopus* embryo, *Lrig3* is expressed around the dorsal blastopore lip, including the prospective neural ectoderm, involuting dorsal mesoderm and anterior dorsal endoderm (Fig. 1F,G). Dorsally restricted expression was verified by RT-PCR (Fig. 1A). As gastrulation progressed, the future neural plate, including the NC anlagen, was stained (Fig. 1H-K). In tailbud and tadpole stages, *Lrig3* was highly expressed in the brain, branchial arches and paraxial mesoderm (Fig. 1L,M). RT-PCR indicated that *Lrig3* is a maternal factor, increases by gastrulation and continues to be expressed through embryogenesis (Fig. 1N). This pattern is consistent with *Lrig3* induction by *Chordin* or a truncated BMP receptor in animal caps (see Fig. S2 in the supplementary material).

The expression pattern of zebrafish *lrig3* was investigated to ask whether the *Xenopus* pattern is conserved. Zebrafish *lrig3* is present maternally, localizes to the shield at gastrula and later shows a complex pattern with strong expression in the branchial arches (Fig. 1O-R).

Lrig3 is required for neural crest differentiation

To study the function of *Lrig3* in *Xenopus* development, we used a splicing morpholino antisense oligonucleotide (hereafter L3MO) to reduce expression of the endogenous protein. The effectiveness of L3MO was checked by RT-PCR, showing that it greatly reduced the level of mature mRNA while leading to the appearance of a 1 kb PCR product corresponding to unspliced RNA (Fig. 2A). Injection of L3MO allowed completion of gastrulation but led to repression of anterior development and bent axis (Fig. 2B-D, Table 2). These phenotypes could

be rescued by co-injection with *Lrig3* mRNA, indicating that the effect of L3MO is specific (Fig. 2E-G, Table 3).

Examination of the expression of various marker genes at different stages in L3MO-injected embryos showed that NC formation was impaired in these embryos. This is illustrated by in situ hybridization with embryos injected with L3MO in one side, the other side acting as control. When examined at around stage 19, *Krox20* is still expressed within rhombomeres 3 and 5, but the stripe of *Krox20*-positive cells that marks the NC stream emanating from rhombomere 5 was strongly inhibited (Fig. 3A-A''), while the retinal marker *Rx2a* was only mildly inhibited (see Fig. S3A-A'' in the supplementary material). Thus, *Lrig3* does not appear to be required for neural induction, as also seen by the lack of inhibition of the pan-neural marker *Sox2* (Fig. 3F-F''), and the resistance of neural induction by Chordin in animal caps to the morpholino (Fig. 3G). However, compensation by maternal *Lrig3* or by other *Lrig* family members has not been excluded. By contrast, severe reduction up to a complete loss of expression was seen in the great majority of L3MO-injected embryos for a panel of NC markers (Fig. 3B-E''; see Fig. S3B-D in the supplementary material; see legend for numbers of embryos affected). Knock-down of *Lrig3* had little effect on the anterior expression domain of *Myc* which marks the border of the neural plate, whereas expression in the NC anlage was strongly reduced (Fig. 3D-D''); this emphasizes the differential nature of the effect. The loss of NC markers could be rescued by co-injection of L3MO with *Lrig3* mRNA, as seen in Fig. 3N-O'' for *Slug* and *Inca*; quantification is shown in Fig. 3P.

The inhibition of NC might possibly be explained by suppression of mesoderm formation; therefore, we examined the expression of *Xbra*, *Chordin*, *Cerberus* and *gooseoid* in embryos targeted dorsally with L3MO at the four-cell stage (see Fig. S4 in the supplementary material). The expression domain and expression level of these mesodermal markers were not substantially affected. Furthermore, we checked the expression of *Slug*, *Inca* and *Sox9* in embryos in which L3MO was injected into one animal-dorsal blastomere at the eight-cell stage to restrict the range of cells that are affected. Strong inhibition of NC was again observed in these embryos (Fig. 3H-J'').

Next, we checked for the presence of differentiated NC derivatives at swimming tadpole stages in *Lrig3*-depleted embryos. NC cells of the branchial arches differentiate into the cartilages of the embryonic facial skeleton (Le Douarin and Kalcheim, 1999). Cartilage formation in *Lrig3* morphants, as visualized by Alcian Blue staining, was strongly inhibited in the injected side, the phenotype ranging from missing a single branchial arch to loss of the entire cranial cartilage (Fig. 3K-M, see legend for the percentage of affected embryos). As NC precursors also give rise to ganglion cells of the peripheral nervous system (Huang and Saint-Jeannet, 2004; Le Douarin and Kalcheim, 1999) we examined trigeminal nerve formation in *Lrig3* morphants by staining with *Xenopus Synuclein* γ , and found it to be strongly reduced (see Fig. S5 in the supplementary material).

