

Churchill, a Zinc Finger Transcriptional Activator, Regulates the Transition between Gastrulation and Neurulation

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Summary

Gastrulation generates mesoderm and endoderm from embryonic epiblast; soon after, the neural plate is established within the epiblast—both events require FGF signaling. We describe a zinc finger transcriptional activator, *Churchill* (*ChCh*), which acts as a switch between different roles of FGF. FGF induces *ChCh* slowly; this activates Smad-interacting-protein-1 (*Sip1*), which blocks further induction of the mesoderm markers *brachyury* and *Tbx6L* by FGF. *ChCh* is first expressed as cells stop migrating through the primitive streak, and we show that it regulates cell ingression. We propose a simple mechanism by which FGF sensitizes cells to BMP signals. These results reveal that neural induction requires cessation of mesoderm formation at the midline in addition to the decision between epidermis and neural plate.

Introduction

During gastrulation, the early embryo establishes three cell layers (ectoderm, mesoderm, and endoderm) by coordinating multiple signaling events with complex cell movements. Then, the ectoderm becomes subdivided into neural and nonneural (mainly epidermis) subregions. A large body of work has revealed that these complex processes are controlled by just a handful of signaling factors, and that the molecular pathways are conserved throughout the Animal kingdom. Among the most important signals are the fibroblast growth factors (FGFs), which are required for mesoderm formation (Kimmel and Kirschner, 1987; Paterno and Gillespie, 1989; Amaya et al., 1991; Christian et al., 1992; Cornell and Kimmel, 1994) and migration (Sun et al., 1999; Ciruna and Rossant, 2001; Yang et al., 2002), for neural induction (Launay et al., 1996; Sasai et al., 1996; Streit et al., 2000; Wilson et al., 2000) and for caudalization of the neural plate (Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol, 1999; Domingos et al., 2001). These processes occur in the ectoderm, very close to each other in time and space: how do the receiving cells decide on the appropriate response?

Previous studies have revealed that FGF signaling is required, but not sufficient for neural induction (Streit et al., 2000; Wilson et al., 2000). FGF8 induces the expression of the early preneural genes *Sox3* and *ERNI*

within 2 hr, but unless cells are exposed to other (unknown) signals, this expression is lost and cells revert to an epidermal fate. We have also found that 5 hr exposure to either organizer-derived (Hensen's node) signals or to FGF8 are required to sensitize cells to BMP antagonists, which then stabilize the expression of *Sox3* (Streit et al., 1998). It therefore became important to define the differences between cells that have or have not been exposed to an organizer for 5 hr.

To achieve this, we designed a differential screen to identify genes whose expression is regulated after 5 hr exposure to the organizer. Here, we report the isolation and functions of one of these genes, *Churchill* (*ChCh*). This encodes a zinc finger protein that acts as a transcriptional activator, yet it represses the induction of mesoderm (*Brachyury* and *Tbx6L*) by FGF, suggesting that at least one of its targets is a transcriptional repressor. We identify one putative target, *Smad-interacting-protein-1* (*Sip1*) and show that *ChCh* is required for normal expression of *Sip1* as well as for neural plate development. *ChCh* also sensitizes cells to neural-inducing signals from the organizer and regulates the cell movements of mesoderm formation. Together with the expression patterns of these components, these results suggest that *ChCh* functions as an important switch between gastrulation (mesoderm/endoderm formation) and neurulation. It also appears to act as a gate separating two different functions of FGF signaling: in mesoderm formation and in neural induction. Finally, this provides a simple explanation for why cells exposed to FGF for 5 hr become sensitive to BMP signaling.

Results

Churchill: a Zinc Finger Gene Expressed in the Developing Nervous System

Chick epiblast cells that are not destined to form neural plate require a minimum of 5 hr exposure to the organizer (Hensen's node) before they become responsive to BMP antagonists (Streit et al., 1998). To uncover the events that take place during these 5 hr, we performed a differential screen between cells that have or have not been exposed to a node graft for 5 hr (Streit et al., 2000). One of the genes isolated encodes a 112 aa protein, containing two putative C4-type zinc fingers. Within each finger, the spacing between the two CXXC motifs (23 aa and 24 aa for the N- and C-terminal fingers, respectively) is different from that of other known C4-type zinc finger proteins (Teakle and Gilmartin, 1998). We named it *Churchill* (*ChCh*; Figure 1A), by analogy to Sir Winston's famous gesture. Northern analysis of RNA from chick embryos from stage 4 (primitive streak) to stage 13 (2 day) shows a single transcript of the predicted size (Figure 1C). Database searches reveal clear homologs in human, rat (and other mammalian species), and Fugu but none of these has been studied to date. Using a combination of cDNA library screening and degenerate PCR, we obtained full-length sequences from mouse, *Xenopus*, and zebrafish. Sequence comparisons

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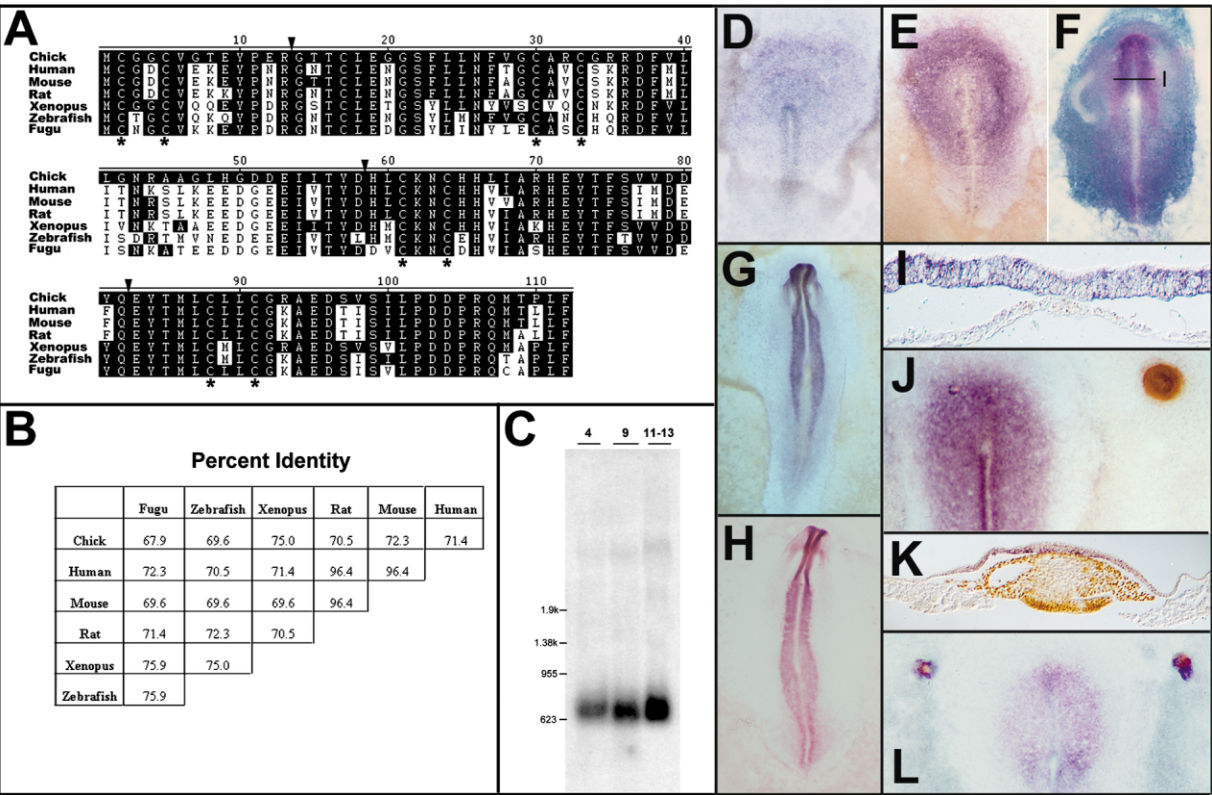


