The primary mouth forms from ectoderm and endoderm at the extreme anterior of the embryo, a conserved mesoderm-free region. In *Xenopus*, a very early step in primary mouth formation is loss of the basement membrane between the ectoderm and endoderm. In an unbiased microarray screen, we defined genes encoding the sFRPs Frzb-1 and Crescent as transiently and locally expressed in the primary mouth anlage. Using antisense oligonucleotides and ‘face transplants’, we show that *frzb-1* and *crescent* expression is specifically required in the primary mouth region at the time this organ begins to form. Several assays indicate that Frzb-1 and Crescent modulate primary mouth formation by suppressing Wnt signaling, which is likely to be mediated by β-catenin.

First, a similar phenotype (no primary mouth) is seen after loss of Frzb-1/Crescent function to that seen after temporally and spatially restricted overexpression of Wnt-8. Second, overexpression of either Frzb-1 or Dkk-1 results in an enlarged primary mouth anlage. Third, overexpression of Dkk-1 can restore a primary mouth to embryos in which Frzb-1/Crescent expression has been inhibited. We show that Frzb-1/Crescent function locally promotes basement membrane dissolution in the primary mouth primordium. Consistently, Frzb-1 overexpression decreases RNA levels of the essential basement membrane genes *fibronectin* and *laminin*, whereas Wnt-8 overexpression increases the levels of these RNAs. These data are the first to connect Wnt signaling and basement membrane integrity during primary mouth development, and suggest a general paradigm for the regulation of basement membrane remodeling.

KEY WORDS: Primary mouth, *Xenopus*, Wnt, sFRP, Frzb-1, Crescent, Basement membrane, Laminin, Fibronectin

INTRODUCTION

The primary mouth is the initial opening that connects the foregut to the outside of the embryo. It originates from a unique and conserved anterior region in the embryo, where endoderm directly contacts ectoderm, without intervening mesoderm (Dickinson and Sive, 2007). In vertebrates, the primary mouth becomes the opening to the pharynx as surrounding tissues form the jaws, teeth, tongue and palate, which together constitute the secondary or adult mouth. We previously defined morphological changes that lead to primary mouth formation in *Xenopus laevis* during early tailbud and hatching stages (Dickinson and Sive, 2006). The earliest step identified is disappearance of the basement membrane between the ectoderm and endoderm, which occurs at early tailbud stage. Later, during tadpole stages, the presumptive primary mouth ectoderm undergoes invagination to form the ‘stomodeum’. Subsequently, this invagination deepens, accompanied by a burst of cell death in the ectodermal layer. Ectoderm and endodermal layers intercalate, leading to thinning of the cell layers in the primary mouth anlage. Finally, the thin covering (the ‘buccopharyngeal membrane’) perforates at swimming tadpole stage to open the primary mouth.

Three regions of the embryo are required to induce formation of the primary mouth. These are the deep anterior endoderm, the anterior neural plate and the lateral mesoderm, including the neural crest (Dickinson and Sive, 2006). These regions are likely to secrete regulatory factors that govern primary mouth development, but these signals and other genes involved are not known, and their identification forms the basis of this paper.

Early during development, substantial data indicate that anterior development in *Xenopus* and other vertebrates requires the inhibition of β-catenin-mediated Wnt signaling (Agathon et al., 2003; De Robertis, 2006; De Robertis et al., 2000; Kemp et al., 2005; Lewis et al., 2008; Niehrs, 1999). Wnt signaling can be inhibited by several secreted antagonists, which target the Wnt co-receptors Frizzled and LRPs (Semenov et al., 2008; Yamamoto et al., 2008). The secreted Frizzled Related Proteins (sFRPs) comprise another class of Wnt antagonists, which contain a cysteine-rich domain with homology to the extracellular domain of Frizzled receptors. sFRPs are believed to bind Wnt ligands, thereby preventing their interaction with Frizzleds (Jones and Jomary, 2002; Kawano and Kypfa, 2003). Some sFRPs also inhibit other pathways, including BMP signaling (Bovolenta et al., 2008; Lee et al., 2006).

Wnt antagonists are required for anterior specification during primary axis formation. For example, during gastrula stages of *X. laevis*, *dkk-1* and the sFRPs *frzb-1*, *crescent*, *sfrp-2* and *sizzled* are expressed in the Spemann organizer and are important for formation of the head (Glinka et al., 1998; Niehrs et al., 2001; De Robertis, 2006; Jones and Jomary, 2002; Kawano and Kypfa, 2003). Later, during *Xenopus* and zebrafish neurulation, Wnt antagonists are expressed anteriorly and are required for formation of the forebrain and placodes (Carmona-Fontaine et al., 2007; Houart et al., 2002). Although it is clear that inhibition of Wnt/β-catenin signaling is important for early stages of anterior patterning, it is not clear whether these antagonists function later during anterior organogenesis, including formation of the primary mouth.
In order to define pathways that regulate primary mouth formation, we used expression microarrays to identify genes with enriched expression in the primary mouth anlage. Through this screen, we isolated two Wnt antagonists, the sFRPs Frzb-1 and Crescent, as potential molecular regulators of primary mouth development. We show that sFRP function is crucial for primary mouth formation, and to locally promote dissolution of the basement membrane. These data are the first to connect Wnt signaling and basement membrane integrity during primary mouth development.

