Current Biology, Vol. 13, 1009–1018, June 17, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S0960-9822(03)00381-6

Negative Feedback Regulation of FGF Signaling Levels by Pyst1/MKP3 in Chick Embryos

Maxwell C. Eblaghie,[1,4] J. Simon Lunn,[1,2,4] Robin J. Dickinson,[3] Andrea E. Münsterberg,[1,5] Juan-Jose Sanz-Ezquerro,[1] Elizabeth R. Farrell,[1] Joanne Mathers,[3] Stephen M. Keyse,[3,*] Kate Storey,[1,2,*] and Cheryll Tickle[1,*]

[1]Division of Cell and Developmental Biology
[2]Neural Development Group
Division of Cell and Developmental Biology
School of Life Sciences
University of Dundee
Dow Street
Dundee DD1 5EH
United Kingdom
[3]Cancer Research UK
Molecular Pharmacology Unit
Biomedical Research Centre
Level 5, Ninewells Hospital
Dundee DD1 9SY
United Kingdom

Summary

Background: The importance of endogenous antagonists in intracellular signal transduction pathways is becoming increasingly recognized. There is evidence in cultured mammalian cells that Pyst1/MKP3, a dual specificity protein phosphatase, specifically binds to and inactivates ERK1/2 mitogen-activated protein kinases (MAPKs). High-level *Pyst1/Mkp3* expression has recently been found at many sites of known FGF signaling in mouse embryos, but the significance of this association and its function are not known.

Results: We have cloned chicken *Pyst1/Mkp3* and show that high-level expression in neural plate correlates with active MAPK. We show that FGF signaling regulates *Pyst1* expression in developing neural plate and limb bud by ablating and/or transplanting tissue sources of FGFs and by applying FGF protein or a specific FGFR inhibitor (SU5402). We further show by applying a specific MAP kinase kinase inhibitor (PD184352) that *Pyst1* expression is regulated via the MAPK cascade. Overexpression of *Pyst1* in chick embryos reduces levels of activated MAPK in neural plate and alters its morphology and retards limb bud outgrowth.

Conclusions: Pyst1 is an inducible antagonist of FGF signaling in embryos and acts in a negative feedback loop to regulate the activity of MAPK. Our results demonstrate both the importance of MAPK signaling in neural induction and limb bud outgrowth and the critical role played by dual specificity MAP kinase phosphatases in regulating developmental outcomes in vertebrates.

*Correspondence: stephen.keyse@cancer.org.uk (S.M.K.); k.g.storey@dundee.ac.uk (K.S.); c.a.tickle@dundee.ac.uk (C.T.)
[4]These authors contributed equally to this work.
[5]Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom.

Introduction

We wish to understand how cell-cell signaling in developing embryos coordinates growth, differentiation, and morphogenesis. Fibroblast growth factors (FGFs) comprise a major family of signaling molecules which are used in many different developmental contexts. Studies in mice, *Xenopus*, *Drosophila*, and *C. elegans* demonstrate that FGF signaling is mediated via tyrosine kinase receptors (FGFRs) that can act through a number of transduction pathways, including the highly conserved Ras-ERK mitogen-activated protein kinase (MAPK) signaling cascade [1–4]. Here we focus on FGF signaling in neural induction and limb bud outgrowth in chick embryos, where downstream pathways that mediate these events are not known.

Recent work has revealed that FGF signaling is negatively regulated by complex intracellular systems which include Sprouty, Sef, Spred, and FRS2α [5–11]. Furthermore, expression of *Sprouty* and *Sef* is induced by activation of the MAP kinase cascade itself, demonstrating that these operate in negative feedback loops [7, 8, 12]. Sef coimmunoprecipitates with FGFR [8] while Sprouty2 prevents activation of raf [13] in some contexts (also see [14]), indicating that there may be multiple points of regulation at different levels within the FGF signaling pathway.

Biochemical assays and studies in cultured mammalian cells have identified phosphatases that specifically act on the ERK1/2 MAP kinases [15]. These include the closely related enzymes Pyst1/MKP3, Pyst2/MKPX, and Pyst3/MKP4, which constitute a distinct subfamily of dual specificity MAP kinase phosphatases (MKPs). Pyst1/MKP3 binds selectively to ERK2, which results in catalytic activation of the phosphatase [16, 17], and expression of *Pyst1* in mammalian cells specifically blocks activation and nuclear translocation of ERK2 [16, 18]. All of these observations strongly suggest that Pyst1 acts as an intracellular brake to signal transduction through the Ras/MAPK pathway in mammalian cells. However, nothing is known about the regulation of *Pyst1* expression or its relationship with MAPK signaling in vivo, and direct evidence for a physiological role for Pyst1 in the regulation of MAPK signaling during vertebrate development or in adult tissues is currently lacking.

Recently we reported that *Pyst1/MKP3* is strikingly expressed in many sites of known FGF signaling in mouse embryos [19]. This association suggests that Pyst1 is involved in the regulation of FGF signaling during vertebrate development. In order to explore the functional role of Pyst1 in the embryo, we isolated the chick homolog of Pyst1 and investigated whether expression of this gene in the developing neural plate and limb buds

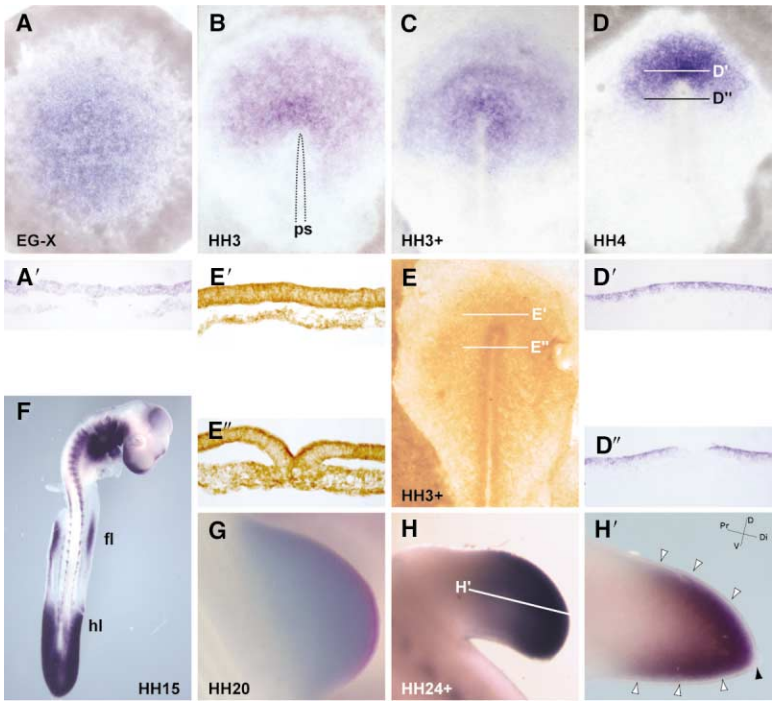

Figure 1. Expression Pattern of Chick *Pyst1*

(A) *Pyst1* expression at Eyal-Giladi stage X. (A′) Transverse section (TS) showing *Pyst1* expression in epiblast layer. (B-D) *Pyst1* expression from HH3 to HH4 is gradually restricted to the neural plate and is absent in primitive streak (ps). (D′ and D″) TS showing *Pyst1* in neural plate and not in primitive streak. (E) Activated MAPK is detected in the neural plate and primitive streak. (E′ and E″) TS. Brown staining in periphery is due to trapping of reagents by yolk and not expression in the extra-embryonic epiblast (see Supplemental Figure S1B). (F) *Pyst1* expression in the presumptive forelimb (fl) and hindlimb (hl), as well as midbrain-hindbrain isthmus region and somites at HH15. (G) Double in situ hybridization showing mesenchymal expression of *Pyst1* (blue) and ectodermal expression of *Fgf8* (magenta) in HH20 limb buds. (H and H′) Restriction of *Pyst1* to distal limb mesoderm of older limb bud at stage HH24+. (H′) TS of limb bud in (H), showing *Pyst1* expression absent in dorsal and ventral ectoderm (white arrowheads) and apical ectodermal ridge (black arrowhead). D, dorsal; Di, distal; Pr, proximal; V, ventral.

depends on FGF-mediated activation of MAPK. Finally, we tested the developmental consequences of overexpressing Pyst1 in these regions.