Lrig3 is required for NC induction in animal caps

To investigate the mechanism of *Lrig3* involvement in NC formation, we used animal caps, in which NC can be induced by the combined action of a canonical Wnt ligand and a BMP antagonist (Saint-Jeannet et al., 1997). Animal caps were dissected from embryos injected with the indicated mRNAs with or without L3MO, cultured to stage 16, and gene expression was examined by RT-PCR. In agreement with previous findings, the neural markers *Otx2*, *Ncam*, *Sox3* and *Sox2* were induced by the injection of *Chordin*, but were suppressed by the co-injection of *Wnt3a*, while the NC markers *Slug*, *Sox9*, *Foxd3*, *Ap2*, *Twist* and *Snail* were induced by the combination of *Wnt3a* and *Chordin* (Fig. 4A,A'). Induction of *Slug*, *Sox9*, *Foxd3* and *Twist* was decreased by co-injection of L3MO, while *Snail* was affected to a lesser extent and *Ap2* was unaffected. *Ap2* is expressed in epidermis in addition to the NC (Luo et al., 2003;

Luo et al., 2002), possibly explaining the reduced effect. In contrast to NC markers, the neural genes *Sox3* and *Sox2*, but not *Otx2* or *Ncam*, were up-regulated moderately by the morpholino, suggesting that the neural fate that had been suppressed by adding *Wnt3a* to *Chordin*-expressing animal caps was partially recovered (Fig. 4A,A'). Overexpression of *Lrig3* alone could not induce neural or NC makers in animal caps, nor could addition of *Lrig3* increase the level of *Slug*, *Sox9* and *Foxd3* over that achieved by *Wnt3a* plus *Chordin* alone (Fig. 4A). These experiments also confirmed that *Lrig3* itself is strongly induced by injection of *Chordin* or a combination of *Chordin* and *Wnt3a*, and that L3MO reduced the level of mature *Lrig3* mRNA (Fig. 4A'). Thus, *Lrig3* is required for the induction of NC in explants as well as in whole embryos.

The *Zic1* and *Pax3* genes play an important role in NC specification by inducing NC markers such as *Slug* and *Foxd3* in the presence of a Wnt signal (Monsoro-Burq et al., 2005; Sato et al., 2005) (for a review, see Steventon et al., 2005). L3MO did not block the expression of *Zic1* and *Pax3* in animal caps injected with *Chordin* and *Wnt3a* (Fig. 4A), suggesting that *Lrig3* functions between *Pax3/Zic1* and *Slug/Foxd3* in the regulatory hierarchy of NC formation. To address this possibility, we examined the L3MO effect on the NC inducing activity of *Pax3* plus *Zic1*. As reported, co-injection of *Pax3* and *Zic1* induced NC markers *Slug*, *Sox9* and *Twist* (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005); this induction was inhibited by L3MO (Fig. 4B). We therefore conclude that *Lrig3* functions downstream of *Pax3* and *Zic1* in NC formation.

Lrig3 acts in NC specification in coordination with a Wnt signal

Lrig3 is required for NC formation in animal caps exposed to a Wnt signal and BMP inhibition. We attempted to determine which of these two pathways interacts with *Lrig3*. Induction of the Wnt target genes *Xnr3* and *Siamois* in animal caps was only slightly enhanced by co-injection of *Lrig3* (Fig. 5A), and *Lrig3* transfection stimulated Wnt-dependent Topflash reporter activity in cultured cells to a modest but significant extent (see Fig. S6 in the supplementary material). In addition to activating dorsal genes such as *Siamois*, canonical Wnt signaling is also capable of inducing mesoderm formation (Schohl and Fagotto, 2003). We found that the slight induction of *Xbra* by *Wnt3a* was greatly enhanced by co-injection of *Lrig3* (Fig. 5B). The combination of *Wnt3a* and *Lrig3* could also induce the NC markers *Slug*, *Foxd3* and *Sox9*, but to a lower level than that obtained after injection of a combination of *Wnt3a*, *Chordin* and *Lrig3* (Fig. 5B). These results indicate that *Lrig3* can enhance canonical Wnt signaling, with the strength of the effect dependent on context.

The role of Wnt signaling in mesoderm formation is correlated with its induction of *Fgf3* (Schohl and Fagotto, 2003). As *Xbra* is a direct target of Fgf signaling (Smith et al., 1991), we tested whether the enhanced *Xbra* expression induced by *Wnt* and *Lrig3* was accompanied by upregulation of *Fgf3*, *Fgf4* (also known as *eFgf*) or *Fgf8*, three genes known to be expressed in early *Xenopus* embryos (Fletcher et al., 2006; Isaacs et al., 1995; Lombardo et al., 1998; Tannahill et al., 1992). Injection of *Lrig3* alone did not induce any of these genes. When harvested at stage 10, animal caps co-injected with *Lrig3* and *Wnt3a* expressed *Fgf8*, while *Wnt3a* alone did not induce any of the three Fgf genes (Fig. 5C). By stage 16, animal caps injected with *Wnt3a* induced a low level of *Fgf3*, but co-injection of *Wnt3a* with *Lrig3* led to the clear increase in the induction of all three Fgf genes tested, consistent with the induction of *Xbra* under the same conditions (Fig. 5C). We conclude that *Lrig3* strongly enhances the ability of canonical Wnt signaling to induce three Fgf genes in *Xenopus* embryonic tissues.