Figure 1. Sequence and Expression of *Churchill*

(A) Alignment of predicted amino acid sequences of chick (AF238863), mouse (XM_126982), human (BG500341), rat (XM_216740), *Xenopus* (AF238862), zebrafish (AL954831), and Fugu (CAAB01002134) *ChCh*.

(B) Degree of amino acid identity between *ChCh* homologs in different species.

(C) Northern blot with chick RNA from different stages, showing a single *ChCh* transcript.

(D–I) Normal expression of chick *ChCh* at stage 4 (D), 4⁺ (E), 6 (F), 7 (G), and 8 (H). A section through the prospective neural plate of the embryo in (F) is shown in (I).

(J and K) A graft of quail Hensen's node induces *ChCh* expression in the area opaca of a chick host. Quail cells (QCPN staining) are shown in brown, *ChCh* RNA in purple. K is a section through the grafted region, showing expression in the chick epiblast overlying the graft.

(L) FGF8b-soaked beads induce *ChCh* expression in the area opaca. This embryo received a graft on each side.

(Figures 1A and 1B) show that the unusual 23–24 aa spacing within the zinc fingers is conserved among all of these; each species appears to have only one gene with these characteristics.

Although the differential screen was performed in the extraembryonic area opaca, any genes involved in neural induction must be expressed in the prospective neural plate of the normal embryo. Consistent with this, in situ hybridization (Figures 1D–1I) reveals that *ChCh* is expressed specifically in the entire prospective neural plate starting at stage 4 (Figure 1D); it continues to be expressed in the neural plate throughout its formation (Figures 1E–1H) until at least stages 11–13 (data not shown).

Churchill Expression Is Regulated by FGF

Some genes (*ERNI*, *Sox3*) are induced very quickly (1–2 hr) in response to a graft of the node (Streit et al., 2000), while others require a longer time (9 hr for *Sox2*). To place *ChCh* within the cascade, we grafted a quail Hensen's node into the area opaca of a stage 3⁺ chick host and assayed for *ChCh* induction in time course: 4 hr contact are required (2 hr: 0/4; 3 hr: 1/11; 4 hr: 6/6;

5 hr: 19/20; Figures 1J and 1K). Node grafts into stage 5 and older hosts which have lost competence for neural induction (Streit et al., 1997) do not induce *ChCh* (0/7). Induction of *ERNI* and *Sox3* by a grafted node requires FGF signaling, and FGF4 or FGF8 alone will induce their expression (Streit et al., 2000). We therefore tested whether these FGFs can also induce *ChCh*. Heparin beads soaked in FGF4 (28/28) or FGF8b (20/23; Figure 1L) induce *ChCh* with the same time course as a node. By contrast, neither control beads (0/4), Chordin (0/12), Noggin (0/18), HGF/SF (0/16), nor Cerberus (0/6) induces *ChCh*. Moreover, FGF8 induces *ChCh* even in the presence of BMP4 (7/8), indicating that this induction is insensitive to BMP signaling.

These results show that the expression of *Churchill*, a gene encoding a zinc finger protein, is regulated within 4–5 hr in response to signals from the organizer and by FGF in a BMP-independent way.

Churchill Represses Mesoderm Markers, but Is a Transcriptional Activator

To determine the intracellular site of action of *ChCh*, we injected myc-tagged *ChCh* mRNA into *Xenopus* em-

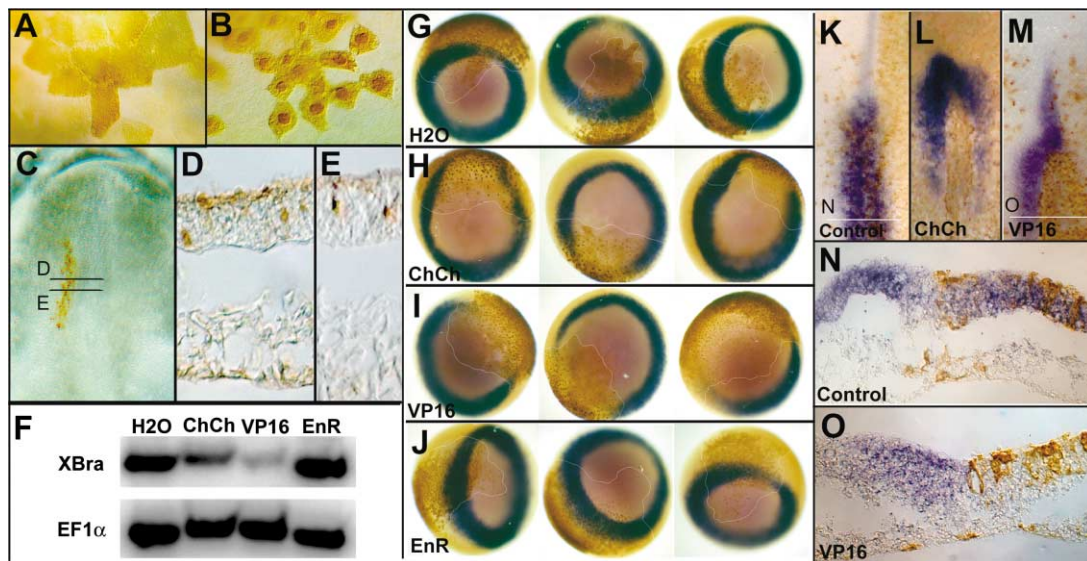


Figure 2. ChCh Is a Transcriptional Activator but Represses the Mesoderm Marker *Brachyury*

(A–E) ChCh protein is localized in the nucleus in *Xenopus* (A and B) and chick (C–E). In *Xenopus*, the protein in cytoplasmic (A) until the start of gastrulation, when it becomes nuclear (B). The chick embryo is shown in whole mount (C) and two sections through the electroporated region (D and E).