Results

Pyst1/MKP3 from chickens is highly similar to homologous proteins in human, mouse, rat, and frog (see Supplemental Figure S1A available with this article online at http://www.current-biology.com/content/supplemental). As in mouse [19], *Pyst1* expression in chick embryos is associated with many sites of FGF signaling. Here we focus on FGF signaling from Hensen's node, which initiates neural development [20–22], and from limb apical ectodermal ridge, which mediates bud outgrowth, accompanied by progressive formation of structures along the long axis of the limb [23]. *Pyst1* expression is first detected in the epiblast layer of pre-streak Eyal-Giladi stage X embryos (Figures 1A and 1A′) and becomes gradually restricted to neural plate in a pattern that resembles the FGF-inducible preneural gene *Sox3* [21]. By Hamilton and Hamburger stages (HH) 4, *Pyst1* is expressed in neural plate close to the node (tip of primitive streak) (Figures 1B–1D, 1D′, and 1D″), which expresses FGF2 and *Fgfs3, 4*, and *8* [21, 24–26]. Many cells responding to FGF signaling in early embryos signal via the Ras/MAPK cascade (e.g., [7, 27]) and, consistent with this, the activated form of MAPK is detected in the primitive streak and overlaps with *Pyst1* in the early neural plate (Figures 1E, 1E′, and 1E″; also see Supplemental Figure S1B).

Pyst1 is expressed in presumptive limb-forming regions in chick embryos (Figure 1F) at a time when *Fgf8* is expressed in neighboring intermediate mesoderm [28]. Then, as limb buds form with an apical ridge at the tip which expresses several different *Fgfs*, including *Fgf8* and *4* [1], *Pyst1* is expressed more or less throughout the mesoderm (Figure 1G). As limb buds elongate, *Pyst1* expression becomes confined to distal limb mesoderm beneath the apical ridge which continues to express *Fgf8* (Figures 1H and 1H′).

This tight correlation between *Pyst1* expression and tissue sources of FGF signaling led us to test whether these cell populations induce and/or maintain *Pyst1* expression in neural plate and limb bud mesoderm, respectively, and whether FGF signaling is involved.

To determine whether the node can induce *Pyst1* expression, we used a well-established neural induction assay in which we transplanted HH3 nodes into host HH3 extra-embryonic epiblast [20, 29]. In nearly all cases, ectopic *Pyst1* expression is induced within 4 hr in epiblast overlying the donor node (20/21 cases; Figures 2A and 2B). Quail donor nodes were used to confirm *Pyst1* induction in chick hosts (7/8 cases; Supplemental Figures S2A, S2A′, and S2B). In contrast, grafts of HH3 posterior primitive streak which is not normally flanked by *Pyst1*-expressing cells only occasionally elicit expression (2/9 cases; data not shown). These findings indicate that the node is a specific source of *Pyst1*-inducing signals and place *Pyst1* in the group of preneural genes expressed as an early response to neural inducing signals (see [21]).

To test whether FGF signals are sufficient to initiate *Pyst1* expression in extra-embryonic epiblast, beads soaked in FGFs 4, 8, or 7 were grafted to this region. FGF4 beads induce *Pyst1* within 1 hr (8/8 cases, 1 hr; 15/19 cases, 2 hr), and this gene is strongly expressed after 4 hr (33/35 cases; Figure 2C), while FGF8, a less potent mitogen than FGF4 [30], elicits ectopic *Pyst1* expression after 2 hr (4/8 cases; Figure 2D). In all cases ($n = 4$), control PBS beads do not induce *Pyst1* (Figure 2E). In contrast, FGF7, which has a restricted FGFR

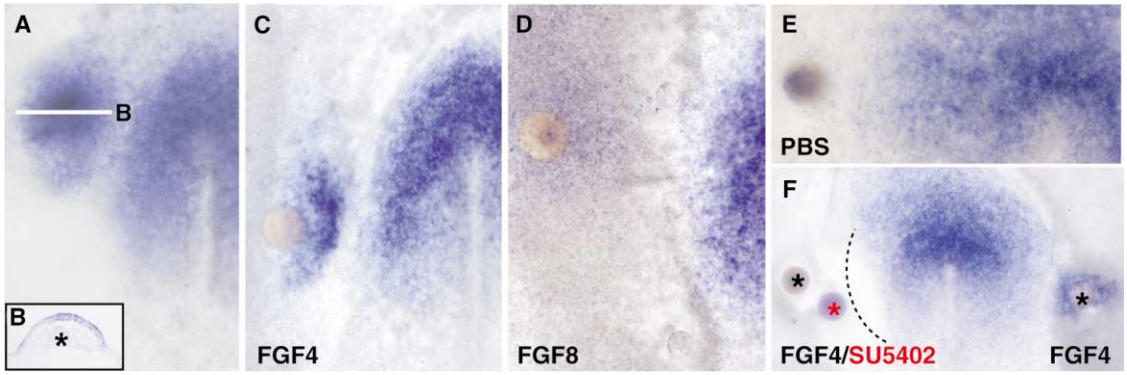

Figure 2. *Pyst1* Induction by Node Is Mimicked by FGF Signaling

Whole-mount in situ hybridization with *Pyst1* probe (purple/blue). (A and B) The node induces ectopic *Pyst1* in extra-embryonic epiblast. (A) Chick donor node (DN) strongly induces ectopic *Pyst1*. (B) TS through chick DN (asterisk) in (A). (C and D) FGF mimics ability of node to induce *Pyst1*. (C) FGF4 bead after 4 hr. (D) FGF8 bead after 2 hr. (E) PBS beads do not induce *Pyst1*. (F) Exposure to FGF4 (black asterisks) and FGFR inhibitor SU5402 (red asterisk) after 4 hr inhibits ectopic induction of *Pyst1* (compare with contralateral FGF4 bead) and reduces endogenous *Pyst1* expression in neural plate (broken black line indicates normal edge of neural plate).

binding profile [30], does not induce *Pyst1* in extra-embryonic epiblast (2 hr, n = 4; 4 hr, n = 8; data not shown). These findings indicate that FGF signaling via a subset of FGFRs can mimic the node's ability to induce *Pyst1*.

The requirement for FGF signaling for ectopic *Pyst1* expression in extra-embryonic epiblast was tested by grafting a bead soaked in the specific FGFR inhibitor SU5402 [31], alongside an FGF4 bead. In most cases, SU5402 locally reduces or abolishes *Pyst1* induction (9/12 cases, 2 hr; 5/5 cases, 4 hr; Figure 2F), while in all cases (n = 9), control DMSO beads have no effect. In five embryos, in which SU5402 beads became displaced toward host neural plate, endogenous *Pyst1* expression is also reduced (Figure 2F). This indicates that, as for other preneural genes [21], FGF signaling is required for *Pyst1* induction and for its maintenance in the neural plate.