In contrast to the interaction seen between *Lrig3* and *Wnt3a*, no interaction was observed with *Chordin*. *Chordin* alone induces neural markers but does not induce NC markers, and addition of *Lrig3* did not change this behavior (Fig. 5B). Likewise, *Chordin* induces *Fgf8* but not the other Fgf genes, and again addition of *Lrig3* had no obvious effect (Fig. 5C). Thus, the role of

Lrig3 in NC induction appears to be related to the Wnt rather than the BMP antagonist arm of the signaling cascade.

Lrig3 can modulate Fgf signaling in mesoderm and NC induction

We investigated the relationship of Lrig3 to the Fgf pathway for three reasons. First, modulation of Wnt signaling in the context of NC induction in explants results in enhanced expression of Fgf genes. Second, mammalian Lrig1 affects EGF signaling, which shares several signal transduction components with the Fgf pathway. Third, Fgf signaling has been implicated in NC formation. We first checked the effect of Lrig3 on Fgf-induced ERK (MAP kinase) phosphorylation in animal caps harvested at an early stage where the effects of Fgf have been well studied. Injection of active Ras or *Fgf4* mRNA into animal caps stimulates ERK phosphorylation (Gupta and Mayer, 1998; Suga et al., 2006; Whitman and Melton, 1992), and this could also be achieved by treatment with bFgf (Fig. 6A). ERK phosphorylation was inhibited by injection of *Lrig3*; similar inhibition was observed with *Sef* or *Mkp3*, known inhibitors of Fgf signaling that were used as controls (Kovalenko et al., 2003; Tsang et al., 2002; Tsang et al., 2004) (Fig. 6A). Using induction of *Xbra* by Fgf (Smith et al., 1991) as an assay for Fgf signaling also showed inhibition by injection of *Lrig3* (Fig. 6B). This inhibition is specific because *Lrig3* could not inhibit *Xbra* induction by activin, while *Smad7*, an inhibitor of activin/nodal signaling (Bhushan et al., 1998), blocked the activin effect (Fig. 6C). Multiple Fgf ligands are expressed in the *Xenopus* embryo, and while the signaling pathways downstream of these ligands are similar, varying consequences of different Fgfs have been reported (Fletcher et al., 2006; Hardcastle et al., 2000). Therefore, we checked the effect of Lrig3 on Fgf8-induced gene activation in animal caps, injecting increasing doses of *Fgf8a* RNA with or without a constant level of *Lrig3* RNA. Induction of *Xbra*, *Msx1* and *Wnt8* increased with the dose of *Fgf8a*, peaked at 100 pg/embryo, and decreased at higher doses. It has been shown previously that overexpression of *Fgf8a* inhibits *Xbra* expression in the whole embryo (Hardcastle et al., 2000). Co-injection of *Lrig3* with *Fgf8a* led to a decrease of the expression of *Xbra*, *Msx1* and *Wnt8* at 100 pg *Fgf8a* RNA, while at higher doses, *Lrig3* had less effect (Fig. 6D). Thus, the level of Fgf at which peak induction is obtained shifted to higher values with the addition of Lrig3. These results suggest that (1) induction of the three mesodermal genes by Fgf8 is dose dependent with a discrete maximum; and (2) Lrig3 attenuates the Fgf signal under these conditions.

We argued that modulation of Fgf signaling might have a role in Lrig3 function during NC induction. Fgf8 is required for NC formation as reduction of *Fgf8* expression by a morpholino inhibits NC formation (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), while injection of *Fgf4* (LaBonne and Bronner-Fraser, 1998) or *Fgf8* (Monsoro-Burq et al., 2003) RNA can induce NC markers in animal caps. Titration experiments in whole embryos showed that Fgf8 has a dual role in this process as a modest increase enhanced, but an excess of Fgf8 inhibited, NC formation (Hong and Saint-Jeannet, 2007). To examine the role of Lrig3 in this system, we injected different levels of *Fgf8a* with or without *Lrig3* into animal caps and tested for induction of NC genes (Fig. 6E). At the lowest dose used, *Fgf8a* RNA induced both *Slug* and *Twist*, and addition of *Lrig3* inhibited the induction. At higher doses, NC marker induction by *Fgf8a* alone was reduced, but it was strongly enhanced by co-injection of *Lrig3*. This is most obvious in the case of *Slug* induction at 1 ng of *Fgf8a*, which changes from undetectable to a robust induction upon addition of *Lrig3*. Even more pronounced than in the case of mesodermal gene induction, addition of Lrig3 shifts the peak of NC gene induction to higher concentrations of Fgf. These results are compatible with the conclusion that an optimal level of Fgf signaling is required for NC induction, and that Lrig3 attenuates Fgf signal transduction to bring the output into optimal range at the higher levels of Fgf signaling.