MATERIALS AND METHODS

Embryos

Xenopus laevis embryos were obtained and cultured using standard methods (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

Microarray analysis

Tissue was collected from three regions of the embryo at stage 25-26. (1) The presumptive primary mouth (PMo), including endoderm and ectoderm, dorsal to the cement gland, ventral to the telencephalon, and central to the hatching gland (Fig. 1A, PMo, red). (2) The anterodorsal (neural) region (AD) (Fig. 1A, dark gray), comprising the central telencephalon, excluding the eyes. (3) The ventral region including the cement gland (V+CG) (Fig. 1A, light gray). One hundred dissections were performed for each of three biological replicates and stored in Trizol (Invitrogen) at −80°C. Total RNA was isolated using Trizol extraction followed by a lithium chloride solution (Ambion) precipitation. Total RNA (100 ng) was used to prepare biotinylated cRNA using the Two Cycle cDNA Synthesis Kit (Affymetrix), according to the manufacturer’s protocol. Briefly, SuperScript II-directed reverse transcription used a T7-OLigo(dt) Promoter Primer to create first strand cDNA. RNase H-mediated second strand cDNA synthesis was followed by MEGAscript T7 (Ambion) directed in vitro transcription, which generated unmodified cRNA. cRNA was used as a template for a second round of cDNA synthesis, followed by a second in vitro transcription reaction, which incorporated a biotinylated nucleotide analog during cRNA amplification. Samples were prepared for hybridization using 15 µg biotinylated cRNA in a 1× hybridization cocktail. Additional hybridization cocktail components were provided in the Affymetrix GeneChip Hybridization Wash and Stain Kit. GeneChip arrays (Xenopus) were hybridized in a GeneChip Hybridization Oven at 45°C for 16 hours at 60 rotations per minute. Washing was done using a GeneChip Fluidics Station 450 according to the manufacturer’s instructions, using the buffers provided in the Affymetrix GeneChip Hybridization, Wash and Stain Kit. Arrays were scanned on a GeneChip Scanner 3000 and images were extracted and analyzed using GeneChip Operating Software v1.4. The generated CHP and CEL files have been deposited in the Gene Expression Omnibus (NCBI, GSE13377). Expression level differences and statistical significance were calculated using Excel, and both were considered in identifying candidate genes.

qRT-PCR

cDNAs were prepared using the Sensiscript Kit (Qiagen). qRT-PCR was performed using ABI Prism 7000 or 7900 (ABI). Fluorescence detection chemistry used the SYBR green dye master mix (Roche). Primers sequences are available on request. The relative amount of product was calculated using ΔCT and products normalized to ef-1-alpha.

In situ hybridization

cDNAs used to transcribe in situ hybridization probes were frzb-1 [GA# U68059 (Wang et al., 1997a; Wang et al., 1997b), crescent (also called frzb-2) (Bradley et al., 2000), XCG (Sive et al., 1989) and nrp-1 [GA#, BC084198 (Richter et al., 1990)]. In situ hybridization was performed as described by Sive et al. (Sive et al., 2000), omitting the proteasine K treatment. Double-staining analysis was performed as described previously (Wiellette and Sive, 2003).

Morpholinos and RNA injections

Antisense morpholinos were purchased from Gene Tools. To design frzb-1 morpholinos, we sequenced the start site of frzb-1 in X. laevis (primer sequences are available on request). Morpholinos included two frzb-1 start site morpholinos (morpholino sequences are available on request), a previously published splice blocking crescent morpholino (Shibata et al., 2005) and a standard control morpholino. For rescue assays, 10 mutations were introduced into the start site of the frzb-1 cDNA, using a site-directed mutagenesis kit (Stratagene). One nanogram per embryo of the mutated frzb-1 mRNA produced an overexpression phenotype comparable to the wild-type construct, as described (Wang et al., 1997a).

F0 transgenics and heat shock

For heat-shock inducible transgenics, we used a construct [pSGH2; IScel-GFP-HSE plasmid (Bajoghli et al., 2004)] with a multimerized heat-shock element (HSE) promoter, a multiple cloning site and IScel sites. The genes dkk-1 [GA# AF030434 (Glinka et al., 1998)], wnt-8b [GA# U22173 (Christian et al., 1991)], chordin [GA#, L35764 (Sasai et al., 1994)], frzb-1 and crescent (see above) were inserted into the multiple cloning site (GFP::HSE::gene of interest). The meganuclease method was used to create F0 transgenics as previously described (Pan et al., 2006). Heat shock was achieved by moving embryos from 15°C to 35-37°C at the appropriate stage, and maintaining embryos at this temperature for 2 hours. The construct minus inserted genes served as a control.

Laminin immunohistochemistry and auto-fluorescent rendering

Specimens were embedded in 4% low-melt agarose (SeaPlaque GTG, Cambrex) and sectioned with a 1000 Series Vibratome at 100 µm. Immunohistochemistry was performed as described (Dickinson and Sive, 2006) using a polyclonal anti-laminin antibody (Sigma, L-9393) diluted 1:150, a goat anti-rabbit Alexa Fluor-conjugated antibody (Molecular Probes) diluted 1:500 and a 0.1% propidium iodide (Sigma) counterstain. Embryos were prepared for optical auto-fluorescent sections as described (Dickinson and Sive, 2006).

Extractions and transplants

Extractions and transplants were performed in 1.0×MBS (Modified Barth’s Solution) in plasticine-lined Petri dishes, using 1-mm diameter capillary tubes pulled to a fine point. Glass bridges were used to hold tissue together while healing. Two types of transplants were performed. (1) ‘Face transplants’, which involve dissecting the ectodermal and endodermal layers of the cement gland and the region just dorsal, which fate maps to the presumptive primary mouth, and transplanting this entire region to a donor embryo in which the same tissue has been removed. (2) ‘Ectoderm transplants’, which involve removing the ectoderm, both superficial and deep, from the presumptive primary mouth and transplanting to a donor embryo in which the same tissue has been removed.