Striking parallels can be drawn with respect to the control of *Pyst1* expression in developing limbs. Removal of the apical ridge from early limb buds reduces endogenous *Pyst1* expression within an hour (data not shown). Three hours after ridge removal, *Pyst1* expression which normally extends throughout the limb bud mesoderm is lost completely (3/4 cases, 3 hr; 4/4 cases, 24 hr; compare Figures 3A and 3B), and a small amount of cell death can be detected at the tip by Nile Blue Sulfate staining (compare Figures 3C, 3C′, 3D, and 3D′; n = 18). Furthermore, FGF4 beads placed in distal limb mesoderm immediately after ridge removal maintained *Pyst1* expression (4/4 cases, 2 hr; 6/6 cases, 10 hr; Figure 3E), while PBS beads had no effect (n = 12; Figure 3F). Exposure to beads soaked in the FGFR inhibitor SU5402 (0.5 mM) also abolishes *Pyst1* expression after 3 hr (4/4 cases; compare Figure 3G with 3G′). Under these conditions, little cell death can be detected (Figure 3G″; n = 8) and *Fgf8* is expressed normally in the apical ridge (compare Supplemental Figures S3A, S3A′, and S3A″; n = 2). With beads soaked in higher concentrations of SU5402 placed in the endogenous *Pyst1* domain at the tip of the limb bud, and/or at longer time points, loss of *Pyst1* expression is associated with increased cell death in distal mesoderm, reduced *Fgf8* expression in the apical ridge, and noticeable stunting of the limb bud (n = 34).

FGFs can also induce ectopic *Pyst1* expression in regions of the limb where *Pyst1* is no longer expressed and in interlimb regions. FGF4 or FGF8 beads placed in proximal mesoderm of older (HH24) limb buds rapidly induce *Pyst1* locally, followed by expansion of *Pyst1* expression throughout the mesoderm (n = 4, weak expression after 15 min; n = 4, stronger expression after 30 min; n = 18, very strong expression 2 hr and 3 hr; compare Figure 3H with control PBS bead, 3I). Application of FGF4 beads to the flank of HH14/15 embryos, a procedure previously shown to initiate limb development [32], induces ectopic *Pyst1* expression within 2 hr in flank lateral plate mesoderm and adjacent somites (n = 2). After 24 hr, induced *Pyst1* expression is more robust and expands into the ectopic limb buds (Figure 3J; 9/15 cases). Thus, as in the neural induction assay, expression of *Pyst1* in limb bud mesoderm and flank is an early response to application of either FGF4 or FGF8.

Signaling via FGF receptors can stimulate a number of downstream pathways including the Ras/MAPK cascade [33]. As *Pyst1* specifically dephosphorylates and inactivates MAPK [16, 34], we speculated that *Pyst1* expression might in turn be regulated via the Ras/MAPK pathway. To investigate this possibility, *Pyst1* expression was examined in embryos following exposure to the specific MAPK kinase (MKK) inhibitor PD184352 [35, 36].

In the neural induction assay, beads soaked in PD184352 or DMSO only were grafted together with a HH3 node into the extra-embryonic epiblast and *Pyst1* expression assessed after 4 hr. *Pyst1* is induced in only 2/7 cases when the node is exposed to PD184352, in comparison with control DMSO beads (Figure 4A; 7/7 cases). FGF4-induced *Pyst1* is also reduced or absent in the vicinity of PD184352 beads (2 hr, 2/2 cases; 4 hr, 13/14 cases), while contralateral FGF4 beads alone induce high-level *Pyst1* (n = 16; Figure 4B). Furthermore, in three cases where the inhibitor bead was displaced

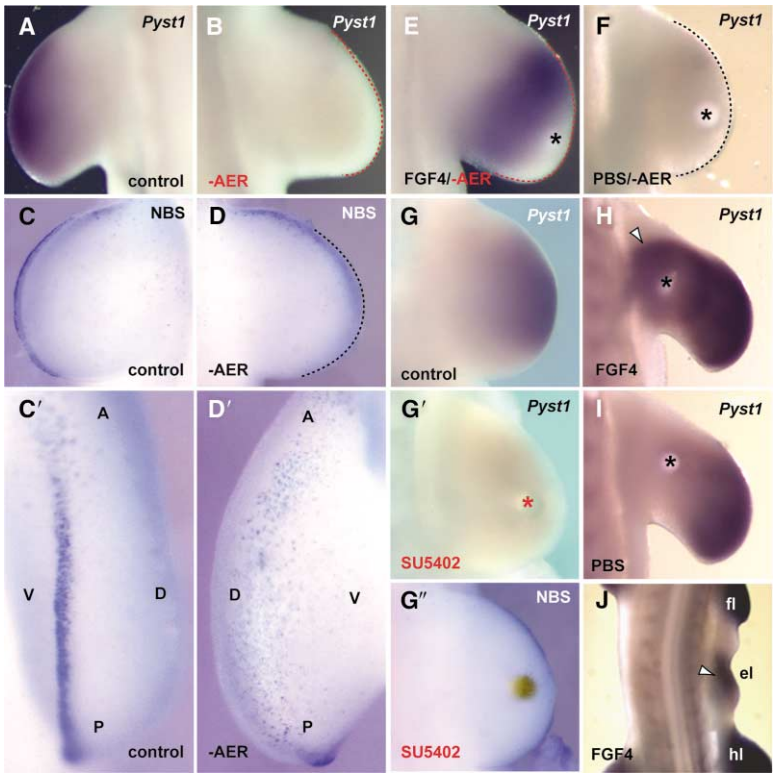

Figure 3. Endogenous *Pyst1* Expression in the Limb Is Maintained by FGF Signaling in the Apical Ridge

(A) Endogenous *Pyst1* expression in control limb bud at HH22. (B) Loss of *Pyst1* expression 24 hr following apical ectodermal ridge removal (-AER; broken red line). (C and C′) Detection of cell death by Nile blue sulfate (NBS) staining in a control untreated HH22 limb bud. A high level of apoptosis normally occurs in apical ridge cells. (D and D′) Apoptosis 6 hr following apical ridge removal (broken black line) is confined to scattered cells in limb tip. (E) Maintenance of *Pyst1* expression by an FGF4 bead (asterisk) 10 hr following ridge removal (broken red line). (F) PBS control bead (asterisk) does not maintain *Pyst1* after ridge removal (broken black line). (G, G′, and G″) Complete loss of *Pyst1* expression following treatment with SU5402 bead. (G) Endogenous *Pyst1* expression in contralateral untreated limb at HH21. (G′) Limb treated with bead soaked in 0.5 mM SU5402 bead (asterisk) for 3 hr. (G″) Same limb as in (G′) stained for cell death with NBS. (H) Ectopic *Pyst1* expression (arrowhead) 3 hr after an FGF4 bead (asterisk) was placed proximally in HH24 limb bud. (I) PBS control bead (asterisk) does not expand distal endogenous *Pyst1* expression. (J) Dorsal view. FGF4 bead implanted in flank of HH15 embryo for 24 hr. *Pyst1* expression in ectopic limb bud (arrowhead). El, ectopic limb bud; fl, wing bud; hl, leg bud.

toward the host neural plate, endogenous *Pyst1* expression is also inhibited (Figure 4B). Coimplantation of PD184352 and FGF8 beads in the limb also locally inhibits ectopic *Pyst1* expression (2 hr and 3 hr, n = 8; Figure 4C). When PD184352 beads were placed at the tip of the limb bud where *Pyst1* is expressed at very high levels, expression decreases dramatically in the posterior region, and outgrowth of the limb bud is markedly reduced leading to an indented shape (8/10 cases; Figure 4D), but expression in the anterior region is relatively unaffected. These results strongly suggest involvement of the Ras/MAPK pathway in regulation of *Pyst1* by FGFs in both neural plate and posterior limb mesoderm.