Lrig3 interacts with Fgfr1 and decreases its level of expression

Lrig3 contains a signal peptide and a transmembrane domain, and a fraction of Lrig3 is localized on the cell membrane (Fig. 1). Lrig1 interacts with all four ErbB receptors as well as Met receptors (Gur et al., 2004; Shattuck et al., 2007). Therefore, we investigated the possible interaction of Lrig3 with Fgf receptors. Lrig3 was co-immunoprecipitated with Xfgr1 when both were co-expressed in 293T cells, showing that these two membrane proteins can bind each other (Fig. 7B; see Fig. S7 in the supplementary material). To determine the Fgfr1-binding domains of Lrig3, we constructed a series of deletion mutants, Δ Cyto (deleting the cytoplasmic domain), Δ LRR (deleting the LRR domains), Δ IgC (deleting the IG C2 domains), Δ EC (deleting the extracellular portion including LRR and IG C2 domains) and Δ CytoTM (deleting the transmembrane and cytoplasmic domains), and performed co-immunoprecipitation assays. Interaction with Xfgr1 was retained in constructs that contained all or even a part of the ectodomains, but was lost when the entire ectodomain was deleted (Fig. 7B). The intracellular and transmembrane domains were not involved in the binding. We conclude that the extracellular region of Lrig3 is responsible for binding of Fgfr1.

As Lrig1 negatively regulates the stabilities of EGFR and the Met receptor (Gur et al., 2004; Shattuck et al., 2007), we next asked whether Lrig3 affects Fgf receptor metabolism. Xfgr1 protein was expressed at a much lower level in cells co-transfected with Lrig3 when compared with controls (Fig. 7C). This result correlates with the reduced ERK phosphorylation in the presence of Lrig3 that was seen in these cells (Fig. 7C) and in animal caps (Fig. 6A). These results suggest a role for Lrig3 in the regulation of FGFR1 availability in the cell.

DISCUSSION

In this paper, we describe the transmembrane protein Lrig3 as a novel factor required for the induction of the NC in *Xenopus* embryos. The *Lrig3* gene is expressed in several regions of the early *Xenopus* and zebrafish embryo, including the NC precursor domain. A splice morpholino that strongly reduces the level of mature *Lrig3* mRNA inhibits the expression of multiple NC marker genes and the formation of NC derivatives such as the cartilages of the branchial arches in the *Xenopus* embryo. We suggest that Lrig3 function is required downstream of *Pax3* and *Zic1* in NC formation, and that Lrig3 exerts its role by modulating signal transduction in the embryo. Lrig3 thus joins the factors known to be required for NC specification, which so far have included transcription factors such as *Slug* and *Myc*, and signaling factors such as Wnt ligands, an attenuated BMP signal and Fgf proteins.

The role of Lrig3 in NC formation

Studies in *Xenopus* have suggested that an intermediate level of BMP signaling is necessary but not sufficient for NC formation, and that activation of the Wnt and Fgf pathways are involved in this process (Steventon et al., 2005). Inhibition of Wnt/ β -catenin signaling by either Frizzled 7 MO (Abu-Elmagd et al., 2006) or *Gsk3b* (Saint-Jeannet et al., 1997) blocks NC formation, while loss of *Wnt1* and *Wnt3a* in mice causes NC deficiencies (Ikeya et al., 1997). Furthermore, activation of Wnt signaling by overexpression of *Lrp6* expands the expression of *Slug* in the embryo (Tamai et al., 2000). In *Xenopus* animal caps, canonical Wnt ligands, including Wnt1, Wnt3a, Wnt7b and Wnt8, together with a BMP antagonist, such as Chordin or Noggin, induce NC markers, but Wnt alone is ineffective (Chang and Hemmati-Brivanlou, 1998; Deardorff et al., 2001; Saint-Jeannet et al., 1997). The importance of Wnt signaling is emphasized by the presence of a TCF-binding site in the *Slug* promoter and its response to Wnt signaling (Vallin et al., 2001). Lrig3 is able to enhance Wnt signaling in animal caps, as most clearly seen in the enhanced induction of several Fgf genes (Fig. 5C). As Fgf signaling can induce NC markers under certain conditions (Fig. 6E), these observations may account for the modest induction of NC genes in *Wnt3a* plus *Lrig3*-injected animal caps (Fig. 5B).

A requirement for *Lrig3* in NC formation was seen by knockdown of *Lrig3* using a splicing MO in whole embryos or animal caps (Fig. 3B-E",H-J", Fig. 4A-B). In animal caps we find that the expression of *Zic1* and *Pax3* is not inhibited by L3MO, while the expression of *Slug*, *Foxd3* and *Sox9* is. In a proposed network of NC formation, *Zic1* and *Pax3* are upstream of the other markers tested (Monsoro-Burq et al., 2005; Steventon et al., 2005), and thus our results can be interpreted by placing *Lrig3* function downstream of *Zic1* and *Pax3*. Indeed, L3MO prevented the expression of NC markers induced by *Zic1* and *Pax3* (Fig. 4B). A Wnt signal is required in combination with *Zic1* and *Pax3* in NC induction, suggesting that *Lrig3* has a role in modulating this signal at this position of the regulatory hierarchy.