JNK inhibition

Embryos were bathed in a 20 µM solution of SP600125 (Sigma) with 1% DMSO in 0.1% MBS from stage 17 to stage 40 in culture dishes at room temperature.

β-Catenin protein levels and activity

β-Catenin protein levels were analyzed by western blotting. Tissue lysates were prepared from pooled samples of 10 embryos. Primary antibodies were anti-β-catenin (Zymed, 71-2700) and anti-β-actin (Sigma, A5441), both diluted 1:1000. Secondary antibodies were HRP-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling), and detection was by chemiluminescence using LuminoGLO Reagent and Peroxide (Cell Signaling). The bands were quantified by densitometry using Photoshop (Adobe).

β-catenin activity was measured using the TOPFLASH system. Briefly, embryos were injected with 10 pg of the TOPFLASH construct (Clevers and van der Wetering, 1997), 10 pg pRL-SV40/TK as a reference plasmid, and 1 ng of frzb-1 RNA. Luciferase assays were performed using the Dual-Luciferase Assay Kit (Promega). The primary mouth and surrounding area were dissected at stage 20-22, suspended in 50 µl of 1× Passive Lysis Buffer and stored at −80°C. Luciferase was detected on a luminometer ( Molecular
Scoring primary mouth formation

We evaluated primary mouth development by examining the perforated opening or by examining the stomodeum, an invagination that forms prior to the opening. Perturbing primary mouth development, in the most severe cases, results in neither a stomodeum nor a primary mouth. In less severe cases, a stomodeum forms with no opening. The stomodeum can also vary in size, which indicates how large the mouth opening will be.

RESULTS
The sFRPs frzb-1 and crescent are expressed in the primary mouth anlage

In order to define genes required for primary mouth formation, we identified those that are differentially expressed in the future primary mouth, relative to surrounding regions. We used an unbiased expression microarray approach and microdissected tissue at stage 24-26, as described in the Materials and methods. Thirty genes were identified, whose expression was highly enriched in the primary mouth (Fig. 1A; see also Table S1 in the supplementary material), and validated by qRT-PCR and in situ hybridization (see Fig. S1A,B in the supplementary material). One of these genes was frzb-1, which displayed 18-fold-enriched expression in the primary mouth relative to the other regions. Relative to other regions used for comparison, the level of frzb-1 expression in the primary mouth is 88% as measured by microarray, and 71% by qRT-PCR (Fig. 1B).

In situ hybridization showed that frzb-1 was expressed in the prechordal plate, presumptive anterior pituitary and primary mouth, as well as in the endoderm lying beneath the cement gland, at neurula stages (stage 17-20; Fig. 2A,A’). At early tailbud stages (stage 24 and 26), expression appeared strong in the deep endoderm of the future anterior pituitary and primary mouth, while fainter expression persisted in underlying endoderm (Fig. 2B-C’). At stage 28, frzb-1 mRNA was present in the developing anterior pituitary (Fig. 2D,D’). By tadpole, stage 32, expression appeared to be absent in the head (data not shown). Increased probe concentrations and longer incubation times revealed a low level of frzb-1 expression in the brain (data not shown), consistent with expression values from the microarray analysis.

Frzb-1 belongs to a large class of Wnt antagonists, the secreted frizzled related proteins (sFRPs). Because our microarray screen consisted of only one time point, and primary mouth formation takes place over many hours, we examined the expression of other sFRPs, including sFRP1, sFRP2, sFRP5 and crescent (see Fig. S2A-L in the supplementary material), to detect redundantly expressed genes. Only crescent was expressed in or near the future primary mouth (Fig. 2E-H’). At stage 17, crescent was expressed in a broader domain than was frzb-1, including the prechordal plate, the presumptive anterior pituitary and primary mouth, and in tissue lying beneath the cement gland (Fig. 2E,E’). At stages 20-24, crescent expression was primarily confined to the prechordal plate, posterior and anterior to the presumptive primary mouth and the anterior pituitary (Fig. 2F,F’). By stage 26, crescent mRNA was no longer detected in the head region (Fig. 2E-H’), although microarray analysis showed very low levels of crescent expression in the future primary mouth at stage 26 (data not shown).

These data indicate that during early neurula stages, crescent and frzb-1 have overlapping expression patterns in the presumptive primary mouth, consistent with a role in the development of this organ.

Frzb-1 and Crescent are required for primary mouth formation

We investigated whether Frzb-1/Crescent function was necessary for primary mouth formation, and whether the function of these sFRPs was redundant, using antisense morpholinos against frzb-1 and/or crescent, as detailed in the Materials and methods. Injection of frzb-1 morpholinos alone resulted in embryos with a small stomodeum (invagination, dotted yellow) but no primary mouth opening, whereas the same amount of control morpholino had no effect on the primary mouth opening (dotted black; Fig. 3A, parts a,b). The injection of crescent morpholinos resulted in a smaller primary mouth opening relative to controls (Fig. 3A, part c). However, co-injection of frzb-1 and crescent morpholinos resulted in morphants with neither stomodeum nor primary mouth opening (Fig. 3A, parts d). The specificity of the phenotype was confirmed by rescue with low levels (200 pg) of mutated frzb-1 mRNA that does not hybridize to the morpholino (see Materials and methods). When injected into control embryos, this amount of frzb-1 mRNA did not alter primary mouth morphology (3A, parts e,f). Ninety-three percent of embryos injected with frzb-1 and crescent morpholinos together with the control GFP mRNA had neither a stomodeum nor a primary mouth opening (Fig. 3A, part g). This phenotype was rescued in 86% of morphant embryos injected with frzb-1 mRNA (Fig. 3A, part h). Although these embryos were not completely normal, possibly because of the effects of crescent loss of function (Shibata et al., 2005), the primary mouth opening was relatively normal in size. crescent mRNA alone was not able to rescue the primary mouth defect (data not shown), and gave a cyclopic phenotype that resulted from effects on prechordal plate migration (Pera and De Robertis, 2000).