FGF can also signal through the PI3 kinase pathway (e.g., [37]). To determine whether this pathway contributes to *Pyst1* induction, we coimplanted a bead soaked in FGF8 or FGF4 with a bead soaked in a PI3K-specific inhibitor [38], LY294002, into the extra-embryonic epiblast or the proximal developing limb. LY294002 beads slightly reduce the ability of FGF4 to induce ectopic *Pyst1* in the neural induction assay (4/6 cases; Figure 4E) in comparison with control DMSO beads which do not affect *Pyst1* expression (n = 5; data not shown). Similarly, in the limb, LY294002 locally inhibits *Pyst1* induction by FGF8 at 4 hr (n = 5; Figure 4F). It should be noted that in this experiment, we applied FGF8, whereas in PD184352 inhibitor experiments in the limb, FGF4 was used. We also implanted LY294002 beads to the tip of developing limb buds or into the neural plate to look at the effects on endogenous *Pyst1* levels. However, in this experiment, no change in *Pyst1* expression is detected in the neural plate after 6 hr with LY294002 (n = 3; data not shown) or after 20 hr in the limb (Figure 4G; 17/19 cases). Thus, although signaling through PI3 kinase may be driven by application of FGF on beads, this pathway does not contribute to the regulation of endogenous *Pyst1* expression.

Although many growth factors signal through the Ras/MAPK pathway, several have recently also been shown to activate the p38/MAPK pathway (e.g., [39–42]). To determine if p38 MAPK activation participates in *Pyst1* induction by FGF, we implanted beads soaked in SB203580, a specific inhibitor of p38/MAPK, [43] into the limb bud. In contrast to the marked reduction in *Pyst1* expression when PD184352 is applied, SB203580 has no effect (compare Figure 4H with control DMSO bead in 4I; n = 10 at 24 hr). In *Drosophila*, expression of *optomotor blind (omb)*, downstream of *decapentaplegic* (*dpp*), is inhibited by SB203580 [44]. In the chick limb bud, anterior expression of *Tbx3*, an ortholog of *omb,* is downstream of *Bmp*, an ortholog of *dpp* [45], and SB203580 inhibits *Tbx3* expression (5/8 cases, 20–24 hr; control DMSO beads showed no effect, n = 2; see Supplemental Figures S4A and S4B). Thus, *Pyst1* expression is not regulated via the p38/MAPK pathway.

These findings show that inhibition of MAPK signaling reduces *Pyst1* expression and interferes with limb bud outgrowth. Pyst1 specifically inactivates MAPK, suggesting that Pyst1 may itself be a crucial component of a negative feedback loop governing levels of MAPK signaling downstream of FGF receptors in vivo. In order to test the role of *Pyst1* in embryonic development, we overexpressed *Pyst1* as an EGFP fusion protein and examined MAPK activity on a cell by cell basis in neural

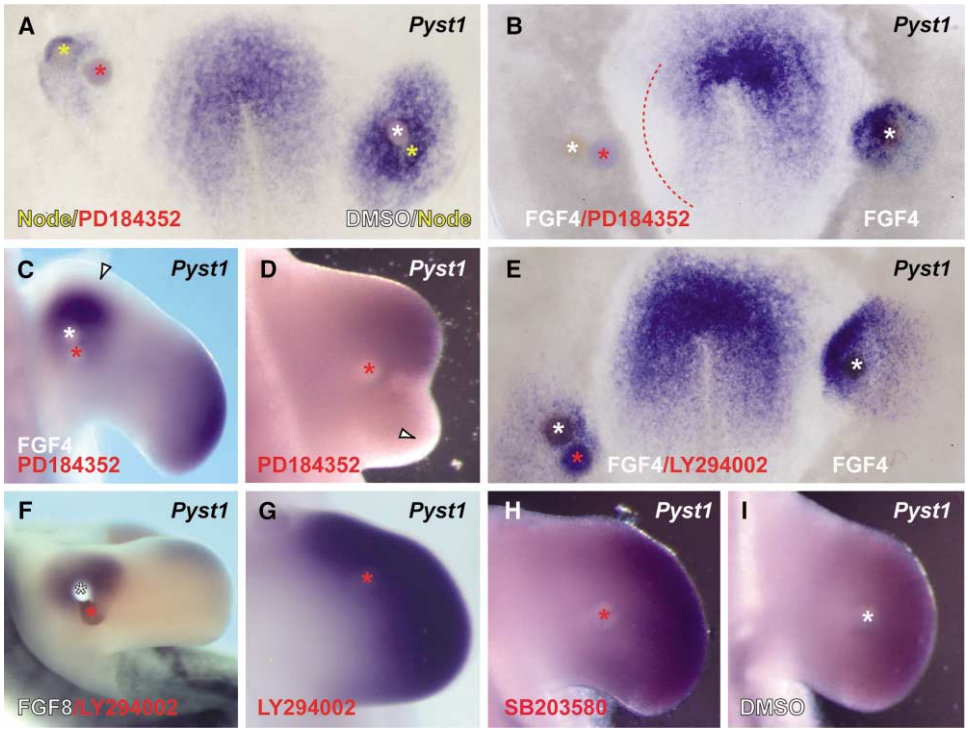

Figure 4. Inhibiting MAPK Signaling Blocks *Pyst1* Expression

(A–D) Beads soaked in MAPK kinase-specific inhibitor, PD184352. (A) Neural induction assay. Ectopic induction of *Pyst1* expression by node (yellow asterisks) is blocked by PD184352 (left; red asterisk), while DMSO control bead (right; white asterisk) has no effect. (B) Four hour exposure to FGF4 beads (white asterisks) induces *Pyst1*, but this is inhibited in the presence of PD184352 bead (left; red asterisk; 4 hr). Blocking signaling via MAPK also downregulates endogenous *Pyst1* (broken red line indicates normal edge of neural plate). (C and D) Limb buds. (C) PD184352 bead (red asterisk) locally inhibits ectopic *Pyst1* induction (arrowhead) at 2 hr when coimplanted with FGF4 bead (white asterisk) in proximal nonexpressing region of HH24 limb buds. (D) PD184352 bead (asterisk) locally inhibits endogenous *Pyst1* expression (arrowhead) in posterior limb bud and produces limb indentation.

(E–G) Beads soaked in the specific PI3 kinase inhibitor, LY294002. (E) LY294002 bead (red asterisk) mildly inhibits ectopic *Pyst1* expression when coimplanted with an FGF4 bead (left; white asterisk) in neural plate compared with Pyst1 induced by FGF4 alone (right; white asterisk). Note absence of effect on endogenous Pyst1 domain. (F) LY294002 bead (red asterisk) locally inhibits ectopic *Pyst1* induction at 3 hr when coimplanted with FGF8 bead (white asterisk) in proximal nonexpressing region of HH24 limb buds. (G) LY294002 bead (asterisk); no inhibition of endogenous *Pyst1* expression detected at 24 hr. (H and I) Beads soaked in the specific p38/MAPK pathway inhibitor, SB203580. (H) SB203580 bead (asterisk) does not inhibit *Pyst1* expression at 24 hr. (I) DMSO control bead (asterisk) has no effect on *Pyst1* expression.

plate and assessed effects on limb bud morphology and outgrowth. As a negative control, we used a vector expressing EGFP alone. We confirmed the ability of the Pyst1-EGFP fusion protein to dephosphorylate activated MAPK in COS cells (Figure 5A).

Activated MAPK is detected in the early neural plate (Figure 1E), and we first confirmed that it can be activated in this tissue downstream of FGF signaling by implanting an FGF4 bead and assaying for expression of activated, dual-phosphorylated MAPK. FGF4 increases levels of activated MAPK in the neural plate (7/8 cases), while PBS beads have no effect (n = 5; Figures 5B and 5C). The Pyst1-EGFP fusion construct was next electroporated into the neural plate and expression of activated MAPK analyzed after 6 hr. Cell by cell analysis of neural plate transfected with Pyst1-EGFP reveals a mosaic of EGFP expressing cells most of which have low levels of activated MAPK (68/75 (90%) cells in three embryos; Figures 5D–5D″). In contrast, fewer control cells (expressing EGFP only) have low levels of active MAPK (68/109 (62%) cells in three embryos; Figures 5E–5E″), and this is similar to the number of cells with low levels of active MAPK in nontransfected neural plate (475/1064 (45%) cells in two embryos). Further, in regions where clusters of Pyst1-EGFP-expressing cells are found, the neural plate is kinked (observed in 48/54 sections in five embryos; Figures 5D–5D″), suggesting that cells expressing high levels of *Pyst1* have different proliferative and/or adhesive properties to their neighbors. Thus, as is the case when *Pyst1* is expressed in cell lines [16], overexpression of *Pyst1* in embryos results in decreased levels of activated MAPK, consistent with the hypothesis that Pyst1 normally regulates MAPK activity during embryonic development.