In addition to the Wnt pathway and an attenuated BMP signal, Fgf signaling is important in NC induction. Gain-of-function experiments indicate that the ectopic expression of *Fgf8* at a level just above physiological leads to expansion of the NC, while an excess of *Fgf8* expression is inhibitory (Hong and Saint-Jeannet, 2007). Furthermore, knockdown of *Fgf8* by MO inhibits NC formation (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), while *Fgf8* is able to induce NC markers in animal cap assays (Monsoro-Burq et al., 2003). We showed in animal caps that *Fgf8* was able to induce NC markers at low but not at high doses, and addition of *Lrig3* shifts peak induction to higher Fgf levels (Fig. 6E). We suggest that *Lrig3* has a role in optimizing the Fgf signal during NC induction in *Xenopus*.

Lrig3 as a modulator of different signaling cascades during embryonic development

The proposed role of *Lrig3* in modulating the Fgf signal is compatible with the observations that *Lrig3* can inhibit Fgf-dependent ERK phosphorylation and mesodermal gene induction in animal caps (Fig. 6). As a transmembrane protein containing leucine-rich repeats, *Lrig3* might be expected to interact with Fgf receptors, and we find that such an interaction can be observed after expressing *Lrig3* and *Fgfr1* in cultured cells (Fig. 7; see Fig. S7 in the supplementary material). *Lrig3* bears some resemblance to *Xflrt3* (Bottcher et al., 2004), which also contains leucine-rich repeats and interacts with *Fgfr1*, but functions as a Fgf signaling enhancer. Human LRIG1 has been reported as an inhibitor of EGF signaling (Gur et al., 2004), and it is possible that *Lrig3* also affects EGF signaling. EGF signaling and the ErbB receptor family regulate mesoderm formation (Nie and Chang, 2006) and gastrulation movements (Nie and Chang, 2007) in *Xenopus*, although a role in NC formation has not been identified. Human LRIG1 negatively regulates the stability of EGFR and Met receptors through different mechanism. *Lrig1* destabilizes EGFR by enhancing its ubiquitination in a Cbl-dependant manner (Gur et al., 2004), while it destabilizes the Met receptor in a Cbl-independent manner (Shattuck et al., 2007). Our data suggest a negative influence of *Lrig3* on *Xfgfr1* expression levels, expanding the range of apparent interactions between *Lrig* family members and RTK receptors.

Although *Lrig3* inhibited the function of Fgf in animal caps, it enhanced the transcription of *Fgf3*, *Fgf4* and *Fgf8* induced by *Wnt3a*. Wnt/ β -catenin signaling has at least two distinct functions in very early *Xenopus* development. First, maternal Wnt signaling is involved in the establishment of the DV axis (Kofron et al., 2007; Tao et al., 2005). Second, Wnt/ β -catenin signaling is required for mesoderm induction through Fgf and Nodal, and promotes ventral/lateral but restricts dorsal development (Schohl and Fagotto, 2003) (Christian and Moon, 1993; Hoppler et al., 1996; Hoppler and Kavanagh, 2007). We observed an effect of *Lrig3* on Wnt signaling mostly in the context of the latter process. Induction of *Siamois* and *Xnr3* by Wnt, representing its role in axis formation, was only slightly affected by *Lrig3*, and overexpression of *Lrig3* in ventral blastomeres did not induce a secondary axis (data not shown). However, induction of *Xbra*, and notably of *Fgf3*, *Fgf4* and *Fgf8* by Wnt was stimulated by *Lrig3* (Fig. 5B), and Topflash reporter activity was moderately enhanced by *Lrig3* (see Fig. S6 in the supplementary material). These observations suggest that *Lrig3* can enhance some but not all outcomes of the Wnt signal transduction pathway.

In this study, we show that *Lrig3* is required for NC formation during early embryonic development by modulating Wnt and Fgf signaling, and we placed *Lrig3* downstream of *Zic1* and *Pax3* in the NC regulatory network. As is well established, an attenuated BMP signal in concert with a canonical Wnt signal is crucial in NC formation. *Lrig3* was induced in this context and stimulated some aspects of Wnt signaling. One of the outputs of the enhanced Wnt signal is the induction of Fgf genes, providing an additional signal required in NC specification. Similar to BMP, which is required in NC formation but must be kept at a low level, an optimum Fgf level must be maintained for effective NC induction. *Lrig3*, through its ability to attenuate Fgf signaling, may assure achievement of this optimal level. In this model, its ability to modulate different signaling cascades is the basis for the requirement for *Lrig3* in NC induction in the embryo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of the *Lrig3* gene