We did not observe abnormal levels of cell death or proliferation in the primary mouth region of morphants at stage 23-24 (see Fig. S3B, parts a-d in the supplementary material). Importantly, despite the absence of the primary mouth at stage 40, this region was correctly specified at stage 23-24, as indicated by the expression of two markers specifically expressed in the primary mouth anlage, pitx3 and wgl-2 (Fig. 3B, parts a-d). Furthermore, the morphology of the morphants appeared to be relatively normal at stage 23-24, with structures around the future primary mouth present, including the eyes and cement gland (see Fig. S3A, part a in the supplementary material). At later times, frzb-1/crescent morphants show a phenotype consistent with a
function in primary axis formation (Bradley et al., 2000; Pera and De Robertis, 2000) (see Fig. S3A, parts b-h in the supplementary material). In agreement with a requirement for Frzb-1/Crescent, removal of the frzb-1 expression domain at early tailbud by extirpation resulted in neither a stomodeum nor a primary mouth (Fig. S4 in the supplementary material).

These data show that expression of both frzb-1 and crescent is required for primary mouth formation, with frzb-1 loss of function giving a stronger phenotype than does crescent loss of function. The function of these genes is required after initial primary mouth specification.

A specific requirement for Frzb-1 and Crescent in the primary mouth region during tailbud stages

Because frzb-1/crescent morphants showed a whole embryo phenotype, we investigated whether gene function is required in the forming primary mouth, or whether the primary mouth phenotype is secondary to earlier defects. To answer this, we performed face transplants to localize morphant tissue specifically to the future primary mouth during early tailbud stages (stage 23-24; see Fig. 3B, part a, and 3C, part a; see also Materials and methods). In the first transplant, donor tissue originated from uninjected sibling embryos (Fig. 3B, part a). Using morphant donor tissue, 83% of embryos did not form a stomodeum or a primary mouth opening and 17% had a smaller stomodeum and no opening; controls all had normal primary mouth morphology (Fig. 3B, parts b-c’). We note that localized loss of Frzb-1/Crescent results in a smaller surrounding face, suggesting that this region may organize other aspects of face development.

In the second transplant, donor tissue originated from uninjected embryos and the recipients were embryos injected with frzb-1 and crescent or control morpholinos (Fig. 3C, part d). All of the controls and 80% of the morphant recipients had a primary mouth opening, albeit of variable size and shape (Fig. 3B, parts e-f’).

These results indicate that Frzb-1 and Crescent are necessary locally in tissue that will form the primary mouth, from the time this organ begins to develop.

Wnt overexpression and loss of Frzb-1/Crescent give similar phenotypes

Previous studies have shown that both frzb-1 and crescent can antagonize Wnt-8 (Bradley et al., 2000; Leyns et al., 1997; Schneider and Mercola, 2001; Wang et al., 1997a). However, sFRPs can also interact with other signaling pathways, such as BMP (reviewed by Bovolenta et al., 2008). In order to determine whether the Wnt signaling pathway is targeted during primary mouth formation, we investigated whether modulators of this pathway could phenocopy the effects of changing Frzb-1/Crescent expression.

First, we tested whether an increase in Wnt signaling would phenocopy the effects of frzb-1/crescent morpholinos. This would be predicted if these sFRPs targeted Wnt signaling (Fig. 4A). Because overexpression of wnt-8 during early development has profound effects on axial patterning, we restricted wnt-8 expression to neurula and tailbud stages by driving the expression of this gene in transgenic embryos under the control of a heat-shock element (HSE) promoter (GFP::HSE::wnt-8). Two periods of heat shock were administered to determine whether the effects of Wnt-8 overexpression correlated with the expression of frzb-1 and crescent in the primary mouth anlage. Maximal overexpression was expected at the end of the 2-hour heat shock. Overexpression of wnt-8, during the time of frzb-1 and crescent expression (stage 17-24), resulted in embryos with a reduced head, and neither a stomodeum nor a primary mouth opening (Fig. 4B,C). This phenotype resembled that observed with loss of Frzb-1/Crescent function in the whole embryo (see Fig. 3A-D). When wnt-8 was overexpressed later (stage 25-28), after frzb-1 and crescent expression is downregulated in the primary mouth anlage, a normal or slightly smaller primary mouth opening formed (Fig. 4D).