These constructs were also electroporated into limb buds of HH21-22 embryos. 51/63 (81%) of the limbs that express Pyst1-EGFP after 12–24 hr display abnormal phenotypes ranging from mild squaring to severe truncation (Figure 6A; compare Figure 6B with 6B′ and 6B″), and the severity appears to correlate with extent of Pyst1-EGFP. When empty pEGFP-N1 vector was used, EGFP expression could be readily detected, and limb buds were normal in nearly every case and no tip indentations were produced (15/17 cases; compare Figure

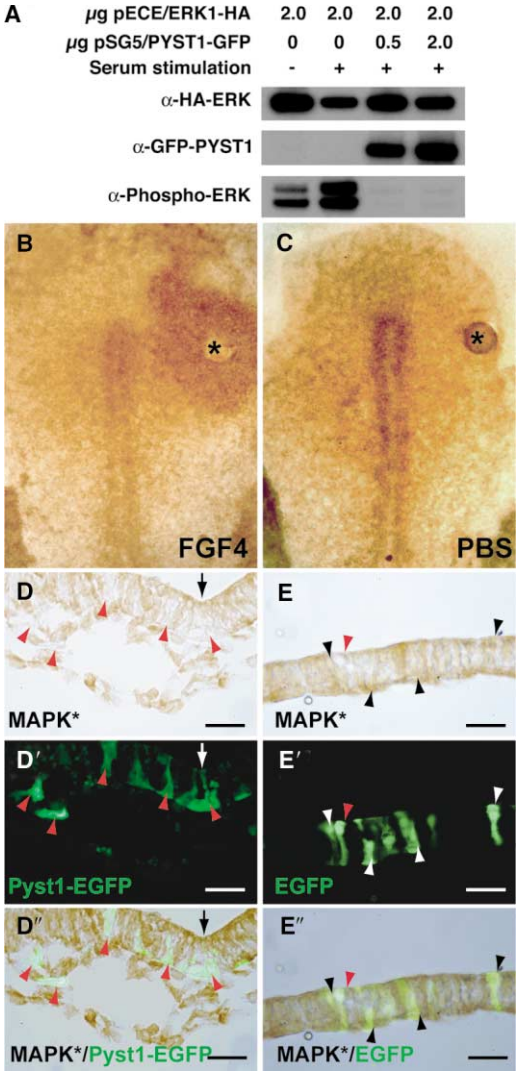

Figure 5. Overexpression of Pyst1 Decreases Levels of Activated MAPK in Neural Plate

(A) EGFP-tagged Pyst1 dephosphorylates ERK in Cos1 cells. (B) FGF4, but not (C) PBS beads (asterisks) locally increase levels of activated MAPK (detected with anti-dual-phosphorylated MAPK antibody, brown labeling) in the neural plate. (D and E) Levels of activated MAPK following electroporation. (D–D″) Cells expressing Pyst1-EGFP (green) and activated MAPK (brown) were scored in TS (red arrowheads, Pyst1-EGFP cells with low activated MAPK). Kinks in neural plate are associated with groups of Pyst1-EGFP-expressing cells (arrow). (E–E″) Control cells in the neural plate expressing EGFP have low (red arrowheads) or high (black and white arrowheads) levels of activated MAPK. Scale bars, 25 μm.

6C with 6C′ and 6C″). Thus, overexpression of *Pyst1* in limb buds can inhibit outgrowth and mimics phenotypes elicited by ridge removal or pharmacological inhibition of FGF or Ras/MAPK signaling. Eight limb buds electroporated with Pyst1-EGFP were assayed for cell death. In all cases, normal regions of programmed cell death including anterior and posterior necrotic zones and the medial opaque patch are slightly expanded (Figure 7A). An increase in cell death in the opaque patch is most common (7/8 cases), while no significant cell death is observed in any of the truncated limb buds in distal regions of the bud expressing Pyst1-EGFP (compare untreated limb in Figures 7B and 7C with Pyst1-EGFP electroporated limb in 7B′ and 7C′). Thus, *Pyst1* overexpression is not accompanied by cell death.

Discussion

These results place a dual specificity MAPK phosphatase in a physiological context in a vertebrate and show that *Pyst1* expression in neural plate and limb is regulated by FGFs. When tissues expressing FGFs are removed or beads soaked in the FGFR inhibitor, SU5402, are applied, *Pyst1* expression is abolished. We have shown in the limb that this change in *Pyst1* expression cannot be accounted for by cell death. Application of FGFs can maintain endogenous *Pyst1* expression in the limb or can induce it very rapidly in ectopic locations in both early embryos and limbs, showing that expression of *Pyst1* is an early response to FGF signaling. Furthermore, using specific inhibitors for the classical MAPK cascade, the PI3 kinase and p38 MAPK pathways, we show that maintenance of endogenous *Pyst1* expression in the neural plate and posterior limb mesoderm by FGF occurs by a direct feedback loop involving the classical pathway. Although PD184352 inhibits *Pyst1* in neural plate and posterior limb, unexpectedly, anterior limb *Pyst1* expression is relatively unaffected. It may be significant that different *Fgfs* are known to be expressed in anterior and posterior apical ridge (reviewed in [1]).

Overexpression of *Pyst1* causes limb bud truncations and defects in neural plate development, suggesting that FGF-inducible expression of *Pyst1* is critical in maintaining appropriate levels of signaling through the Ras/MAPK cascade. In the neural plate, *Pyst1* behaves like a preneural gene and its dependence on MAPK signaling downstream of node/FGF signaling suggests that this pathway regulates the onset of neural development in higher vertebrates, as observed in invertebrate (ascidian) embryos [46, 47]. Furthermore, later differentiation of the neural plate is also dependent on MAPK signaling since preliminary results show that PD184352 beads inhibit expression of the later neural marker *Sox2* (S.L. and K.S., data not shown). Thus, Pyst1 activity is likely to be a key regulator of neural induction in the chick. In the limb, truncations produced by treatment with a specific MAPK inhibitor and by overexpression of *Pyst1* demonstrate that FGF signaling mediates limb bud outgrowth via the MAPK pathway. In regions of the limb in which ectopic *Pyst1* was expressed, there was little cell death, although the normal zones of programmed cell death were expanded.

The role of *Pyst1* in determining precise levels of MAPK activity in vertebrate embryos is reminiscent of that of the *puckered* phosphatase in determining the precise levels of JNK/MAPK activity required to achieve dorsal closure during *Drosophila* embryogenesis [48]. Our results also underscore the importance of phosphatases in regulating developmental outcomes of MAP kinase signaling. Other intracellular antagonists of FGF signaling, Sprouty, Sef, and Spred, have been identified [6–10] (Figure 8). Overexpression of *Sprouty*, which in