(F–J) ChCh inhibits induction of *Brachyury* by eFGF in *Xenopus*. (F) shows the results of an animal cap assay. The animal pole was injected with water (H₂O), *ChCh*, *ChChVP16*, or *ChChEnR* RNA. At the blastula stage, the animal cap was excised and treated with eFGF. Expression of *Brachyury* (*XBra*) and *EF1α* as a loading control were examined by RT-PCR. (G–H) show the effects of misexpressing *ChCh* by injection of RNA into the region fated to express *XBra*. (K–O) show the results of a similar experiment by electroporation in the chick, where brown staining reveals GFP and purple is *Brachyury* mRNA. (K–M) show details of the primitive streak and emerging head process (anterior to the top) in whole mount. (K) is a control embryo (*ires-GFP*). (N) and (O) are sections through the levels indicated in (K) and (M). In all experiments (F–O), the ChCh and the VP16-fusion construct inhibit *Brachyury* (the latter more strongly), while EnR-constructs do not.

bryos. At or before stage 8 (Figure 2A), staining is detected in the cytoplasm; however, the protein becomes strongly concentrated in the nucleus at stage 8½ after midblastula transition (Figure 2B). Likewise, chick embryos electroporated with a tagged expression construct reveal nuclear localization in the epiblast at stage 6 (Figures 2C–2E). Since the predicted protein sequence does not include a nuclear localization signal, and since the protein does not localize to the nucleus until mid-to-late blastula, it is likely that ChCh is carried to the nucleus by another protein that appears at this time.

What is the function of ChCh? As an initial approach, we injected *ChCh* mRNA into 2–4 cell stage *Xenopus* embryos and grew them to neurula stage. The gross appearance of these embryos (data not shown) was very similar to the phenotype of embryos injected with a dominant-negative FGF receptor, having severe body curvature and axial truncations (Amaya et al., 1991). This suggested that ChCh may inhibit FGF signaling. To test this more directly, we examined the effect of ChCh misexpression on the induction of the mesoderm marker *Brachyury* (*XBra*), an “immediate-early” target of FGF signaling in *Xenopus* (Smith et al., 1991), in animal cap assays. Indeed, *ChCh* RNA injection represses the induction of *XBra* by eFGF (Figure 2F).

To determine whether ChCh is a transcriptional activator or a repressor, we fused *ChCh* to the VP16-activator or the engrailed-repressor (EnR) domains. In animal cap assays, the VP16-activator construct also represses *XBra* induction by eFGF, while the EnR construct does not (Figure 2F). Likewise, when injected into the pre-

sumptive marginal zone (the future *XBra* expressing region), *ChCh* (23/34 = 68%; Figure 2H) and *ChCh-VP16* (42/45 = 95%; Figure 2I) but not control injections (0/36) or *ChCh-EnR* (0/29; Figure 2J), inhibit *XBra* expression in intact embryos. Similar results were obtained by electroporation of *ChCh* in the chick: *ChCh* and *ChChVP16* repress both *cBra* (Figures 2K–2O) and the related mesodermal gene, *Tbx6L* (Knezevic et al., 1997) (for *Brachyury*: *ChCh*-5/10 = 50%; *ChChVP16*-17/21 = 81%; *ChChEnR*-0/5; control *GFP*-2/17 = 12%; for *Tbx6L*: *ChChVP16*-4/7 = 57%; *GFP*-0/8). Analysis of several other markers in the electroporated cells revealed no change in the expression of neural (*Sox2*, *Sox3*) epidermal (*GATA2*, *GATA3*, E-cadherin) or neural plate border (*Msx1*) markers apart from downregulation of *cBra* and *Tbx6L*. In all of these experiments, the VP16 construct had a stronger effect than the wild-type protein, again suggesting that ChCh may normally act with a cofactor.

These results suggest that Churchill may be a DNA binding protein. Using an in vitro DNA binding selection assay, we determined the optimal binding sequences for ChCh protein from a pool of random oligonucleotides flanked by constant sequences. Comparison of the selected sequences yielded an optimal binding site of CGGG(GAT(GAC) (Figure 3A). This consensus is similar, but not identical to binding sites reported for other zinc finger proteins. To test this, we performed gel mobility shift and competition experiments, which confirmed that ChCh can bind specifically to this sequence (Figures 3B and 3C). Thus, although ChCh can repress the mesoderm markers *Bra* and *Tbx6L*, the above results suggest

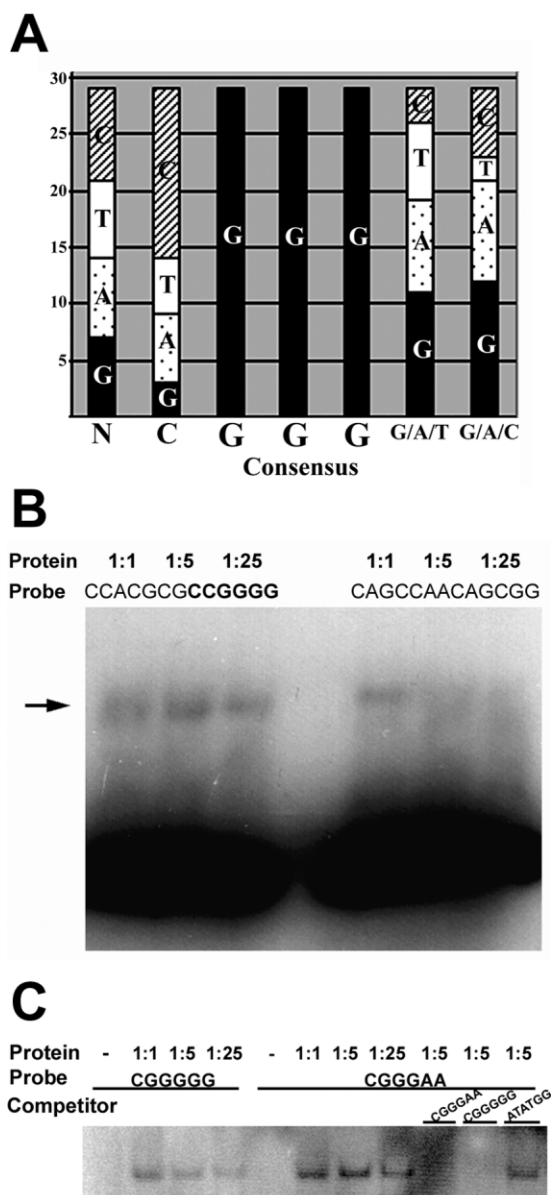


Figure 3. Identification of the Target Sequence of ChCh
(A) Consensus binding sequence revealed by the Selex assay.
(B) Gel mobility shift assay confirming the results of the selection: ChCh binds the sequence CGGGRR.
(C) Gel shift with consensus sites CGGGGG and CGGGAA (lanes 1–8). Binding is abolished by coinubation with unlabeled consensus (lanes 9–10) but not by mutated sequences (lane 11).

that it may function as a transcriptional activator, raising the possibility that other genes are upregulated in response to ChCh action (e.g., via CGGGRR DNA motifs) that can block *Bra* and *Tbx6L* expression.

