In the frog, *Xenopus laevis*, fibroblast growth factor (FGF) signaling is required for both mesoderm formation and the morphogenetic movements that drive the elongation of the notochord, a dorsal mesodermal derivative; the coordination of these distinct roles is mediated by the *Xenopus Ctr1* (Xctr1) protein: maternal Xctr1 is required for mesodermal differentiation, while the subsequent loss of Xctr1 promotes morphogenesis. The signaling cascade activated by FGF in the presence of Ctr1 has been well characterized; however, the Xctr1-independent, FGF-responsive network remains poorly defined. We have identified *Xenopus Marginal Coil* (*Xmc*) as a gene whose expression is highly enriched following Xctr1 knockdown. Zygotic initiation of Xmc expression in vivo coincides with a decrease in maternal Xctr1 transcripts; moreover, Xmc loss-of-function inhibits Xctr1 knockdown-mediated elongation of FGF-treated animal cap explants, implicating Xmc as a key effector of Xctr1-independent gastrular morphogenesis. 

Key words: Xctr1; Xmc; FGF; Xenopus; morphogenesis

Accepted 29 June 2009

INTRODUCTION

Vertebrate development is driven largely by inductive interactions, during which one group of cells influences the fate of another, often by means of the activity of secreted extracellular ligands. A striking feature of embryonic induction is the prominence of a small set of ligands in these varied interactions, from germ layer formation and early patterning to morphogenesis and organogenesis. This observation raises an important question regarding signal interpretation; that is, how does the embryo ensure that a given signal elicits a response that is specific, and biologically appropriate, to the responding cell? At least two distinct scenarios are plausible. On the one hand, one could envision that a single “core” pathway induced by a given ligand could be modified, at or near the cascade’s terminus, by a set of transcriptional effectors specific to one or a few cell types. Alternatively, a single ligand-receptor pair could induce largely distinct signaling cascades, depending upon the cellular context in which the induction takes place.

Members of the fibroblast growth factor (FGF) family play a critical role during innumerable steps of embryogenesis. In the frog, *Xenopus laevis*, FGF is essential for maintaining the differentiation of the mesodermal germ layer, first induced by transforming growth factor-β (TGFβ) signaling (Heasman, 2006). Shortly after the initiation of gastrulation, FGF receptor activation no longer supports mesodermal differentiation; instead, FGF signaling promotes the dramatic lengthening of both the notochord, a dorsal mesodermal derivative, and the caudal neural tube, by means of the morphogenetic processes of convergence and extension (Nutt et al., 2001). FGF mediated-mesoderm induction results from activation of the “canonical” Ras/MAPK cascade (Heasman, 2006); FGF-mediated morphogenesis, however, results instead from the activation of Ras/MAPK-independent “noncanonical” FGF signaling (Nutt et al., 2001). These distinct FGF-dependent pathways are distinguished not only by their activating components, but by their distinct re-
pressors, as well: the *Xenopus* Spred proteins block Ras/MAPK signaling; while the related Sprouty proteins inhibit Ca$^{2+}$ and PKC$\gamma$ “noncanonical” signaling (Sivak et al., 2005). Thus, the shift in the biological consequence of FGF signaling is due to a shift in the signaling cascades stimulated by FGF receptor activation.

A key mechanism by which early embryonic cells “translate” FGF receptor activity into a specific signaling cascade and subsequent biological activity hinges on the presence or absence of the Ctrl protein within the plasma membrane-associated FGF signaling complex. The maternal *Xenopus* Ctrl (Xctr1) protein facilitates phosphorylation of the FGF receptor-associated docking protein SNT-1/FRS2a by the FGF receptor and the Laloo nonreceptor tyrosine kinases, as well as the phosphorylation and activation of ERK, the latter event a defining step in “canonical” FGF signaling (Hama et al., 2001; Heasman, 2006; Haremaki et al., 2007). Consistent with this biochemical activity, Xctr1 misexpression enhances FGF-mediated mesoderm induction, and inhibits convergent extension, both in explants and in vivo (Haremaki et al., 2007). Conversely, loss of Xctr1 inhibits mesoderm differentiation and instead promotes mesodermal and neural morphogenesis (Haremaki et al., 2007).