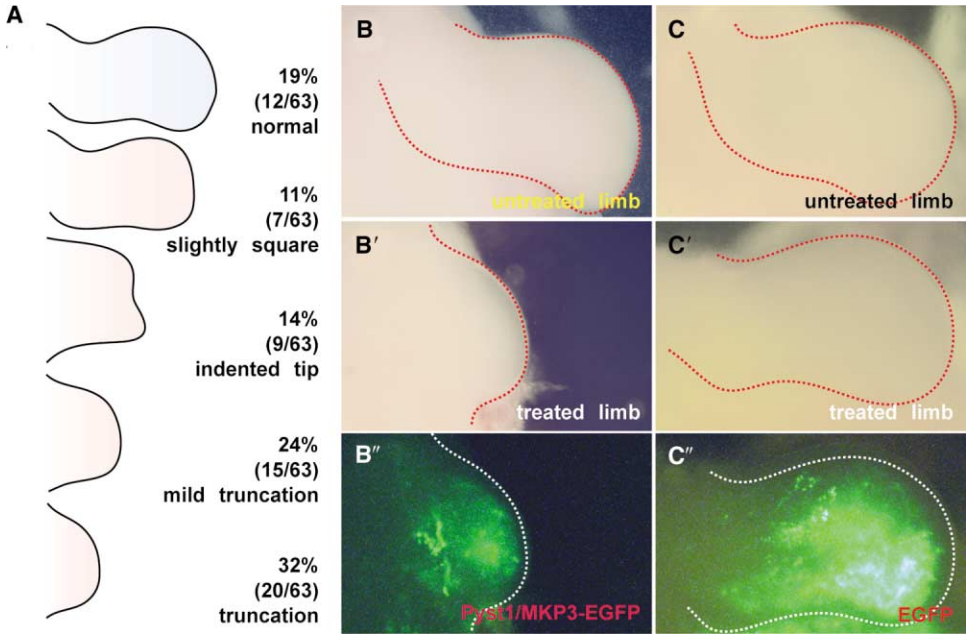

Figure 6. Overexpression of Pyst1 Inhibits Limb Bud Outgrowth

(A–C) Limb electroporations. (A) Schematic diagrams and percentages of limbs with abnormal phenotypes (n = 63). (B, B′, and B″) Overexpression of Pyst1-EGFP fusion construct. (B) Normal untreated (left) limb at HH25/26 (broken red line marks limb outline). (B′) Treated (right) limb bud 24 hr after electroporation showing resulting limb truncation (broken red line). (B″) Same limb as in (B′), viewed under UV, showing extensive EGFP expression (broken white line marks limb outline). (C, C′, andC″) Control experiment. (C) Normal morphology of untreated (left) limb bud at HH25/26 viewed under brightfield (broken red line marks limb outline; yellow color due to filter). (C′) Treated (right) limb bud, viewed under brightfield, electroporated 24 hr earlier with the pEGFP-N1 vector showing normal morphology. (C″) Same limb as in (C′), viewed under UV, showing extensive EGFP expression (broken white line marks limb outline).

some contexts prevents activation of raf (e.g., [13]) thus decreasing the level of MAPK activity, can also lead to limb bud truncations [49]. Sprouty and Sef are induced by FGF and belong to the FGF synexpression group [5] to which we can now add Pyst1. Sprouty and Sef are also known to be regulated via the MAPK cascade and as Pyst1 regulates MAPK activity directly, Pyst1 could be pivotal in controlling expression of these antagonists which act at different levels in the pathway (Figure 8). However, subsets of this FGF synexpression group operate in specific tissues [49] and, for example, chick neural plate expresses *Pyst1* but not *Sprouty2,* which is detected in the primitive streak [50]. These intracellular controls might not only integrate different intracellular transduction pathways downstream of FGF signaling [14], but also coordinate inputs from other signaling pathways.

Recent mathematical modeling of protein kinase signal transduction has provided insights into possible ways in which cells in the embryo may respond to FGF signaling. These models stress the importance of protein phosphatases in control of timing and duration of MAPK signaling and how interpretation of agonist signaling can change over time [51, 52]. They predict that the initial activation of MAPK in response to activation of tyrosine kinase receptors is "switch-like," while sustained activation and engagement of feedback loops leads to different responses. This could provide a mechanism for linking different cellular activities which occur over different time scales, for example, coordination of limb bud outgrowth and patterning. The models also suggest that cells expressing high levels of *Pyst1* can respond proportionally to agonist concentration, and this would provide a mechanism for cells to interpret an FGF gradient. Another possible outcome of negative feedback loops in a signaling network is an oscillatory output which could allow cells to measure time; indeed a clock mechanism has been proposed for proximo-distal patterning of the limb.

Experimental Procedures

PCR Cloning of Chicken Pyst1

Three overlapping ESTs (GenBank accession numbers BU235949, BU250216, and BM490647) were identified by database searching using the human *Pyst1* sequence and used to generate a contiguous sequence assembly corresponding to the full open reading frame of chicken *Pyst1*. A cDNA fragment corresponding to nucleotides 772–1149 of this cDNA was then obtained by RT-PCR using total RNA isolated from HH16-24 chick embryos as template and the following oligonucleotide primers 5′-CTGGATCCAAATCCCCATCT CGGATCACTGG-3′ and 5′-CCTCGAGTCTCGTAGATTGCAGAG AGTCC-3′. Following capture of the resulting PCR product in pCRBlunt (Invitrogen), this was excised as an EcoRI-XhoI fragment and subcloned into pBSSK+ (Stratagene).

Whole-Mount In Situ Hybridization

Chick embryos were staged according to Eyal-Giladi and Hamilton and Hamburger, and in situ hybridization was carried out using standard techniques.

Immunocytochemistry

Standard whole-mount immunocytochemistry was used to detect quail cells with QCPN (DS Hybridoma Bank) (1:5). To reveal activated

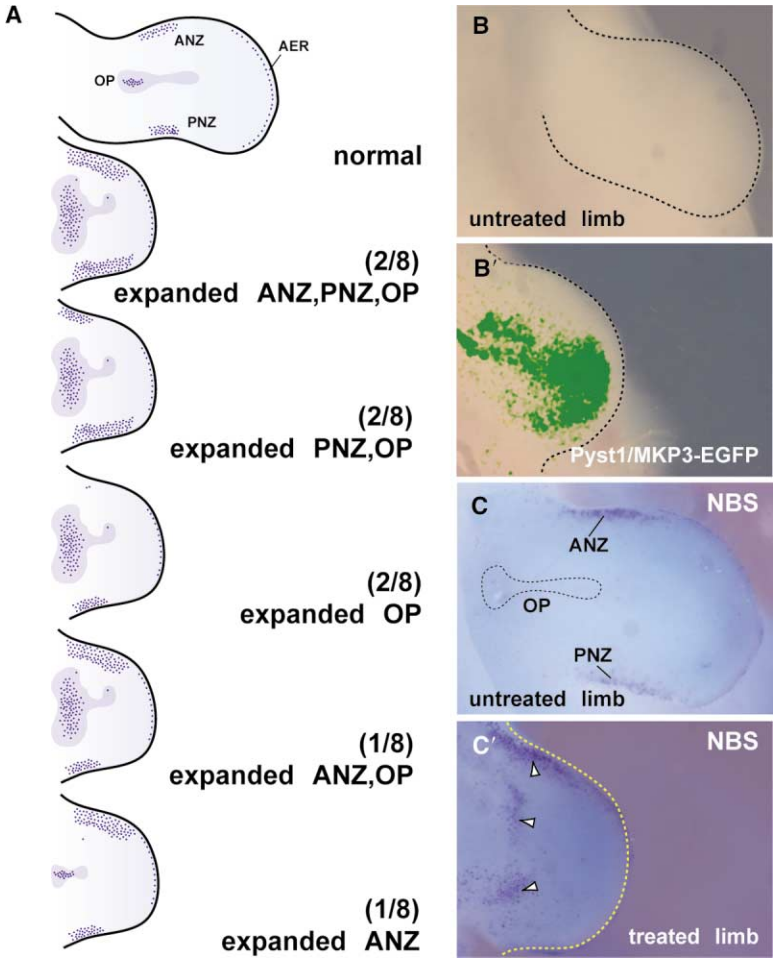

Figure 7. Cell Death following Overexpression of *Pyst1*

(A–C) Overexpression of Pyst1-EGFP fusion construct. (A) Schematic diagrams of limb buds with normal and abnormal patterns of programmed cell death (n = 8). (B and B′) Pyst1-EGFP overexpression. (B) Normal morphology of untreated (left) limb bud at HH24/25 viewed under brightfield (broken black line marking outline of limb bud). (B′) Treated (right) limb bud showing extensive EGFP expression (false green color; broken black line marking limb outline), electroporated 14 hr earlier with the Pyst1-EGFP fusion construct. (C and C′) Cell death assay. (C) Normal untreated (left) limb at HH24/25, same limb as in (B), stained with NBS showing normal regions of programmed cell death including anterior (ANZ) and posterior necrotic zones (PNZ) and medial opaque patch (OP). (C′) Treated (right) limb bud 14 hr after Pyst1-EGFP overexpression with resulting limb truncation (same limb as in [B′]; broken yellow line marking outline of limb) stained with NBS showing slight expansion of OP and ANZ (arrowheads).