Because Wnt-8 has many functions in the whole embryo, we examined the effect of overexpression exclusively in the presumptive primary mouth by performing ‘ectoderm transplants’. These transplants involved a smaller region than the face transplants described (see Materials and methods) to limit the cells exposed to Wnt-8 (Fig. 4E). Expression was controlled temporally using the HSE promoter. Donor embryos were heat shocked at stage 17-24 or stage 25-28, and were transplanted into host embryos at stage 24 or stage 28, respectively. In recipient embryos, where heat shock was administered at stage 17, and Wnt-8 expressing tissue was transplanted at stage 24, neither a stomodeum nor a primary mouth opening formed (Fig. 4F,G). The timing of this experiment correlates with the time of frzb-1/crescent expression in the presumptive primary mouth. When heat shock was administered later (stage 24) and wnt-8 expressing tissue was transplanted at stage 28 (when frzb-1 and crescent are no longer expressed in the future primary mouth) a normal opening formed (Fig. 4H).

These data show that temporally and spatially restricted Wnt-8 overexpression phenocopies loss of Frzb-1/Crescent function, suggesting that these sFRPs target the Wnt pathway. Because Wnt-8 predominantly activates β-catenin-mediated signaling (Darken and Wilson, 2001), these data suggest a role for Frzb-1 and Crescent in modulating the Wnt/β-catenin pathway.
Frzb-1 and Crescent may inhibit the function of one or several Wnt proteins during primary mouth formation. Data gathered in our microarray screen indicate that the expression of wnt-8, wnt-8b, wnt-3a, wnt-2 and wnt-4 is lower in the primary mouth than in the surrounding regions (see Fig. S1 in the supplementary material). This suggests that other mechanisms may downregulate Wnt gene expression at the RNA level.

**Overexpression of Dkk-1 or Frzb-1 lead to similar phenotypes**

We extended this analysis by asking whether Dkk-1 overexpression, which inhibits Wnt signaling (Yamamoto et al., 2008), phenocopies the effects of Frzb-1 or Crescent overexpression. Using a heat-shock element (HSE) promoter, we overexpressed frzb-1 (GFP::HSE::frzb-1) during late neurula and early tailbud stages (Fig. 5A, part a) in transgenic embryos. This resulted in a very large stomodeum (Fig. 5A, part b-d’), indicating that Frzb-1 is sufficient to expand the size of the primary mouth, consistent with the mRNA overexpression effects (Pera and De Robertis, 2000). A similar, yet less profound, phenotype is observed when Crescent is overexpressed at neurula and tailbud stages (data not shown).

Overexpressing Dkk-1 under a heat-shock element promoter (GFP::HSE::dkk-1) in transgenic embryos, during neurula and tailbud stages, to prevent an early patterning phenotype (Glinka et al., 1998) mimics Frzb-1 overexpression (Fig. 5A, part c). These results further suggest that Frzb-1/Crescent function to inhibit Wnt signaling, similar to Dkk-1.

Another prediction is that Dkk-1 overexpression would rescue the frzb-1/crescent morphant phenotype. We tested this by injecting dkk-1 or GFP mRNA together with frzb-1/crescent morpholinos and performing face transplants (Fig. 5B, part a) to localize the control or morphant tissue. Ninety percent of embryos receiving donor tissue containing the morpholinos and control GFP mRNA form neither a stomodeum nor a primary mouth, and 10% form a small
Fig. 4. Temporal and spatial overexpression of wnt-8 under control of a heat-shock promoter element (HSE) and using ectoderm transplants. Frontal views are shown, assayed at stage 40 in two to three independent experiments. Open primary mouth, black dotted line; cg, cement gland; pSGH2, Sceil-GFP-HSE plasmid. Arrows indicate the primary mouth or region where it would form. Scale bars: 250 μm. (A) Schematic showing prediction that if frzb-1/Crescent inhibits Wnt signaling, increased Wnt-8 would phenocopy loss of Frzb-1/Crescent. (B) Injected with GFP::HSE followed by heat shock at either stage 17 or 25 has no effect on primary mouth formation (90%, n=50). (C) Injection of GFP::HSE::wnt-8 and heat shock administered at stage 17 results in neither a stomodeum nor a primary mouth (97%, n=65). (D) Injection of GFP::HSE::wnt-8 and heat shock administered later (stage 25-28) results in a normal primary mouth (47%) or a slightly smaller one (53%, n=32). (E) Schematic of experimental design using ectoderm transplants. (F) Control (GFP::HSE): when heat shock was administered at stage 17 and transplants performed at stage 24, recipients form a primary mouth (100%, n=12). The same is true if heat shock is administered at stage 24 and transplants are performed at stage 28 (n=10). (G) GFP::HSE::wnt-8: when heat shock was administered at stage 17 and transplants performed at stage 24, 83% of the recipients do not form a stomodeum or a primary mouth (n=12). (H) When the experiment in G was performed later, 88% formed a normal primary mouth (n=9).

Frzb-1/Crescent do not target the JNK or BMP signaling pathways
sFRPs can antagonize Wnt signaling mediated by both β-catenin and c-Jun NH2-terminal kinase [JNK; planar cell polarity (PCP)], as well as the BMP pathway (reviewed by Bovolenta et al., 2008). We therefore investigated whether perturbation of the Wnt/PCP and BMP pathways gave a similar primary mouth phenotype to that caused by Frzb-1 overexpression.

Both Frzb-1 and Crescent can inhibit Wnt/PCP signaling (Qian et al., 2007; Shibata et al., 2005) by activation of the JNK pathway. If Frzb-1 functions to inhibit Wnt/PCP signaling in the primary mouth, inhibition of JNK would phenocopy the Frzb-1 overexpression phenotype (Fig. 6A). However, treatment of late neurula stage embryos (stage 17) with a JNK chemical inhibitor, SP600125 (Han et al., 2001), led to an absence of a stomodeum and a primary mouth opening, which was opposite to the Frzb-1 gain-of-function phenotype (Fig. 6B,C). Thus, although the Wnt/PCP pathway seems to be important for primary mouth formation, Frzb-1 probably does not regulate this pathway. In addition to acting through the JNK pathway, non-canonical Wnt signaling can also use other signaling pathways, including those involving calcium and Src (van Amerongen et al., 2008). It is possible that Frzb-1 and Crescent can target these other pathways during primary mouth formation.