Evidence for Smad-Interacting Protein (Sip1) as a Target of Churchill

The consequences of ChCh misexpression are very similar to the phenotype of *Xenopus* embryos injected with Smad-interacting-protein-1 (Sip1; also known as ZEB-2 and ZFH1B)— a direct transcriptional repressor of

XBra (Verschuere et al., 1999; Lerchner et al., 2000; Papin et al., 2002). We therefore cloned a fragment (encoding amino acids 582–936) of chick *Sip1* (Figure 4). Its expression pattern is indistinguishable from that of *ChCh* (compare Figures 1D–1I with Figures 5A–5G). We examined putative regulatory regions of human and mouse *Sip1* to see if they contain target sequences for ChCh. Thirteen such sequences, highly conserved between the two species, are found tightly clustered in a putative 480 bp enhancer immediately upstream of the translation initiation codon of *Sip1*. More extensive analysis of a 4 Kb region including exons 1 and 2 revealed that the number (120) of ChCh sites found in this region is statistically nonrandom (Monte Carlo analysis, log-L ratio 478.72, $P < 0.001$; see Supplemental Data available at <http://www.cell.com/cgi/content/full/115/5/603/DC1>).

As a more direct test that *Sip1* is a target of Churchill, we electroporated a fluorescein-labeled morpholino oligonucleotide (MO) against chick *ChCh* into the future rostral neural plate of stage 4 chick embryos. By stages 7–9, not only was *Sip1* expression absent (Figures 5J, 5K, 5N, 5O, and 5Q), but the neural plate had either not formed (Figures 5N and 5O) or was greatly diminished in thickness (Figure 5R) in the electroporated region (9/15 = 60% with this result), consistent with the effects of gain and loss of *XSip1* in *Xenopus* (Eisaki et al., 2000; Papin et al., 2002). By contrast, electroporation of either a MO against a related sequence (11 bp changes) or of an unrelated “standard control” (Gene Tools) (0/10 and 0/16 respectively; Figures 5H, 5I, 5L, 5M, and 5P) had no effect on *Sip1* expression or neural development. As additional controls, we tested the specificity of the MOs by their ability to knock down translation of chick ChCh protein. Tagged *cChCh* mRNA was coinjected into *Xenopus* embryos, either together with the chick *ChCh* MO or with the closely related MO. Staining for the tagged protein revealed that the chick *ChCh* MO specifically reduced translation of chick ChCh protein, while the related MO did not (Supplemental Figure S1 available on Cell website). In conclusion, *Sip1* is a likely target of ChCh, which can account for the finding that ChCh represses *Brachyury*.

Churchill Regulates Cell Ingression through the Primitive Streak

Brachyury is an important regulator of mesoderm formation during gastrulation (Wilson et al., 1995; Wilson and Beddington, 1997; Tada and Smith, 2000). We therefore investigated whether ChCh affects gastrulation movements through the primitive streak. Cells in the epiblast near the primitive streak (stage 3–3⁺) were electroporated with *ChCh-IRES-GFP*, *ChChVP16-IRES-GFP*, or control constructs *IRES-GFP* or *ChChMutVP-IRES-GFP* (containing mutated zinc fingers), and cell movements followed in time lapse (Figures 6A–6D and Supplemental Movies S1 and S2 available on Cell website). Cells expressing either of the two control constructs moved to the primitive streak, ingressed through it, and emerged migrating anterolaterally in the mesoderm underneath (Figure 6A). By contrast, cells expressing *ChCh* (Figure 6B) or *ChChVP16* (Figure 6C) moved normally toward the primitive streak, but failed to ingress and to move

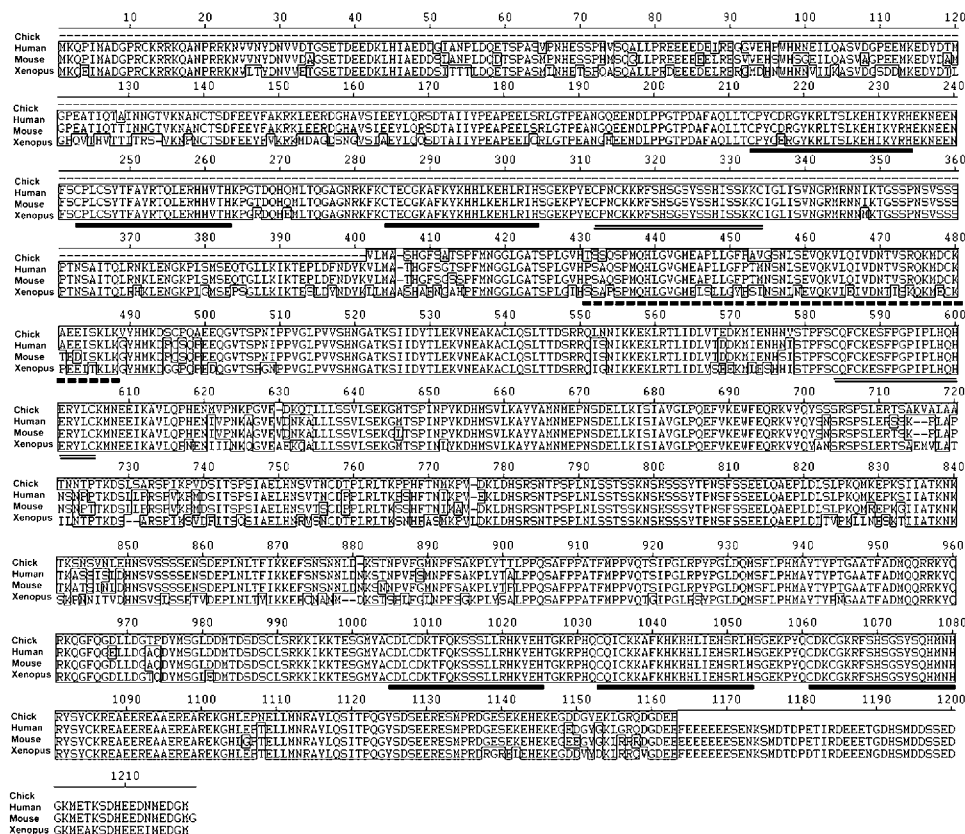


Figure 4. Sequence of chick Sip1

Alignment of amino acid sequences of chick, mouse, human, and *Xenopus* Sip1 (*Sip1* sequences in chick EST database: ChEST166d5, ChEST448o9, ChEST66f11, ChEST63p12, and ChEST320p12). C2H2 zinc fingers are thickly underlined and C3H fingers are double-underlined. The dashed line marks the Smad-interacting domain.

away from the streak to form mesoderm (see Supplemental Movies [control and Churchill] available on Cell website). This suggests that ChCh functions to control mesoderm formation at the primitive streak by regulating

cell movements. The onset of normal expression of *ChCh* at the end of stage 4 is consistent with this role, since ingression of epiblast through the anterior parts of the streak to form mesoderm and endoderm ends