While it is clear that loss of Xctr1 both inhibits differentiation and promotes gastrular morphogenesis, little is yet known of the “noncanonical” signaling cascade that is activated by FGF in the absence of Xctr1. In an attempt to address the mechanisms by which Xctr1 elimination promotes coordinated tissue movements, we performed gene array studies to identify transcripts that were robustly enriched by FGF in the absence of Xctr1. We have identified several genes that are highly expressed following Xctr1 knockdown. One of these, *Xenopus Marginal Coil (Xmc)*, a gene previously described as an FGF-responsive transcript, was selected for further study (Frazzetto et al., 2002). Xmc expression initiates shortly after the initial clearing of maternal Xctr1 transcripts in the *Xenopus* embryo, and Xmc loss-of-function inhibits Xctr1 knockdown-mediated elongation by FGF, indicating that Xmc is necessary for FGF-stimulated, Xctr1-independent morphogenesis.

**RESULTS**

We have previously demonstrated that Xctr1 loss-of-function promotes FGF-mediated elongation of *Xenopus* explant cultures (Haremaki et al., 2007). While several signaling components have been implicated in caudal neural and dorsal mesodermal morphogenesis, little is known about the regulation of these events at the level of gene expression. To characterize the transcriptional response to Xctr1 knockdown, we performed microarray analyses to compare the expression profiles of FGF-treated animal caps from embryos injected with either Xctr1-specific (Xctr1MO) or 5-base pair mismatch- (Xctr1MM) morpholinos; Xctr1MM does not inhibit Xctr1 translation, in vitro (Haremaki et al., 2007). A representative experiment is shown in Figure 1; the lack of effect on “housekeeping” genes (e.g., *EF1-a*) and the reduction of dorsal mesoderm-specific genes (e.g., *muscle actin*) following Xctr1 knockdown point to the quality of these data sets (Fig. 1; Mohun et al., 1984; Krieg et al., 1989).

Our aim in these studies was to identify genes whose expression was strongly induced following Xctr1 knockdown. We reasoned that, because both neural and mesodermal differentiation were inhibited by Ctrl1 elimination, the genes we identified as “up-regulated” in our analysis were not likely to represent molecular markers of induced cell fates and were, thus, reasonable candidate effectors of Ctrl1 knockdown-mediated morphogenesis. We identified 25 annotated genes with greater than threefold elevated expression, following Xctr1 knockdown in FGF-treated neurula stage animal cap explants, in each of two independent microarray experiments (Table 1). Candidates selected for independent verification were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR; Table 1, bold, Fig. 2, and data not shown). Strikingly, exogenous FGF was not required for enhanced expression of these transcripts; knockdown of Xctr1 was alone sufficient to stimulate this response (Fig. 2).

To narrow the pool of candidate effectors, we reasoned that mediators of Xctr1-independent morphogenesis might also show a reduction in transcript abundance following Xctr1 misexpression, as Xctr1 inhibits elongation of both dorsal mesoderm and caudal neurectoderm (Haremaki et al., 2007). We therefore performed an additional microarray study to select for transcripts down-regulated by Xctr1 in animal cap explants. Figure 3A shows the effects of Xctr1 misexpression on transcripts shown to be up-regulated in the Xctr1 knockdown microarray studies. Of the transcripts enriched following injection of Xctr1 morpholino oligos (Fig. 3A, left panel, red), only two were significantly down-regulated following misexpression of Xctr1 RNA (Fig. 3A, right panel, arrows pointing to bright green bands). Both of these bands correspond to *Xenopus marginal coil (Xmc)*. The FGF-inducible Xmc gene encodes a coiled-coil protein previously implicated in the regulation of gastrulation movements (Frazzetto et al., 2002); it was thus chosen for further analysis. RT-PCR was used to confirm that Xctr1 knockdown enhances Xmc expression in the presence of FGF (Fig. 3B). Xmc transcripts are also enriched following Xctr1 knockdown in the absence of FGF (Fig. 3C). We next confirmed, using RT-PCR, that Xctr1 misexpression down-regulates Xmc in animal caps (Fig. 3D,E, lanes 1 and 2). FGF protein induces robust expression of Xmc in animal cap explants, presumably reflecting mesodermal Xmc expression; Activin protein induces levels similar to those seen with FGF (Fig. 3E, compare lanes 2, 3, and 5; Frazzetto et al., 2002). Ectopic Xctr1 does not, however, inhibit Xmc expression in either FGF- or Activin-treated caps (Fig. 3E). These data demonstrate that Xctr1 loss-of-function enhances Xmc expression in both ectoderm and mesoderm, and that Xctr1 gain-of-function suppresses Xmc expression in ectoderm, but not mesoderm.