The sFRP Sizzled inhibits the Xolloid-like protease, which is essential for BMP regulation (Lee et al., 2006). If Frzb-1 acts similarly and inhibits Xolloid-like in the primary mouth anlage, then overexpression of the BMP inhibitor Chordin should phenocopy the Frzb-1 overexpression phenotype (Fig. 6D). However, contrary to this prediction, overexpression of Chordin under the HSE promoter (GFP::HSE::chrd) in transgenic embryos led to a very small stomodeum and no opening (Fig. 6E,F). A similar phenotype was observed by overexpression of a dominant-negative BMP receptor during neurula stages (data not shown). Therefore, although the BMP signaling pathway is likely to be important for primary mouth development, it does not appear to be a target of Frzb-1 and Crescent.

Frzb-1 is likely to be the major sFRP regulating primary mouth formation owing to the higher level and longer period of expression of the mRNA in the presumptive primary mouth. Therefore, we tested whether increased frzb-1 mRNA could decrease Wnt signaling in the primary mouth region by using β-catenin protein and activity as a readout. We found that overexpression of Frzb-1 significantly decreased β-catenin protein and activity levels in the presumptive primary mouth and flanking tissues (see Fig. S5 in the supplementary material). Furthermore, the microarray screen reveals that β-catenin, and Wnt ligands associated with the Wnt/β-catenin pathway, are expressed at lower levels in the presumptive primary mouth than in surrounding regions (see Fig. S1C in the supplementary material).

Together, these data suggest that Frzb-1 and Crescent primarily act to inhibit β-catenin-mediated Wnt signaling, rather than the Wnt/PCP or BMP pathways, during primary mouth formation.

Loss of Frzb-1 and Crescent, or increased Wnt-8 expression, results in a persistent basement membrane
One of the earliest morphological changes during primary mouth formation is loss of the basement membrane (Dickinson and Sive, 2006) between ectoderm and endoderm when frzb-1 and crescent...
are expressed. We therefore hypothesized that Frzb-1/Crescent regulate basement membrane dissolution. Consistent with this, in frzb-1/crescent morphants, expression of Laminin, a basement membrane protein, persisted in the presumptive primary mouth, whereas control embryos lacked Laminin in this region (Fig. 7A, parts a-b'). This phenotype was specific, as co-injecting a small amount of frzb-1 mRNA restored the loss of Laminin staining to 70% of the morphants (Fig. 7A, parts d,d'). This level of frzb-1 mRNA did not alter primary mouth morphology (not shown), nor normal basement membrane loss (Fig. 7A, parts c,c').

In order to address the spatial requirement for frzb-1/crescent expression during basement membrane dissolution, we localized morpholinos by performing face transplants (Fig. 7B, part a; see also Materials and methods). Transplant of frzb-1/crescent morphant tissue to uninjected recipients led to a persistence of Laminin staining in the primary mouth region, whereas control transplants showed the normal absence of Laminin (Fig. 7B, parts b-c'). These data indicate that Frzb-1/Crescent expression is specifically required in the presumptive primary mouth for basement membrane dissolution.

We further investigated whether Wnt signaling can regulate basement membrane breakdown by performing ectoderm transplants (Fig. 7C, part a; see also Materials and methods), where a small piece of presumptive primary mouth ectoderm was transplanted from donor embryos transgenic for wnt-8::HSE::GFP to uninjected recipient embryos (Fig. 8C, part a). With donor tissue overexpressing Wnt-8, Laminin immunoreactivity persisted in the primary mouth region compared with controls (Fig. 7C, parts b-c'). Thus, dissolution of the basement membrane in the presumptive primary mouth region requires the inhibition of Wnt signaling.
Specifically, we expressed membrane proteins at the level of RNA expression (Fig. 7D).

We next tested whether Wnt signaling modulates basement membrane dissolution. Increased primary mouth region at early tailbud (stage 20-22), prior to the control of the HSE promoter and isolated the presumptive basement membrane dissolution. Increased primary mouth region at early tailbud (stage 20-22), prior to the control of the HSE promoter and isolated the presumptive primary mouth region (87%, n=10). Note that similar phenotypes were seen in morphants injected without GFP mRNA. Control morpholino and frzb-1 mRNA (200 pg) results in a normal absence of Laminin in the presumptive primary mouth (n=10).

(d) Co-injection of frzb-1/crescent morpholinos and frzb-1 mRNA restores the absence of Laminin in 70% of morphants (n=10).

We examined apoptosis in the primary mouth region of embryos injected with GFP::HSE and GFP mRNA levels (Fig. 7D, part a). Conversely, increased frzb-1 results in fibronectin mRNA that is 45% of the control level, laminin-γ1 mRNA that is 48% of the control level and β1-integrin mRNA that is 112% of the control level (n=20). Temporally increased wnt-8 results in fibronectin mRNA that is 232% of the control level, laminin-γ1 mRNA that is 170% of the control level and β1-integrin mRNA that is 93% of the control levels (n=20).