MAPK, rapid fixation in 4% PFA was required, followed by dehydration to MeOH and rehydration. Embryos were incubated serially with intervening washes in activated MAPK antibody (raised in rabbit) (1:50) (Cell Signaling Technology), biotinylated anti-rabbit antibody (1:1000) (Jackson), and StreptAvidin-HRP conjugate (1:50) (Becton Dickinson) and then underwent a standard DAB reaction. Levels of active MAPK were scored on a cell by cell basis in 20 μm transverse sections.

Tissue Manipulations

Removal of the apical ridge was carried out at HH20 with sharpened tungsten needles. Nodes were grafted in contact with the extra-embryonic epiblast, see [29].

Bead Implantations

Heparin beads (Sigma H-5263) were incubated in FGF4, FGF8, or FGF7 (R&D systems) (1 mg/ml in the limb; 50 μg/ml in the early embryo) for 1 hr at RT. PBS soaked beads were used as controls. AG-1 X2 (BioRad Laboratories) formate-derivitized beads were incubated in one of the following inhibitors: 0.5–10 mM (unless stated otherwise) SU5402, 20 mM PD184352, 20 mM LY294002, and10 mM SB203580 (kind gifts from Sir Philip Cohen) for 1hr at RT. DMSO beads were used as controls. Beads were implanted in the limb and interlimb regions or in contact with the extra-embryonic epiblast.

Nile Blue Staining

Nile Blue staining on whole embryo limbs was performed essentially as described in [53]. Briefly, embryos were dissected in PBS and immediately incubated in a 1:5000 solution of Nile Blue A (Sigma) in PBS for 15–20 min at 37°C. Embryos were then rinsed in cold PBS (on ice) for 15 min at 4°C, photographed, then fixed in 4% PFA/PBS for subsequent in situ hybridization.

Plasmid Constructs

To express wild-type human Pyst1 as an NH2-terminal fusion to EGFP, the open reading frame was amplified by PCR using the primers 5′-CCCAAGCTTATGATAGATACGCTCAGACCCGTGCCCT TCG -3′ and 5′-CGCGTCGACGTAGATTGCAGAGAGTCCACCTGGT ATAC-3′ and subcloned into the mammalian expression vector pEGFP-N1 (Clontech) as a Hind III-Sal I fragment.

Cos Cell Transfection and Western Blots

Cos1 cells were cotransfected with 2 μg pECE-p44MAPK-HA (a kind gift from Dr. Anne Brunet) and increasing amounts (0, 0.5, or 2 μg) of pEGFP-N1-PYST1 using the calcium phosphate method. After 48 hr, cells were serum starved for 18 hr and either left untreated or stimulated by addition of foetal bovine serum (15% v/v final concentration) for 15 min. Cells were lysed and 5 μg of cell lysate per transfection analyzed by SDS-PAGE. Levels of HA-tagged p44MAPK, Pyst1-EGFP, and phosphorylated MAPK were determined by Western blotting using antibodies against HA (12CA5, Cancer Research UK), EGFP (sc-8334, Santa Cruz), or phosphorylated MAPK (#9101S, Cell Signaling Technologies), respectively.

In Vivo Electroporation

Pyst1-EGFP fusion DNA construct or empty pEGFP-N1 (Clontech) vector was electroporated into HH21-22 chick wing bud or into HH3+ neural plate. For limb, multiple injections of plasmid (1–3 μg/μl, 1:40 (v/v) 0.4% fast green/distilled H_2O) were made into mesenchyme. Electroporation was then carried out using two electrodes

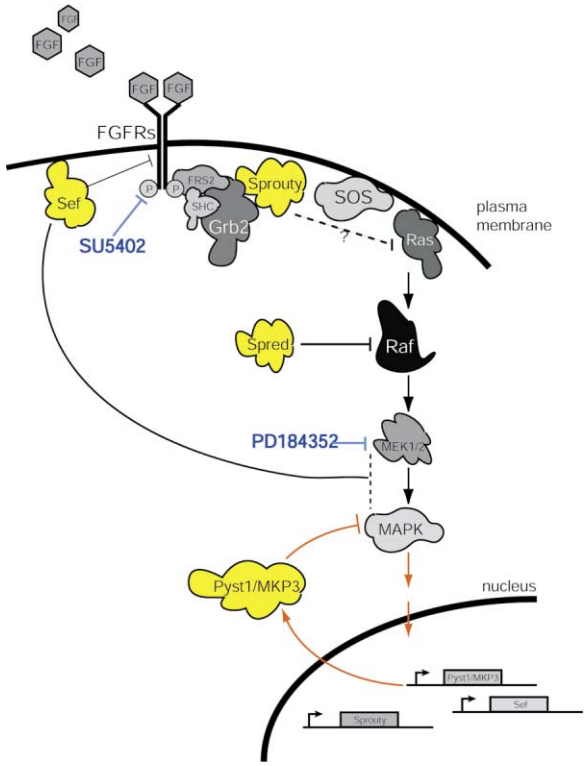

Figure 8. Inhibitory Feedback Loops Downstream of FGF Signaling

Factors regulating signal propagation via MAP kinase cascade downstream of FGF receptor activation. Previously identified inhibitory factors Sef [7–9], Spred [10], and Sprouty [6] (but see [14]) (yellow) act at different levels within FGF signaling pathway, although the biochemical basis of these inhibitory activities is not yet clear. In contrast, PYST1/MKP3 is a known specific inhibitor of MAPK signaling through dephosphorylation of the T-E-Y MAPK activation motif [16, 34]. As with Sef and Sprouty, it is expressed downstream of FGF-induced MAPK signals and thus constitutes a novel feedback loop governing activity of this pathway (red arrows). Points of action for pharmacological inhibitors SU5402 and PD184352 (blue) are indicated at level of FGF receptor and MEK, respectively.

(2.5 mm apart) placed at anterior and posterior edges of wing bud and 30–50V administered in 2–3 pulses of 50 ms using a CUY-21 pulse generator. For neural plate, embryos prepared in vitro (following EC culture methods) were electroporated in a custom built chamber (a kind gift of Ivor Mason) and exposed to 2 pulses of 50 ms × 10V using an Intracel Intracept TSS10 pulse generator.

Supplemental Data

Supplemental Figures for this article are available at http://www.current-biology.com/content/supplemental.

Acknowledgments

We are grateful for the technical assistance of Graham Christie, Fiona MacIsaac, and Pam Halley. M.C.E. is supported by a studentship from the Anatomical Society of Great Britain and Ireland and the British Council. J.S.L. is a BBSRC Student. R.J.D. is supported by a studentship from the Biomedical Research Centre and by Cancer Research UK. A.E.M. was a Wellcome Trust RCDF. J.M. is supported by a studentship from the Imperial Cancer Research Fund (now Cancer Research UK). S.M.K. is supported by Cancer Research UK. K.S. is an MRC Senior Research Fellow. C.T. is a Royal Society Research Professor and supported by the MRC.