Our studies suggest that Xctr1 knockdown may promote morphogenesis by means of induction of Xmc. Consistently, we find that initiation of Xmc expression coincides with the decline in maternal Xctr1 expression (Fig. 4; Frazzetto et al., 2002; Haremaki et al., 2007). Coupled with the
gain- and loss-of-function analyses described above, these correlative data provide further evidence for regulation of Xmc by Xctr1, in vivo. We observed no effects on cell fate determination or morphogenesis from Xmc misexpression in ectodermal or mesodermal explants, including animal caps treated with FGF and/or Activin, or in intact Xenopus embryos, consistent with earlier published studies (data not shown; Frazzetto et al., 2002), suggesting that Xmc gain-of-function is not sufficient to alter morphogenesis. We next sought to address the potential requirement for Xmc in mediating Xctr1-independent morphogenesis. Knockdown of Xmc function by means of Xmc-specific antisense morpholino oligonucleotides has been shown to affect axial elongation in Xenopus (Frazzetto et al., 2002); morpholino studies were thus used to further address the relationship between Xmc and Xctr1 during gain- and loss-of-function analyses described above, these correlative data provide further evidence for regulation of Xmc by Xctr1, in vivo.

We observed no effects on cell fate determination or morphogenesis from Xmc misexpression in ectodermal or mesodermal explants, including animal caps treated with FGF and/or Activin, or in intact Xenopus embryos, consistent with earlier published studies (data not shown; Frazzetto et al., 2002), suggesting that Xmc gain-of-function is not sufficient to alter morphogenesis. We next sought to address the potential requirement for Xmc in mediating Xctr1-independent morphogenesis. Knockdown of Xmc function by means of Xmc-specific antisense morpholino oligonucleotides has been shown to affect axial elongation in Xenopus (Frazzetto et al., 2002); morpholino studies were thus used to further address the relationship between Xmc and Xctr1 during
morphogenesis. Injection of an Xmc-specific morpholino (XmcMO) results in a dose-dependent translational block of a co-expressed Myc epitope-tagged Xmc construct (Xmc-Myc) in gastrula stage embryos (Fig. 5; Frazzetto et al., 2002). This effect cannot be mimicked with either a control, “scrambled” morpholino (CMO), or with an Xmc-specific morpholino containing 5 base pair mismatches (XmcMM; Fig. 5). Injection of XmcMO has been previously shown to generate three phenotypic classes by the tadpole stage: embryos with a kinked axis, embryos with a short axis, and embryos with an open blastopore (Class I, II, and III, after Frazzetto et al., 2002). In our hands, dorsal marginal injection of 25 ng of XmcMO at the four-cell stage results in 87% of embryos with Class II defects, with the remainder exhibiting Class III defects (N = 15). This dose of XmcMO was used for subsequent Xmc loss-of-function studies.

Explant assays were used to test for the potential requirement for Xmc in Xctr1 knockdown-mediated morphogenesis. Elongation of FGF-treated animal cap explants is enhanced following Xctr1 knockdown (Fig. 6A,B) (Haremaki et al., 2007); this elongation is significantly inhibited by co-injection of the Xmc-specific morpholino XmcMO (Fig. 6A,C). Inhibition of Xctr1 knockdown-mediated elongation is not seen after injection of a 5 base pair mismatch Xmc morpholino (XmcMM; Fig. 6D); moreover, the block to inhibition by XmcMO can be partially rescued by co-injection of XmcRNA lacking the XmcMO binding site (Xmc), pointing to the specificity of XmcMO for the Xmc transcript in these assays (Fig. 6A,E). Xmc knockdown does not, however, inhibit elon-