Fig. 7. Laminin persists in the primary mouth region when Frzb-1/Crescent are depleted or when wnt-8 is overexpressed. Sagittal sections (anterior to the left) assayed at stage 35-37, in 2-3 independent experiments. Laminin is immunolabeled green, nuclear propidium iodide, red; cement gland is outlined by a dotted gray line. Panels denoted by primes are tracings of Laminin immunolabeling (green). Bracket indicates the presumptive primary mouth; cg, cement gland. Scale bars: 170 μm. (A) Laminin persistence in frzb-1/crescent morphants is specific. (a) Control morpholino and GFP mRNA results in a normal absence of Laminin in the presumptive primary mouth (100%, n=10). (b) In frzb-1/crescent morphants (also injected with control GFP mRNA), Laminin persists in the primary mouth region (87%, n=10). Note that similar phenotypes were seen in morphants injected without GFP mRNA. (c) Control morpholino and frzb-1 mRNA (200 pg) results in a normal absence of Laminin in the presumptive primary mouth (n=10). (d) Co-injection of frzb-1/crescent morpholinos and frzb-1 mRNA restores the absence of Laminin in 70% of morphants (n=10).

(B) Laminin persists in embryos locally depleted of frzb-1/crescent. (a) Schematic of the experimental design. (b) Recipients receiving tissue injected with control morpholino and GFP mRNA have a normal absence of Laminin (100%, n=7). (c) Eighty-nine percent of recipients receiving tissue from frzb-1/crescent morphants have persistent Laminin (n=9). (C) Temporal and spatial overexpression of Wnt-8 results in persistent Laminin. (a) Schematic of the experimental design. (b) Recipients receiving tissue injected with GFP::HSE have a normal absence of Laminin (91%, n=11). (C) Seventy-five percent of recipients receiving tissue from embryos injected with GFP::HSE::wnt-8 have persistent Laminin (n=12).

(D) Wnt signaling regulates the expression of basement membrane components. Schematic depicts experimental design. Results are an average of two independent experiments. (a) Increased frzb-1 results in fibronectin mRNA that is 45% of the control level, laminin-γ1 mRNA that is 48% of the control level and β1-integrin mRNA that is 112% of the control level (n=20). (b) Temporally increased wnt-8 results in fibronectin mRNA that is 232% of the control level, laminin-γ1 mRNA that is 170% of the control level and β1-integrin mRNA that is 93% of the control levels (n=20).

We next tested whether Wnt signaling modulates basement membrane proteins at the level of RNA expression (Fig. 7D). Specifically, we expressed frzb-1 mRNA or a wnt-8 construct under the control of the HSE promoter and isolated the presumptive primary mouth region at early tailbud (stage 20-22), prior to basement membrane dissolution. Increased frzb-1 mRNA resulted in fibronectin and laminin-γ1 mRNA levels that were approximately 50% of the control levels, with little effect on integrin-β1 mRNA levels (Fig. 7D, part a). Conversely, overexpression of Wnt-8 from mid-neurula (stage 17) to harvest, resulted in fibronectin mRNA levels that were 232% of the control level, and laminin mRNA levels that were 170% of the control level, while having little effect on integrin-β1 mRNA levels (Fig. 7D, part b). These results indicate that Frzb-1 can regulate basement membrane dissolution by downregulating laminin and fibronectin mRNA expression.

Are other steps of primary mouth morphogenesis impacted by the loss of Frzb1/Crescent function? We examined apoptosis in the frzb-1/crescent morphants at stage 34-35 and could not detect the burst of cell death normally observed (data not shown). However, we cannot rule out the possibility that cell death does occur, but at a different time in the morphants compared with the wild type. Consistent with the absence of cell death, histology revealed that the stomodeum does not thin in frzb-1/crescent morphants, as it does in normal embryos (see Fig. 7A, part b, 7B, part c). Together, these results suggest that Frzb-1/Crescent function, and possibly basement membrane dissolution, is necessary for subsequent steps in primary mouth development.
DISCUSSION

A local sFRP expression domain is required for primary mouth development

Formation of the primary mouth requires multiple signaling regions, including endoderm of the anterior foregut, lateral tissue including branchial arches, and anterior dorsal tissue that will form the telencephalon and placodes (Dickinson and Sive, 2006). The present study identifies a fourth domain, which expresses the sFRPs frzb-1 and, to a lesser extent, crescent. Removal of this domain ablates primary mouth formation, and antisense assays show that this is due to loss of Frzb-1 and Crescent expression, rather than because the domain contains the future primary mouth. In *Xenopus*, this domain expresses both frzb-1 and secreted Wnt inhibitors, including dkk-1 and wnt inhibitory factor-1 (wif-1), suggesting that there is a requirement for low Wnt function in this region (Glinka et al., 1998; Hsieh et al., 1999) (see Fig. S1 in the supplementary material). Interestingly, when frzb-1/crescent expression was inhibited locally in the future primary mouth region, the entire face was smaller than controls, suggesting that the frzb-1/crescent domain may organize other aspects of the face.

Roles of early and late Wnt signaling modulation

Wnt inhibition is an important aspect of early anterior determination (Agathon et al., 2003; De Robertis, 2006; De Robertis et al., 2000; Kemp et al., 2005; Lewis et al., 2008; Niehrs, 1999). Our data indicate that local inhibition of Wnt signaling is also crucial to primary mouth formation at the extreme anterior of the embryo. Gene expression analyses indicate that a primary anteroposterior axis is in place by the end of gastrulation (Gamse and Sive, 2001; Zaraisky et al., 1995). Primary mouth specification begins many hours later, by late neurula/early tailbud, when a discrete region can be fate mapped as the future primary mouth, when region-specific gene expression is activated, and when the basement membrane between the ectoderm and endoderm breaks down locally (Dickinson and Sive, 2006). Our data demonstrate that inhibition of Wnt signaling is required for anterior organogenesis after the primary anteroposterior pattern is formed. It is not clear whether this represents a sustained requirement for inhibition of Wnt signaling to maintain ‘anteriorness’, or a new period that involves more local inhibition of this signaling pathway.