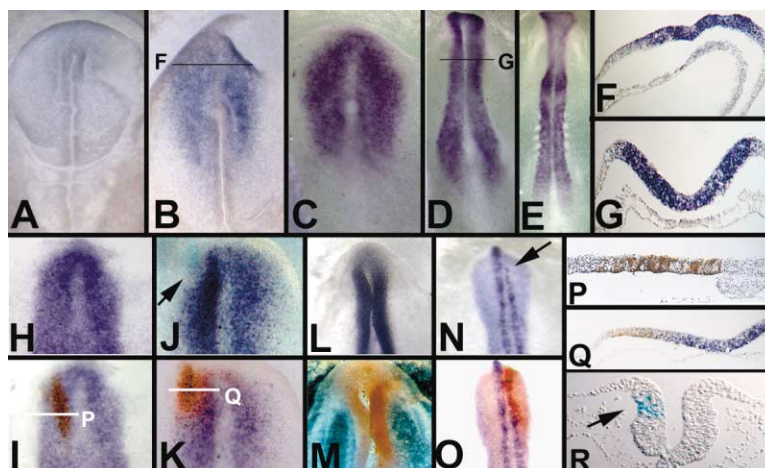


Figure 5. ChCh Is Required for Sip1 Expression

(A–G) Normal expression of *Sip1* in chick at stages 4 (A), 4⁺ (B), 5 (C), 7 (D), and 8 (E). Sections through the embryos in (B) and (D) at the levels indicated are shown in (F) and (G), respectively. (H–R) *ChCh* morpholino (J, K, N, O, and Q) inhibits *Sip1* expression while control morpholino (H, I, L, M, and P) does not. The pairs of whole mounts H:I, J:K, L:M, and N:O show the same embryo after in situ hybridization for *Sip1* (blue) and after subsequent staining for the fluorescein-tagged morpholino (brown) respectively. (P–R) are sections through MO-electroporated embryos showing downregulation of *Sip1* (Q) and a decrease in thickness of the neural plate (R, arrow) with *ChCh*-MO as compared to controls (P). In all cases, MO is visualized (antifluorescein staining) with red except in (R) where it appears turquoise. Note that in older embryos (N and O) not only is *Sip1* expression lost, but the neural fold fails to form (arrow in N).

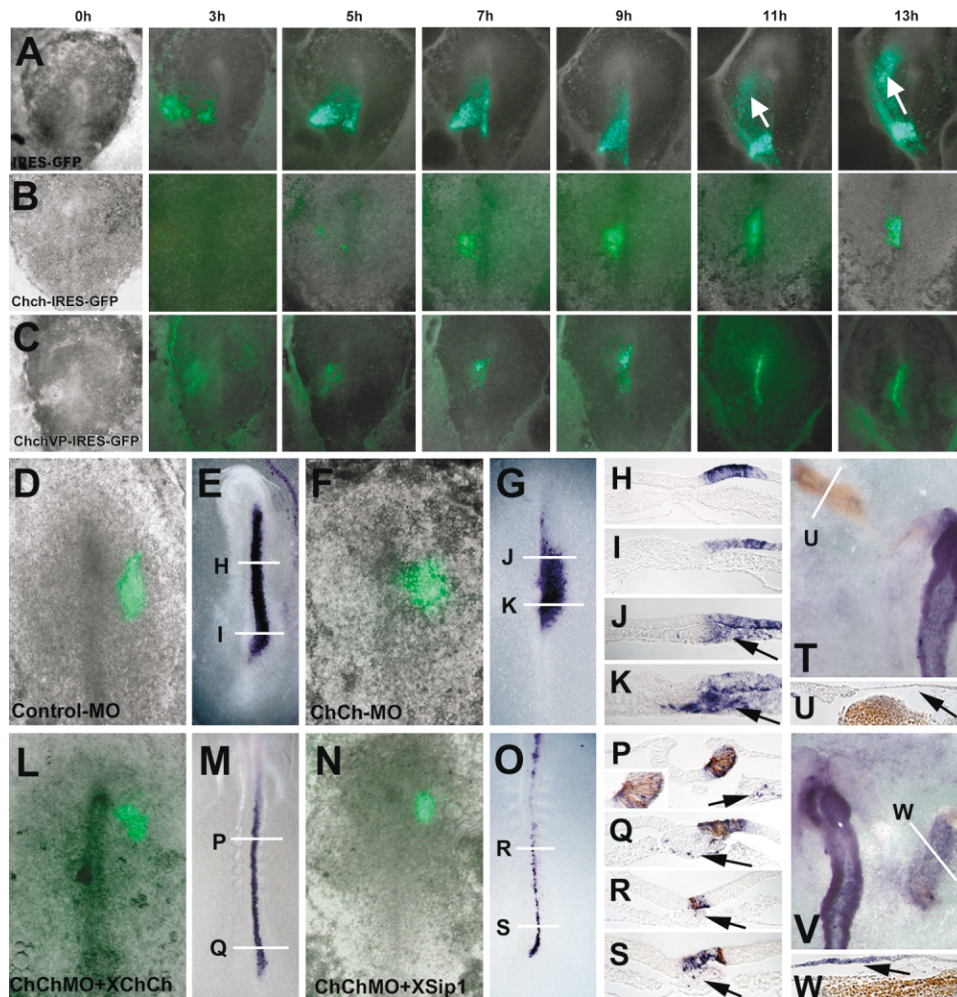


Figure 6. ChCh Regulates Ingression through the Primitive Streak and Sensitizes Cells to Neural-Inducing Signals

(A–C) ChCh prevents cell ingress. Time lapse sequences showing the movements of cells electroporated with *IRES-GFP* (control, A), *ChCh-IRES-GFP* (B), and *ChChVP16-IRES-GFP* (C). In (A), cells move normally toward and out (white arrows) of the primitive streak to form mesoderm. In (B) and (C) cells, converge to the streak but become trapped within it and do not emerge as mesoderm. (D–S) Downregulation of ChCh promotes cell ingress. Cells electroporated at stage 4⁺ with a control-MO (D at t = 0, green) do not ingress and contribute only to neural plate (E, H, I; blue). Cells with ChCh-MO (F at t = 0, green) ingress through the streak and contribute to mesoderm (G, J, K; blue, arrows). This effect can be rescued by coelectroporation of *Xenopus* ChCh (L, M, P, and Q) or of *Xenopus* Sip1 (N, O, R, and S). In (P–S), note that the blue cells (which received MO alone) still migrate into mesoderm (arrows) while cells that received the rescuing construct (brown) remain in the epiblast, where they integrate normally into the neural tube (P and inset). (T–W) A quail node graft into the area opaca at stage 5 does not induce the neural marker *Sox2* (T and U) unless the epiblast (arrows) has previously been electroporated with ChCh (V and W). Quail cells in brown, *Sox2* in purple. All images are whole mounts except (H–K), (P–S), (U), and (W), which are sections.

soon afterward (Nicolet, 1965, 1970; Rosenquist, 1966, 1971, 1972; Gallera, 1975; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Psychoyos and Stern, 1996; Joubin and Stern, 1999).