Received: December 23, 2002
Revised: April 17, 2003
Accepted: April 17, 2003
Published: June 17, 2003

References

1. Martin, G.R. (1998). The roles of FGFs in early development of vertebrate limbs. Genes Dev. *12*, 1571–1586.
2. Umbhauer, M., Marshall, C.J., Mason, C.S., Old, R.W., and Smith, J.C. (1995). Mesoderm induction in Xenopus caused by activation of MAP kinase. Nature *376*, 58–62.
3. Michelson, A.M., Gisselbrecht, S., Buff, E., and Skeath, J.B. (1998). Heartbroken is a specific downstream mediator of FGF receptor signalling in Drosophila. Development *125*, 4379–4389.
4. Borland, C.Z., Schutzman, J.L., and Stern, M.J. (2001). Fibroblast growth factor signaling in Caenorhabditis elegans. Bioessays *23*, 1120–1130.
5. Niehrs, C., and Meinhardt, H. (2002). Modular feedback. Nature *417*, 35–36.
6. Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M.A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell *92*, 253–263.
7. Furthauer, M., Lin, W., Ang, S.L., Thisse, B., and Thisse, C. (2002). Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. Nat. Cell Biol. *4*, 170–174.
8. Tsang, M., Friesel, R., Kudoh, T., and Dawid, I.B. (2002). Identification of Sef, a novel modulator of FGF signalling. Nat. Cell Biol. *4*, 165–169.
9. Kovalenko, D., Yang, X., Nadeau, R.J., Harkins, L.K., and Friesel, R. (2003). Sef inhibits fibroblast growth factor signaling by inhibiting FGFR1 tyrosine phosphorylation and subsequent ERK activation. J. Biol. Chem. *278*, 14087–14091.
10. Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001). Spred is a Sprouty-related suppressor of Ras signalling. Nature *412*, 647–651.
11. Lax, I., Wong, A., Lamothe, B., Lee, A., Frost, A., Hawes, J., and Schlessinger, J. (2002). The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. Mol. Cell *10*, 709–719.
12. Ozaki, K., Kadomoto, R., Asato, K., Tanimura, S., Itoh, N., and Kohno, M. (2001). ERK pathway positively regulates the expression of Sprouty genes. Biochem. Biophys. Res. Commun. *285*, 1084–1088.
13. Yusoff, P., Lao, D.H., Ong, S.H., Wong, E.S., Lim, J., Lo, T.L., Leong, H.F., Fong, C.W., and Guy, G.R. (2002). Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. J. Biol. Chem. *277*, 3195–3201.
14. Nutt, S.L., Dingwell, K.S., Holt, C.E., and Amaya, E. (2001). Xenopus Sprouty2 inhibits FGF-mediated gastrulation movements but does not affect mesoderm induction and patterning. Genes Dev. *15*, 1152–1166.
15. Keyse, S.M. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr. Opin. Cell Biol. *12*, 186–192.
16. Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S., and Keyse, S.M. (1996). Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J. *15*, 3621–3632.
17. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkinstall, S. (1998). Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. Science *280*, 1262–1265.
18. Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. *18*, 664–674.
19. Dickinson, R.J., Eblaghie, M.C., Keyse, S.M., and Morriss-Kay, G.M. (2002). Expression of the ERK-specific MAP kinase phosphatase PYST1/MKP3 in mouse embryos during morphogenesis and early organogenesis. Mech. Dev. *113*, 193–196.

20. Storey, K.G., Goriely, A., Sargent, C.M., Brown, J.M., Burns, H.D., Abud, H.M., and Heath, J.K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. Development *125*, 473–484.
21. Streit, A., Berliner, A.J., Papanayotou, C., Sirulnik, A., and Stern, C.D. (2000). Initiation of neural induction by FGF signalling before gastrulation. Nature *406*, 74–78.
22. Wilson, S.I., Graziano, E., Harland, R., Jessell, T.M., and Edlund, T. (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr. Biol. *10*, 421–429.
23. Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G.R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell *75*, 579–587.
24. Riese, J., Zeller, R., and Dono, R. (1995). Nucleo-cytoplasmic translocation and secretion of fibroblast growth factor-2 during avian gastrulation. Mech. Dev. *49*, 13–22.
25. Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C., and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. Development *121*, 1399–1410.
26. Shamim, H., and Mason, I. (1999). Expression of Fgf4 during early development of the chick embryo. Mech. Dev. *85*, 189–192.
27. Christen, B., and Slack, J.M.W. (1999). Spatial response to fibroblast growth factor signalling in Xenopus embryos. Development *126*, 119–125.
28. Crossley, P.H., Minowada, G., MacArthur, C.A., and Martin, G.R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. Cell *84*, 127–136.
29. Storey, K.G., Crossley, J.M., De Robertis, E.M., Norris, W.E., and Stern, C.D. (1992). Neural induction and regionalisation in the chick embryo. Development *114*, 729–741.
30. Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. J. Biol. Chem. *271*, 15292–15297.
31. Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B.K., Hubbard, S.R., and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science *276*, 955–960.
32. Cohn, M.J., Izpisua Belmonte, J.C., Abud, H., Heath, J.K., and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. Cell *80*, 739–746.
33. Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell *89*, 693–702.
34. Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A., and Arkinstall, S. (1996). The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. J. Biol. Chem. *271*, 27205–27208.
35. Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., et al. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. *5*, 810–816.
36. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105.
37. Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J., and Lax, I. (2001). Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc. Natl. Acad. Sci. USA *98*, 6074–6079.
38. Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H–1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.
39. Hayama, M., Inoue, R., Akiba, S., and Sato, T. (2002). ERK and p38 MAP kinase are involved in arachidonic acid release induced by H_2O_2 and PDGF in mesangial cells. Am. J. Physiol. Renal Physiol. *282*, F485–F491.
40. Recio, J.A., and Merlino, G. (2002). Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. Oncogene *21*, 1000–1008.
41. Shimoke, K., and Kudo, M. (2002). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine has a transient proliferative effect on PC12h cells and nerve growth factor additively promotes this effect: possible involvement of distinct mechanisms of activation of MAP kinase family proteins. Brain Res. Dev. Brain Res. *133*, 105–114.
42. Morooka, T., and Nishida, E. (1998). Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J. Biol. Chem. *273*, 24285–24288.
43. Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. *364*, 229–233.
44. Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y., and Matsumoto, K. (1999). p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol. Cell. Biol. *19*, 2322–2329.
45. Tumpel, S., Sanz-Ezquerro, J.J., Isaac, A., Eblaghie, M.C., Dobson, J., and Tickle, C. (2002). Regulation of Tbx3 expression by anteroposterior signalling in vertebrate limb development. Dev. Biol. *250*, 251–262.
46. Kim, G.J., and Nishida, H. (2001). Role of the FGF and MEK signaling pathway in the ascidian embryo. Dev. Growth Differ. *43*, 521–533.
47. Hudson, C., Darras, S., Caillol, D., Yasuo, H., and Lemaire, P. (2003). A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian Ciona intestinalis. Development *130*, 147–159.
48. Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A.M., and Martinez-Arias, A. (1998). *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes Dev. *12*, 557–570.
49. Minowada, G., Jarvis, L.A., Chi, C.L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M.A., and Martin, G.R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development *126*, 4465–4475.
50. Chambers, D., and Mason, I. (2000). Expression of sprouty2 during early development of the chick embryo is coincident with known sites of FGF signalling. Mech. Dev. *91*, 361–364.
51. Heinrich, R., Neel, B.G., and Rapoport, T.A. (2002). Mathematical models of protein kinase signal transduction. Mol. Cell *9*, 957–970.
52. Bhalla, U.S., Ram, P.T., and Iyengar, R. (2002). MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. Science *297*, 1018–1023.
53. Sanz-Ezquerro, J.J., and Tickle, C. (2000). Autoregulation of Shh expression and Shh induction of cell death suggest a mechanism for modulating polarising activity during chick limb development. Development *127*, 4811–4823.