Frzb-1/Crescent regulate β-catenin-mediated Wnt signaling

Several assays indicate that Frzb-1 and Crescent antagonize Wnt signaling in the primary mouth anlage. For example, overexpression of Dkk-1, a Wnt inhibitor, gives a similar phenotype to overexpression of Frzb-1/Crescent, and can substitute for these sFRPs in regulating primary mouth formation. Our data suggest that these sFRPs target β-catenin-mediated Wnt signaling. Thus, overexpression of Wnt-8, a known Wnt/β-catenin ligand, mimics the phenotype seen after loss of Frzb-1/Crescent, whereas Frzb-1 overexpression decreases β-catenin levels in the presumptive primary mouth (see Fig. S5 in the supplementary material). Further indication that Frzb-1/Crescent targets Wnt/β-catenin signaling in the primary mouth region comes from the examination of putative promoters for the basement membrane genes laminin and fibronectin, whose expression is inhibited by Frzb-1/Crescent. In the *X. tropicalis* genome, one TCF/LEF/β-catenin-binding site lies within 5 kb upstream of each of the fibronectin and the laminin start sites (data obtained using Transfac software; not shown). These data suggest that Wnt signaling regulates the transcription of basement membrane genes, via the modulation of β-catenin.

Frzb-1/Crescent do not appear to target either the Wnt/PCP pathway, or the BMP pathway, as neither BMP nor JNK inhibition could mimic the Frzb-1 overexpression phenotype. However, the inhibition of BMP and JNK results in a very small or absent primary mouth, suggesting that both pathways regulate other aspects of primary mouth development.

Baseline membrane dynamics and Wnt signaling

Baseline membrane dissolution is important in many developmental contexts and may be necessary for invagination, cell death and intercalation (Davidson et al., 2004; Ingber, 2006; Svoboda and O’Shea, 1987). For example, during cavitation of embryoid bodies, loss of contact with the basement membrane initiates cell death (Murray and Edgar, 2000). Baseline membrane breakdown is required for changes in cell polarity and movement during chick gastrulation (Nakaya et al., 2008). Basement membrane dissolution may promote subsequent steps in primary mouth formation; however, this possibility has not yet been addressed.

This study provides the first connection between Wnt signaling and basement membrane modulation during primary mouth development in any species. This connection has not been made extensively in any embryonic context. In organ culture of mouse lung, addition of Dkk-1 protein resulted in depressed levels of Fibronectin protein and aberrant lung branching (De Langhe et al., 2005). In addition to in *Xenopus*, sFRPs and other Wnt inhibitors are expressed in anterior domains at a time that could influence primary mouth development in zebrafish, chick and mouse (Chapman et al., 2004; Duprez et al., 1999; Hoang et al., 1998; Houart et al., 2002; Tylzanowski et al., 2004).
In cancer, basement membrane breakdown is pivotal to metastasis (Spaderna et al., 2006), and connections have been made between Wnt signaling and the expression of basement membrane components. For example, β-catenin controls the expression of laminin during tumor progression (Hlubek et al., 2001), and increased frzb-1 (sFRP3) expression correlates with decreased Fibronectin protein (Guo et al., 2008). It is not clear whether inhibition of Wnt signaling is the sole mediator of basement membrane dissolution in the primary mouth region. Other mechanisms downstream of Frzb1/Crescent, or in an independent pathway, may contribute. These could include proteolytic degradation of extracellular matrix components by metalloproteases (Page-McCaw et al., 2007), and we are presently investigating this possibility.

**Model: local repression of Wnt signaling leads to basement membrane dissolution in the primary mouth anlage**

Previous data have shown that loss of the basement membrane in the primary mouth region is a very early step in the formation of this organ (Dickinson and Sive, 2006). Our data identify a molecular mechanism that locally regulates basement membrane dissolution (Fig. 8). Specifically, the Wnt antagonists Frzb-1 and Crescent act redundantly to inhibit β-catenin activity in cells adjacent to the basement membrane in the primary mouth anlage. We suggest that the transcription of pivotal basement membrane genes, including laminin and fibronectin, is dependent on β-catenin, and therefore decreases after Wnt inhibition. After synthesis of basement membrane proteins ceases, the basement membrane breaks down. Without Wnt inhibition, the basement membrane is maintained. Loss of the basement membrane may be required for subsequent steps in primary mouth development, including invagination, cell death, intercalation and perforation. However, these putative connections have not been explored in detail. In addition to the local inhibition of Wnt signaling, other mechanisms may regulate the fine spatial control of basement membrane dissolution. We suggest that the modulation of Wnt signaling is a widespread regulator of basement membrane remodeling during development.

We thank members of the Sive lab, especially J. Gutzman, E. Graeden, A. Carmona-Fontaine, C., Acuna, G., Ellwanger, K., Niehrs, C. and Mayor, R. (2007). Neural crests are actively precluded from the anterior neural fold by a novel inhibitory mechanism dependent on Dickkopf1 secreted by the prechordal mesoderm. Dev. Biol. 309, 208-221.