To test this hypothesis, we electroporated fluorescein-labeled control or ChCh morpholinos lateral to the anterior streak at stage 4⁺ (when these cells should no longer ingress) and followed their movements. Cells with control-MO remained in the epiblast, underwent convergent extension, and contributed exclusively to neural plate ($n = 5$; Figures 6D, 6E, 6H, and 6I), while large numbers of cells with ChCh-MO ingressed through the streak and gave rise to paraxial mesoderm ($n = 7$; Figures 6F, 6G, 6J, and 6K). To test the specificity of the

morpholino, we coelectroporated *Xenopus* ChCh together with the cChCh-MO; this leads to a mosaic of cells receiving XChCh, cChCh-MO, or both. Cells that only received cChCh-MO enter the mesoderm as in the MO experiment (blue in Figures 6L, 6M, 6P, and 6Q) while cells receiving XChCh or both (brown in Figures 6L, 6M, 6P, and 6Q) remain in the epiblast and give rise to neural plate. Likewise, coelectroporation of *Xenopus* Sip1 with cChCh-MO rescues the phenotype of the morpholino (Figures 6N, 6O, 6R, and 6S). In both cases, the rescued cells not only remain in the epiblast but also participate normally in neural tube formation (e.g., Figure 6P and inset). Furthermore, Sip1 is sufficient to overcome the thinning of the neural plate that results from

loss of ChCh function (cf. Figures 5R and 6S). These results show that ChCh function is required to stop gastrulation movements through the anterior primitive streak. Since Sip1 is sufficient to rescue the effects of ChCh loss of function, Sip1 is likely to be the major effector of ChCh in controlling cell movements through the primitive streak.

Churchill Regulates Competence to Neural-Inducing Signals

Epiblast cells surrounding Hensen's node and the anterior part of the primitive streak that escape ingression through the streak and remain on the surface of the embryo eventually contribute to the nervous system. Could ChCh play a role in sensitizing these cells to neural-inducing signals emanating from the node? Since the nonneural epiblast loses competence to respond to neural-inducing signals from the node between stages 4 and 4⁺ (Gallera and Ivanov, 1964; Dias and Schoenwolf, 1990; Storey et al., 1992), sensitization can be tested by assessing whether ChCh can maintain the responsiveness of epiblast to node signals beyond stage 4⁺. We therefore electroporated *ChCh-IRES-GFP* into one side of the epiblast of the area opaca at stage 4, incubated the embryo to stage 5 and grafted a quail node in contact with the electroporated cells. As a control, a second node was transplanted into the contralateral side. In 6/9 cases, the grafted node induced the neural marker *Sox2* in the host chick epiblast on the electroporated side (Figures 6V and 6W) but not on the control side (0/9) (Figures 6T and 6U). This suggests that ChCh can maintain the competence of the epiblast to respond to neural-inducing signals from the node.

Discussion

Gastrulation involves multiple processes: establishment of the germ layers (ectoderm, mesoderm, endoderm), orchestration of cell movements and ingression of the epiblast to form mesoderm and endoderm, and subdivision of the ectoderm into future epidermal and neural subdomains. Our results implicate Churchill in all of these processes (Figure 7).

Churchill Separates Different Functions of FGF Signaling

FGFs have multiple functions during the early stages of vertebrate development (see Introduction). These functions are incompatible with one another, yet occur very close to each other in time and space. How do the receiving cells decide how to respond? Our findings suggest a mechanism that can switch between mesoderm- and neural-inducing activities of FGF (Figure 7). Ectoderm cells exposed to FGF (perhaps in conjunction with Nodal and/or Wnt; (Bertocchini and Stern, 2002) turn on the mesodermal markers *Brachyury* and *Tbx6L*, and ingress through the primitive streak to generate mesendoderm. After 4 hr exposure to FGF, however, Churchill is induced; this in turn induces Sip1 which blocks further induction of *Brachyury* and *Tbx6L* by FGF, as well as cell ingression through the primitive streak and thus mesendoderm formation. Cells remaining in the epiblast which express Churchill can now be exposed

to further signals from the organizer; these signals will stabilize the "preneural" state established in the epiblast by earlier exposure to FGF (Stern, 2001), by maintaining or enhancing the competence of the responding epiblast to other neural-inducing signals emanating from the node. This mechanism would effectively separate the processes of gastrulation and neurulation, both of which require FGF but which are mutually incompatible.

Role of Churchill in Mesoderm Formation

A significant feature of the expression pattern of *ChCh* (and of *Sip1*) in the chick is that transcripts first appear in the epiblast adjoining the anterior primitive streak at the same stage as ingression of the epiblast to form mesendoderm ceases. Churchill misexpression does not prevent convergence of epiblast cells toward the primitive streak, but it does block the emergence of mesendoderm from it. Conversely, knockdown of ChCh after the end of gastrulation causes cells to continue to ingress. The former effect resembles those of Sip1 misexpression in the frog (Verschuere et al., 1999; Papin et al., 2002), and the phenotype of mice mutant for *brachyury* (Wilson et al., 1995), *Tbx6* (Chapman and Papaioannou, 1998), *FGF8* (Sun et al., 1999), or *FGF receptor-1* (Ciruna et al., 1997). The roles of FGF8 in gastrular ingression have recently been ascribed to chemorepulsion for the emerging mesoderm (Yang et al., 2002). However, the FGF targets *Brachyury* and *Tbx6* are also essential for cell movements through the primitive streak (Wilson et al., 1995; Chapman et al., 1996; Wilson and Beddington, 1997; Chapman and Papaioannou, 1998; Ciruna and Rossant, 2001), even though they are unlikely to be involved in repulsion, and FGF4, which does not act as a repellent (Yang et al., 2002), induces *ChCh* just as well as does FGF8.

It was recently shown (Postigo, 2003; Postigo et al., 2003) that Sip1 binds to the activated forms of both Smad1/5 (BMP targets) and Smad2/3 (TGF β /activin/nodal targets) and that it inhibits activin-dependent *Brachyury* expression and thus mesoderm formation, which requires activin/nodal-related signals in cooperation with FGFs (Kimelman et al., 1992; LaBonne and Whitman, 1994; Cornell et al., 1995). Churchill/Sip1 could therefore act in three separate ways in mesendoderm formation: changing the responses of cells to BMP signaling, blocking mesendoderm induction by TGF- β /activin/nodal + FGF, and ending ingression movements through the primitive streak.

Roles of Churchill in Neural Induction

Node grafts induce the early neural markers *ERNI*, *Sox3*, and *Otx2* in 1–2 hr in the area opaca, but if the graft is removed before about 13 hr, this expression is lost and the cells revert to a nonneural fate (Gallera, 1971). Inhibition of BMP by Chordin after 5 hr exposure to a node will stabilize the expression of *Sox3* (Streit et al., 1998). The differential screen that identified Churchill was designed to understand why 5 hr signaling from the organizer is required for cells to become sensitive to BMP (Streit et al., 2000). The conclusion that *Sip1* is a target of Churchill provides an attractive explanation for the initial observation. *Sip1* was identified in a screen designed to find partners of Smad1, a protein required for