(A) Regional expression of *Lrig3* in stage 10 embryos. *Lrig3* was expressed strongly in dorsal explants, but barely detected in other regions. The indicated genes were assayed to test quality of dissection. St10, stage 10 embryo; AC, animal cap; Veg, vegetal explant; RT-, control without reverse transcriptase. (B) Schematic drawing of the protein structure of *Xenopus* and zebrafish *Lrig3*. SP (yellow), signal peptide; LRRNT (blue box), leucine-rich repeat N-terminal domain; LRR TYP, leucine-rich repeats, typical; LRR, leucine-rich repeats; LRRCT (blue oval), leucine rich repeat C-terminal domain; IG C2 (green oval), immunoglobulin C-2 Type; TM (purple), transmembrane domain. Sequence identity between zebrafish and *Xenopus* *Lrig3* is indicated. (C) Dendrogram of the *Lrig3* family including Kekk1 of *Drosophila*. (D,E) Subcellular distribution of *Lrig3* after transfection into COS7 cells. (D) Transfected *Lrig3*-Flag

(red) co-localized with the cis Golgi apparatus marker GM130 (green); the nucleus was stained with DAPI (blue). (E) A small proportion of Lrig3-Flag co-localized with the early endosome marker EEA1 (green). **(F-M)** Expression pattern of *Lrig3* in *Xenopus*. Vegetal view at stage 10, expression in the organizer (F); stage 10 section (G). (H,I) Stage 12 (H, posterior view; I, lateral view). (J,K) Stage 15 (J, dorsal view; K, lateral view). Tailbud (stage 24, L) and tadpole (stage 32, M); expression is seen in brain, eye, somites and branchial arches. **(N)** Temporal expression of *Lrig3* during *Xenopus* development. **(O-R)** Expression of *lrig3* in zebrafish. Transcripts are present maternally (O), become localized in the forming organizer at 30% epibody (P) and subsequently in the shield (Q), and were found in brain, eye and branchial arches at 24 hours (R). (P-R) Lateral views.

Fig. 2. Suppression of *Lrig3* by a splicing morpholino disturbed embryonic development
 (A) RT-PCR shows that injection of L3MO decreased the mature *Lrig3* mRNA while inducing the predicted ~1 kb unspliced band, using intron-spanning primers for pseudoalleles *Lrig3A* and *Lrig3B*. ODC was loading control. (B-D) Phenotypes induced by L3MO. The morphants showed severe anterior defects, delayed or failed neural fold closure, and shortened axis. (C,D) Embryos were injected with 30 ng L3MO; uninjected control in B. (E-G) The phenotype was rescued by co-injection of *Lrig3* mRNA. (E) Uninjected control; (F) L3MO (30 ng); (G) 30 ng of L3MO plus 20 pg *Lrig3* mRNA.

Fig. 3. Knockdown of *Lrig3* results in defects of the NC and its derivatives

(A-F'') In situ hybridization with the indicated markers for uninjected or two examples ('') of L3MO-injected embryos. One dorsal blastomere at the four-cell stage was injected with 15 ng L3MO and 100 pg *lacZ* mRNA, and fixed at about stage 19. The expression of hindbrain marker *Krox20* (rhombomeres 3 and 5) (A-A''), NC markers *Slug* (B-B''), *Sox9* (C-C''), *Myc* (D-D'') and *Inca* (E-E''), and pan-neural marker *Sox2* (F-F'') were examined. The injected side was traced by *lacZ* staining. The stream of *Krox20*-positive cells extending from the hindbrain was absent in 97% (28 of 29 embryos) on the injected side. The expression of NC markers was reduced on the injected side in the following percentage of embryos: *Slug* (58%, 14 of 24), *Sox9* (79%, 15 of 19), *Myc* (83%, 20 of 24) and *Inca* (65%, 17 of 26). (G) Neural induction by overexpression of *Chordin* (*Chd*) in animal caps was barely affected by L3MO. *Chd* (100 pg) or *Chd* (100 pg) plus L3MO (30 ng) were injected, caps dissected at stage 9, and assayed at equivalent stage 22. Pan neural markers *Sox2* and *Ncam* were examined, and interference with *Lrig3* splicing was verified (see also Fig. 2A); arrow indicates the predicted unspliced band (lane 4). (H-J'') NC markers, including *Slug*, *Inca* and *Sox9*, were examined in embryos injected with 7.5 ng L3MO in one animal dorsal blastomere at the eight-cell stage. Strong inhibition was observed for *Slug* (95%, 18 of 19), *Inca* (89%, 24 of 27) and *Sox9* (73%, 19 of 26). (K-

M) Cranial cartilages were reduced by knockdown of *Lrig3*. Ventral views of Alcian Blue stained cartilage from embryos injected with control morpholino (K) or L3MO (L,M). ba, basihyal; br, branchial; ch, ceratohyal; m, Meckel's. Percentages of embryos with reduced head cartilage are 82% (38 of 47) at 7.5 ng L3MO and 97% (31 of 32) at 15 ng L3MO. **(N-P)** Rescue of L3MO effect. All embryos were injected with 15 ng L3MO, and embryos in N-O" were co-injected with 20 pg *Lrig3* mRNA; in situ hybridization with *Inca* (N-N") and *Slug* (O-O"). Percentages of strongly affected (examples in N,O), mildly affected (N',O') and normal (N",O") are shown in P.