| ID            | Symbol    | Name                                             | Fold change |
|---------------|-----------|--------------------------------------------------|-------------|
| XI.5274.1.S1_at | DasraA    | chromosomal passenger complex protein Dasra A    | 7.80        |
| XI.585.1.S1_at | ESR-6e    | enhancer of split related epidermal protein-6   | 6.74        |
| XI.1262.1.S1_at | LOC397921 | nucleoplasm                                       | 5.83        |
| XI.1268.1.S1_at | Otx2      | homeobox protein                                  | 4.80        |
| XI.973.1.S1_at | LOC397824 | homeobox protein Xgbx-2                          | 4.34        |
| XI.5454.1.S2_at | Xmc       | marginal coil Xmc                                 | 4.15        |
| XI.446.1.S1_at | xZPA      | vitelline envelope 69/64 kDa glycoprotein         | 3.88        |
| XI.5888.2.S1_at | rGAP      | rho-GTPase activating protein                     | 3.67        |
| XI.869.1.S2_at | LOC397936 | POU-domain protein                                | 3.65        |
| XI.11672.1.A1_at | Otx2    | homeobox protein                                  | 3.65        |
| XI.798.1.S1_at | B4        | histone H1-like maternal protein                  | 3.56        |
| XI.7590.2.A1_at | LOC397951 | XDCoH                                             | 3.44        |
| XI.5288.1.S1_at | LOC398163 | cyclin B4                                         | 3.42        |
| XI.25540.3.A1_at | LOC398660 | KH domain RNA-binding protein Sam68               | 3.41        |
| XI.699.1.S1_a_at | LOC397948 | VENT-2 transcription factor                      | 3.41        |
| XI.2582.1.S1_at | rgma      | repulsive guidance molecule A protein phosphatase  | 3.31        |
| XI.8340.2.A1_a_at | ppm1d    | isofrom                                           | 3.27        |
| XI.15540.2.A1_at | atf5      | activating transcription factor                   | 3.23        |
| XI.8719.1.S1_at | dynI2     | dynein light chain 2                              | 3.20        |
| XI.17481.1.A1_at | mthd2    | methylene tetrahydrofolate dehydrogenase         | 3.13        |
| XI.15450.1.A1_at | atf5      | activating transcription factor                   | 3.13        |
| XI.1592.1.A1_at | LOC397824 | homeobox protein Xgbx-2                          | 3.12        |
| XI.8701.1.S1_a_at | aldoc    | aldolase C, fructose-bisphosphatase               | 3.11        |
| XI.7590.2.A1_x_at | LOC397951 | XDCoH                                             | 3.07        |
| XI.25306.1.S1_at | cldn6     | Claudin 6                                         | 3.05        |

*List of Xenopus laevis transcripts with expression up-regulated at least threefold in fibroblast growth factor (FGF)-treated animal cap explants from embryos injected with a Ctrl-specific morpholino (XctrlMO). Only annotated genes are listed.
gation of animal caps by Activin (Fig. 6F); thus, Xmc is required for FGF-mediated, Xctr1-independent morphogenesis.

**DISCUSSION**

The presence or absence of the transmembrane protein Ctr1 dictates the interpretation of FGF receptor activation as, respectively, either a pro-differentiation or a pro-morphogenesis cue. Here, we demonstrate that Xmc is a critical component of the signaling cascade activated by FGF in the absence of Xctr1, as Xmc loss-of-function inhibits Xctr1 knockdown-mediated morphogenesis. This functional link is buttressed by the temporal patterns of expression of the two genes: the initiation of Xmc expression at gastrula stages correlates with the initial decline in maternal Xctr1 expression levels, and also with the initiation of FGF-mediated morphogenesis. Xmc represents one of more than two dozen genes whose expression is induced following Xctr1 knockdown; it was selected for further analysis because it was the only transcript that was also suppressed following Xctr1 misexpression in ectodermal explants. We have found, however, that Xctr1 misexpression, despite inhibiting explant and embryo elongation, does not block Xmc expression in the presence of FGF or Activin (this study, and Haremaki et al., 2007). This suggests that, although Xmc is required for Xctr1-independent, FGF-mediated elongation, Xmc suppression does not underlie Xctr1-dependent inhibition of mesodermal elongation; early suppression of Xmc may, however, play a role in the inhibition of neural morphogenesis by Xctr1. Other candidates from our screens, perhaps including factors that are not required for mediating Xctr1-independent morphogenesis, may be involved in the suppression of premature mesodermal morphogenesis downstream of Xctr1 (Haremaki et al., 2007); we are beginning to test, in gain- and loss-of-function assays, microarray targets whose expression is altered by Xctr1 RNA, but not Xctr1 morpholino, injection.