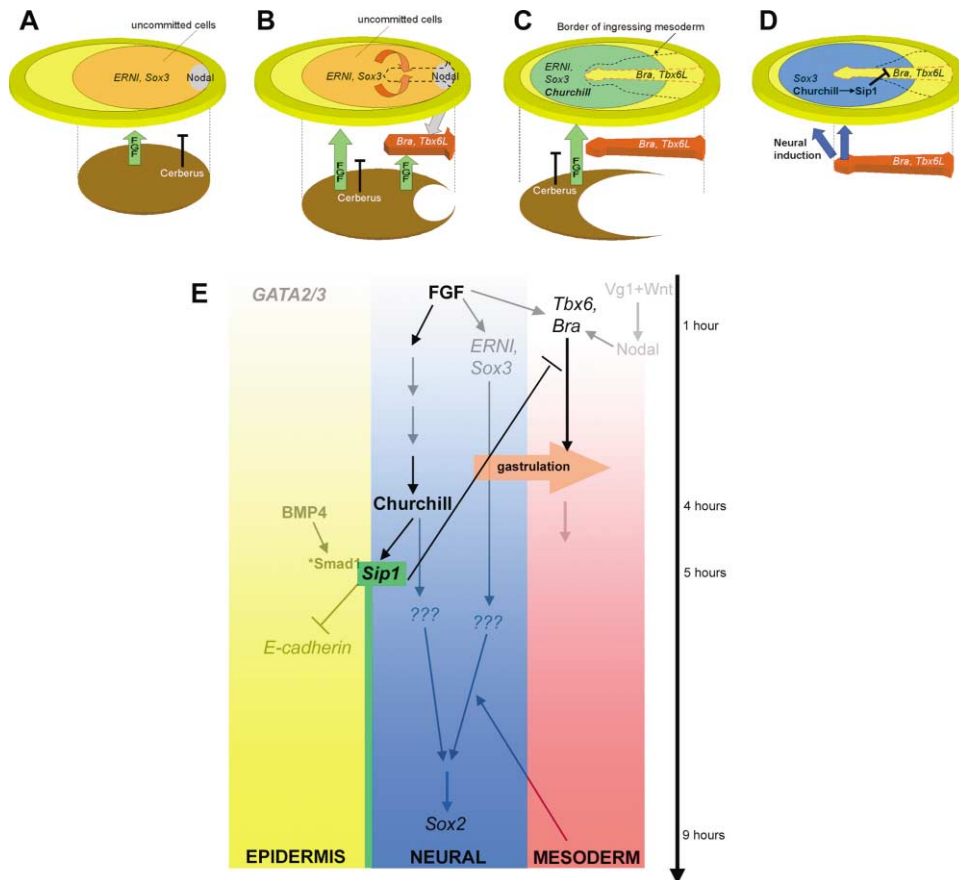


Figure 7. Model Summarizing the Regulation and Functions of ChCh during Early Development

(A–D): the embryologist's view; (E): the geneticist's view. In (A–D), embryos are shown at four stages, with their germ layers exploded. (A) At stages XI–XII, the hypoblast (brown) emits FGF8, which induces the early preneural genes *ERNI* and *Sox3* (orange) in the overlying epiblast (yellow), but the cells in this domain are still uncommitted. At this stage *Nodal* is expressed in the posterior (right) epiblast but is inhibited by Cerberus secreted by the hypoblast. (B) At stages XIII–2, the hypoblast is displaced from the posterior part of the embryo by the endoblast (white) which allows nodal signaling, in synergy with FGF, to induce *Brachyury* and *Tbx6L* and ingression (red arrows) to form the primitive streak (red). (C) At stages 3⁺–4, continued FGF signaling now induces *Churchill* in a domain of the epiblast (turquoise). The border of the epiblast territory destined to ingress to form mesoderm is shown with a dashed black line. (D) At the end of stage 4, *Churchill* induces *Sip1*, which blocks *Brachyury*, *Tbx6L*, and further ingression of epiblast into the streak. The epiblast remaining outside the streak (blue) is now sensitized to neural-inducing signals emanating from the node (blue arrows). (E) The same model shown as a genetic cascade. Interactions described in this paper are shown as black lines; those from the literature are faint. The time axis runs vertically, wherein the color gradients indicate progressive commitment to epidermis (yellow), neural (blue), and mesoderm (red). BMP/Smad/Sip1 interactions regulate the epidermis-neural plate border, while ChCh/Sip1/FGF/Bra/Tbx6 regulate the mesoderm-neural decision.

BMP4 activity (Verschuere et al., 1999). The association of Sip1 and Smad1 only takes place when Smad1 is activated by phosphorylation (Verschuere et al., 1999; Postigo, 2003; Postigo et al., 2003), raising the possibility that Sip1 acts as a sensor for the status of BMP signaling in the cell. Our findings therefore suggest a simple mechanism by which the node (or FGF) might sensitize cells to BMP antagonists after 5 hr (Streit et al., 1998): induction of ChCh by either a node or by FGF requires 4 hr, then, Sip1 is induced by ChCh, providing cells with a sensor for the status of BMP signaling (Figure 7E). The mechanism by which *Sox3* transcription is stabilized is not clear, but Sip1 may play an important role. Two possibilities worth exploring are that association of Sip1 with phospho-Smad1 modulates activating and repressing activities of Sip1 so as to regulate *Sox3* transcription,

and that the complex activates proteins that affect the turnover of *Sox3* mRNA.

Although *Churchill* is clearly not a neural-inducing factor, our results implicate it in several aspects of this process. By blocking ingression of epiblast through the primitive streak at the end of gastrulation, it allows prospective neural plate cells to remain in the ectoderm, separating the processes of gastrulation and neurulation. As a necessary regulator of Sip1, it controls neural plate and neural crest development (Eisaki et al., 2000; Cacheux et al., 2001; Wakamatsu et al., 2001; Yamada et al., 2001; Van de Putte et al., 2003). It also provides a simple and plausible mechanism by which 5 hr exposure to FGF can sensitize cells to BMP signaling as predicted by previous work (Streit et al., 1998). Finally, by maintaining or enhancing the competence of epiblast

to inducing signals from the node, it sensitizes to neural-inducing signals and may contribute to confine their effects to the prospective neural plate (where *ChCh* is expressed). Importantly, a recent study of the regulatory regions of *Sox2* revealed several binding sites for *Sip1* (Uchikawa et al., 2003), and *Sip1* misexpression in *Xenopus* induces neural markers (Papin et al., 2002; Postigo et al., 2003).

Neural induction has classically been viewed as a choice between neural and epidermal fates, mainly because it has been studied through grafts of the organizer into prospective epidermal domains. Our results suggest that in the normal embryo a major fate decision is between neural and mesodermal fates, by establishing the boundary that limits cell ingression during gastrulation. This clarifies the otherwise surprising finding that mice mutant for *Tbx6* lack paraxial mesoderm and instead make an excess of nervous system (Chapman and Papaioannou, 1998).