Fig. 4. Lrig3 is required for NC formation in animal caps downstream of Pax3 and Zic1
 (A,A') L3MO inhibits NC marker induction by *Chordin* (*Chd*) plus *Wnt3a* in animal caps. Embryos were injected with 300 pg *Chd*, 1 ng *Lrig3*, 30 ng L3MO, 300 pg *Chd* + 300 pg *Wnt3a*, 300 pg *Chd* + 300 pg *Wnt3a* + 30 ng L3MO, or with 300 pg *Wnt3a* + 300 pg *Chd* + 1 ng *Lrig3*. Gene expression was assayed by RT-PCR. (B) NC induced by co-injection of *Pax3* and *Zic1* was inhibited by L3MO. *Pax3* (200 pg) and *Zic1* (200 pg) each, alone or with 30 ng L3MO were injected, and assayed as in A. AC, uninjected animal caps. RT-, without reverse transcriptase.

Fig. 5. Lrig3 is involved in NC formation by modulating Wnt and Fgf signaling

(A) Lrig3 slightly enhanced *Xnr3* and *Siamois* induction by Wnt3a. Animal cap assay at stage 10, embryos injected with 300 pg *Wnt3a* or 300 pg *Wnt3a* + 1 ng *Lrig3*. (B) NC markers were moderately induced by the combination of Lrig3 and Wnt3a. Embryos were injected with *Lrig3* (1 ng), *Wnt3a* (300 pg), *Wnt3a* and *Lrig3*, *Chd* (300 pg), *Chd* and *Lrig3*, and *Chd*, *Wnt3a* and *Lrig3*, and animal caps were assayed at equivalent stage 16. (C) Lrig3 enhanced the induction of *Fgf3*, *Fgf4* and *Fgf8* by Wnt3a. RNAs were injected at the concentrations listed in B, and animal caps were assayed at stages indicated.

Fig. 6. Lrig3 attenuates Fgf signaling

(A) Lrig3 inhibited ERK phosphorylation. Embryos were injected with active Ras (HaRas, 100 pg), *eFgf* (1 pg), *Lrig3* (1 ng), zebrafish *sef* (500 pg) or *mkp3* (500 pg). Animal caps were treated with bFgf (20 ng/ml) for 3 hours as indicated. Cell lysates were analyzed for diphosphorylated (dp)-ERK and total (pan)-ERK. AC, untreated. (B) Overexpression of *Lrig3* inhibits *Xbra* induction by bFgf. Animal caps were treated with bFgf (50 ng/ml) for 4 hours. *Xbra* expression was induced by bFgf treatment in uninjected (lane 4), but not in *Lrig3* (1 ng) injected caps (lane 5). (C) *Lrig3* did not attenuate *Xbra* expression induced by activin (250 pM). Injection of *Smad7* (500 pg), but not of *Lrig3* (1 ng), inhibited *Xbra* induction. (D) *Lrig3* inhibits Fgf8 activity in animal caps. Embryos were injected with the indicated dose of *Fgf8a* alone or with 1 ng of *Lrig3*. Expression of *Xbra*, *Xwnt8* and *Xmsx1* was examined at equivalent stage 11.5. (E) Differential effect of *Lrig3* on *Slug* and *Twist* induction by different doses of *Fgf8a*. *Fgf8a* at increasing doses, as indicated, was injected alone or with a constant amount (1 ng) of *Lrig3*, and induction of *Slug* and *Twist* was assayed at equivalent stage 22.

Fig. 7. Lrig3-Xfgr1 interactions

(A) The domain structure of wild-type Lrig3 and its deletion mutants; all constructs were tagged with Myc. LRRNT (blue box), leucine-rich repeat N-terminal domain; LRR TYP, leucine-rich repeats, typical; LRRCT (blue oval), leucine-rich repeat C-terminal domain; IG C2 (green oval), immunoglobulin C-2 Type; SP (yellow), signal peptide; TM (purple), transmembrane domain. (B) Lrig3 binds to Xfgr1 through its ectodomains. Co-immunoprecipitation was carried out in extracts of 293T cells co-transfected with Xfgr1 and wild type or mutants of Lrig3 using anti-Xfgr1 antibody, and blotted with anti-Myc antibody. (C) Lrig3 decreases Xfgr1 levels. 293T cells were co-transfected with Xfgr1 and Lrig3 or GFP, which was used to adjust the amount of DNA. Xfgr1 and endogenous diphospho-ERK and Pan-ERK were detected by western blotting. Actin was employed as a loading control. IP, immunoprecipitate; WCL, whole cell lysate; WB, western blot.