**EXPERIMENTAL PROCEDURES**

**Gene Chip Analysis**

RNA from 80 animal cap explants, cultured to stage 20, were used to generate hybridization probes for use on Affymetrix GeneChip *Xenopus laevis* Genome Arrays; hybridization was performed with the help of the Mount Sinai Microarray Shared Research Fa-
REGULATION OF MORPHOGENESIS BY XCTR1 AND XMC

Myc, 16 base pairs of 5’XhoI sites of pCS2+/+H11001/H11001 gene was obtained from Open Biosystems (IMAGE ID: 6634521; accession no. BC072122). The full coding sequence of Xmc was amplified by PCR and cloned into the pCS2+ MT vector; this construct contains the complete XmcMO binding site. The Xmc construct used for morpholino rescue experiments lacks all 5’-UTR and thus much of the XmcMO binding site.

Xmc Cloning and Mutant Construction
An Image clone containing the Xenopus marginal coil (Xmc) gene was obtained from Open Biosystems (IMAGE ID: 6634521; accession no. BC072122). The full coding sequence of Xmc was amplified by PCR and cloned into the EcoRI and XhoI sites of pCS2+/+. For Xmc-Myc, 16 base pairs of 5’-untranslated region (UTR) and thus much of the XmcMO binding site. The Xmc construct used for morpholino rescue experiments lacks all 5’-UTR and thus much of the XmcMO binding site.

RNA Preparation, Explant Dissection, and Cell Culture
RNA was synthesized in vitro in the presence of cap analog using the mMessage mMachine kit (Ambion). Microinjection, explant dissection, and cell culture were performed as described (Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995).

RT-PCR
RT-PCR was performed as described (Wilson and Hemmati-Brivanlou, 1995). Primers used in this study are as follows: Xgbx2-U: 5’-CCCTTGACAGAT-TAGATCGG; Xgbx2-D: 5’-CTCGCA-CTATACCTTCTGTC; Nucleosamin-D: 5’-TAGACTGCG- AATCGGACCTG; Dasraa-U: 5’-ACTGTCACCACAGGTTGC; Dasraa-D: 5’-CTGTATCTCTTCTCAGTACC; ESR-6e-U: 5’-GGCAGGCAATAC-TGTGGT; ESR-6e-D: 5’-CCCCACTTGG-GCAATATGCT; Vent-2U: 5’-ACC- ACTAAATGAAAACCTGT; Vent-2D: 5’-CACATGCGGCAATATAGGCC; Otx2U: 5’-CGGGAATGGATTTGGTCA; Otx2D: 5’-TTGAAACCAGACCTGGACT; Xmc-U: 5’-CTGTGTTCATAGAACAACATGGG; Xmc-D: 5’-ACCTGCTTCTTTGCACTC; Xctr1-U: 5’-GTGTGTCTGGCTGAAATCTTCAG; ODC-U: 5’-ATTGGATTTCAGAGACCA; ODC-D: 5’-CACAAGCTAAGTTGACG.

Morpholinos
Morpholino oligos (Gene Tools) were heated for 5 min at 65°C, then quenched on ice, before injection at the two- or four-cell stage. XmcMO: TG-AATGGATTTCAGAGACCA; ODC-D: 5’-CACAAGCTAAGTTGACG.

ACKNOWLEDGMENTS
We thank S. Sokol and P. Wilson for gifts of reagents. D.C.W. was funded by a PHS grant.

REFERENCES
Basses T, Paris J, Omilli F, Dorel C, Osborne HB. 1990. Post-transcriptional regulation of ornithine decarboxylase in Xenopus laevis oocytes. Development 110:955–962.

Frazzetto G, Klingbeil P, Bouwmeester T. 2002. Xenopus marginal coil (Xmc), a novel FGF inducible cytosolic coiled-coil protein regulating gastrulation movements. Mech Dev 113:3–14.

Hama J, Xu H, Goldfarb M, Weinstein DC. 2001. SNT-1/FRS2alpha physically interacts with Lalo and mediates mesoderm induction by fibroblast growth factor. Mech Dev 109:195–204.