Churchill May Act with a Cofactor

Several observations suggest that although Churchill is an essential regulator of *Sip1*, it requires one or more cofactors. First, misexpression of *Churchill* far from its normal domain of expression (e.g., in the area *opaca*) does not induce *Sip1* even though *Sip1* contains numerous binding sites for ChCh and despite the finding from morpholino experiments that ChCh is required for normal expression of *Sip1*. Second, ectopic expression of *Churchill* in either chick or frog embryos does not induce or repress any of a large panel of markers analyzed (including the neural markers *Sox3*, *ERNI*, and *Sox2*, the epidermal markers E-cadherin, *GATA-2* and *-3*, and the neural plate border markers *Msx1* and *BMP4*). Third, in *Xenopus*, ChCh protein localizes to the nucleus only shortly before gastrulation, suggesting that nuclear localization is facilitated by a cofactor that is not present at earlier stages. Finally, fusion of ChCh with the VP16 activator domain enhances the effects of wild-type ChCh. By analogy with GATA factors (Fox et al., 1999; Morin et al., 2000; Newton et al., 2001; Patient and McGhee, 2002), one possibility is that one of the zinc fingers binds DNA while the other associates with other proteins, and that these are required for its biological activity.

Upstream of Churchill

Since a 4 hr lag for induction by either a node or by FGF is a relatively long period, this induction must be indirect. Preliminary examination of genomic sequences of human, mouse, and rat homologs of *ChCh* revealed a highly conserved 480 bp sequence immediately upstream of the coding region, which contains putative binding sites for 12 transcription factors, of which at least five (*GATA-3*, *SP1/SP5*, *C/EBP*, *CREB*, and *Oct-1*) are present in early embryos. Future experiments will establish whether the convergence of several pathways could account for the slow induction of *ChCh* by FGF.

Conclusions

Our findings strongly implicate Churchill as a key regulator of the position and timing of subdivision of the epi-

blast into neural and ingressing mesendoderm domains during gastrulation. Since Churchill is induced slowly by FGF yet inhibits some of its targets, this provides a mechanism by which the early embryo can separate, in time and space, two different functions of FGF: a role in mesendoderm formation and a function in early neural development. In addition, FGF4 and -8 can have different activities in different assays, yet both regulate ChCh expression in our system. Thus, FGFs presumably act by multiple different mechanisms in their various roles related to mesoderm induction and migration and the switch to neural induction. The ChCh/*Sip1* module reflects one part of a mechanism whose complexity we are only beginning to understand.

Experimental Procedures

Chick Embryology

Fertile hens' eggs (White Leghorn: SPAFAS, U.S.A.; Henry Stewart & Co., UK) and quails' eggs (Strickland, U.S.A.) were incubated at 38°C to the desired stages. For electroporation and grafts, embryos were explanted and cultured using a modified New culture method (Stern and Ireland, 1981). Methods for Hensen's node grafts (Storey et al., 1992), implantation of sources of secreted factors (FGF4- or FGF8b-soaked heparin beads: Streit et al., 2000; HGF-SF-soaked AG1X2 beads: Streit et al., 1997; or cells expressing Chordin, Noggin, Cerberus, or BMP4: Streit et al., 1998; in situ hybridization and whole mount immunocytochemistry: Stern, 1998) followed published protocols. Fluorescein-tagged morpholinos (*ChCh-MO*: CGT GCCACACAGCCCCGACATC; *control-ChCh*: CCTGCTGGACG CAGCCTCCGACAT and "standard control"; Gene Tools) were electroporated into chick embryos (see below). After in situ hybridization, morpholinos were revealed by antifluorescein immunostaining.

Cloning and Sequence Analysis

The differential screen for early response genes to Hensen's node was described previously (Streit et al., 2000); this identified an initial clone of chick *Churchill* containing the entire open reading frame. This was confirmed by screening two chick cDNA libraries (stages 2–4 and stages 12–15). *Xenopus ChCh* cDNA was obtained from a *Xenopus* stage 10 cDNA library (kind gift of A. Hemmati-Brivanlou). Human, rat, and mouse *ChCh* sequences were obtained from database searches, and were confirmed by PCR (for mouse and human) and genomic DNA analysis (for mouse). Zebrafish and Fugu *ChCh* sequences were obtained from database searches and confirmed by in situ hybridization (in zebrafish). For Northern analysis, 5 µg of total RNA from chick embryos at various stages were electrophoresed and the blot probed with a radiolabeled *ChCh* probe containing the entire open reading frame.

Selection

A GST-ChCh fusion protein was made by cloning the chick ChCh coding sequence into pGEX-2T. The fusion protein (attached to Glutathione-agarose beads) was used for in vitro binding sequence selection. Selection was carried out in 50 mM Tris [pH 7.5], 10 mM NaCl, 1 mM ZnCl₂, 10% glycerol, 0.0125% Triton X-100, 20 µg/ml BSA, and 2 µg/ml dl-dC. 1 µg of annealed oligonucleotides (random 12 mer with constant regions at both ends) were mixed with 100 ng of fusion protein in 500 µl selection buffer. After incubation (4°C, 1 hr), the beads were washed three times with ice-cold buffer. Beads were then resuspended in 30 µl water and heated at 95°C degrees for 2 min. The oligonucleotides were used for PCR amplification (30 s at 94°C, 30 s at 40°C, and 30 s at 72°C for 25 cycles). 10 µl of PCR product was used for the next round of selection, and this repeated five times. PCR products were cloned using pGEM-T-Easy. About 30 clones from the selection and mock selection procedures were sequenced.

Xenopus Embryology

Xenopus oocytes were fertilized in vitro and embryos staged according to Nieuwkoop and Faber (1967). To make capped mRNA,

the *Xenopus* ChCh coding sequence was cloned into p- β UT3 for wild-type ChCh, p- β UT3-EnMT for engrailed repressor, and p- β UT2-VP16 (vectors kind gift of R. Patient) for VP16 fusion proteins. Capped mRNA was made with mMessage mMachine kits (Ambion). Microinjection was performed as described (Hemmati-Brivanlou and Harland, 1989). For animal cap assays, cap explants from injected embryos were isolated and cultured in $0.5\times$ MMR. RT-PCR primers for *Xbra* were designed as described (Smith et al., 1991). The *Xbra* probe was kindly provided by C. Chang (A. H. Brivanlou lab). In situ hybridization was carried out as described (Harland, 1991).

Electroporation and Time Lapse Microscopy

pCAB-GFP (kind gift of A. Lumsden), encoding GFP driven by the chick β -actin promoter, was used to construct pCAB-IRES-GFP, used for all electroporations. An internal ribosomal entry site (IRES) was cloned into a ClaI site in the polylinker (and the 3' ClaI site subsequently destroyed). Chick embryos at stage 3–3⁺ were placed in a plastic chamber containing a Pt cathode embedded in the bottom, exposed to the saline through a 1.5 mm window. The embryo was positioned over the window (dorsal side up), and DNA applied between the dorsal side of the embryo and an adjustable anode (sharpened Pt wire). Three 50 ms pulses of 4–6 volts were given with a TSS10 pulse generator (Intracel). Embryos were then grown to the desired stages. For time lapse microscopy, embryos were photographed every 2 hr using a fluorescence-dissecting microscope; movies were made with a Princeton Instruments cooled CCD camera and Metamorph software (Universal Imaging).

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Accession Numbers

The chick and *Xenopus* sequences have been deposited in the Protein Data Bank under ID codes AF238863 and AF238862, respectively.