Table 1

Primers used for RT-PCR

Gene name	Sequence (upstream and downstream, respectively)	Reference
Ap2	TCCGACCCGAACGAGCAAGTA ATCAGGACCGGCAATGTCTA	This work
Bmp4	GATCTCAGACTCAACGGCAC GCATGTACGGATAAGTCGATC	De Robertis *
Chordin	CCTCCAATCCAAGACTCCAGAG GGAGGAGGAGGAGCTTTGGGACAAG	De Robertis *
Cerberus	GCTGAACTATTTGATTTCACC ATGGCTTGTATTCTGTGGGGCG	De Robertis *
Fgf3	GTCATTTGTTTCCAGACTTC TATCTGTAGGTGGTACTTAG	This work
Fgf4	TTACCGGACGGAAGGATA CCTCGATTTCGTAAGCGTT	This work
Fgf8	CTGGTGACCGACCAACTAAG ACCAGCCTTCGTACTTGACA	This work
Foxd3	GGAGGGAGGGGGCAATGCAC CCCCGAGCTCGCCTACT	(Pohl and Knochel, 2001)
Frzb-1	GACCACTGAATGTAGCCAGGAC GGAGATGCAGACTCCTCTGTCA	De Robertis *
Lrig3	GTGCAGTGTACAAGGCCTCA TTGGAATGTACACGGACTGC	This work
Lrig3A intron	CGGCTAGACTAGAGTGTGCT AGCATCTTCAAGGTCTGTGT	This work
Lris3B intron	CAGCTAGATTAGAATGTGCAGCG CCATTAATGGCCGTAGAAACGATG	This work
Msx1	GCTAAAAATGGCTGCTAA AGGTGGGCTGTGTAAAGT	De Robertis *
Odc	CAGCTAGCTGTGGTGTGG CAACATGGAAACTCACACC	De Robertis *
Pax3	CTACCTCGGTTTCTTGACTG TGGTCAATCCTTCTTAATGG	This work
Siamois	CAGGTTTGTTTCAGAACAG TTCAGGCCAGTTGAATGTAT	This work
Slug	TCCCGCACTGAAAATGCCACGATC CCGTCCTAAAGATGAAGGTATCCTG	(LaBonne and Bronner-Fraser, 1998)
Snail	AAGCACAATGGACTCCTT CCAATAGTGATACACACC	(LaBonne and Bronner-Fraser, 1998)
Sox2	GAGGATGGACACTTATGCCCCAC GGACATGCTGTAGGTAGGCGA	De Robertis *
Sox3	TGATGCAGGACCAGTTGGGC	

Gene name	Sequence (upstream and downstream, respectively)	Reference
	TGAAGTGAAGGGTCGCTGGC	(Ishibashi and Yasuda, 2001)
Sox9	AAGCAGAATGTCCTCTGTGA	This work
	AAGGCCAGATTCAGTTCTTC	
Sox17 β	GTCATGGTAGGAGAGAAC	De Robertis [*]
	ATCTGTTTAGCCATCACTG	
VegT	CAAGTAAATGTGAGAAACCG	De Robertis [*]
	CAA ATACACACACATTTCCC	
Xbra	TTAAGTGCTGTAATCTCTTCA	De Robertis [*]
	GCTGGAAGTATGTGATGGAG	
Xlim-1	GAAGGATGAGACCACTGGTGG	De Robertis [*]
	CACTGCCGTTTCGTTTCATTC	
XTwist	AGTCCGATCTCAGTGAAGCGCA	(LaBonne and Bronner-Fraser, 1998)
	TGTGTGTGGCCTGAGCTGTAG	
XVent-1	TTCCCTTCAGCATGGTTCAAC	(Gawantka et al., 1995)
	GCATCTCCTTGGCATATTTGG	
Zic1	ATGAAGGTCCACGAAGCATC	(Nakata et al., 1998)
	CGTGCTGTGATTGGACGTGT	

* <http://www.hhmi.ucla.edu/derobertis/>

Table 2

Phenotype induced by L3MO

L3MO dose	<i>n</i>	Normal	Head repression, short and bent axis	
			Mild	Severe
10 ng	48	46 (95.8%)	2 (4.2%)	0
20 ng	32	17 (53.1%)	15 (46.8%)	0
30 ng	97	7 (7.2%)	13 (13.4%)	77 (79.4%)
40 ng	68	0	9 (13.2%)	59 (86.7%)

Table 3
Rescue of L3MO phenotype by co-injection of *Lrig3* mRNA

	<i>n</i>	Normal	Head repression, short and bent axis	
			Mild	Severe
L3MO 30 ng	82	7 (8.5%)	24 (29.3%)	51 (62.2%)
L3MO+Lrig3 (30 ng + 20 pg)	80	50 (62.5%)	24 (30%)	4 (5%)