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

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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.

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...
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 with 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 embryo 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
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', 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 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).
is detected in the neural plate after 6 hr with LY294002. Similarly, in the limb, LY294002 locally inhibits the limb bud where, ever, in this experiment, no change in expression by FGF8 at 4 hr (n = 10, 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 test the role of 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

![Image of embryos and control samples](current-biology.com)
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
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 Sprad, have been identified [6–10] (Figure 8). Overexpression of Sprouty, which in

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 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′–E′) Cells expressing Pyst1-EGFP (green) and activated MAPK (brown) were scored in TS limb buds 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.

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
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 proximodistal 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 [14], but also coordinate inputs from other signaling pathways.

Whole-Mount In Situ Hybridization

Chick embryos were staged according to Eyal-Giladi and Hamilton, 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 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’, and C”) 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).
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 (1:10000) (Jackson), and StreptAvidin-HP 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 \( \mu \text{m} \) 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 \( \mu \text{g} / \text{ml} \) in the early embryo) for 1 hr at RT. PBS soaked beads were used as controls. 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 Pyst1 as an NH2-terminal fusion to EGFP, the open reading frame was amplified by PCR using the primers 5'-CCCCAGCTTATGATAGATACGCTCAGACCCGTGCCCT 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 \( \mu \text{g} \) pECE-p44MAPK-HA (a kind gift from Dr. Anne Brunet) and increasing amounts (0, 0.5, or 2 \( \mu \text{g} \)) 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 fetal bovine serum (15% v/v final concentration) for 15 min. Cells were lysed and 5 \( \mu \text{g} \) 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 \( \mu \text{g} / \mu \text{l} \), 1:40 (v/v) 0.4% fast green/distilled H\(_2\)O) were made into mesenchyme. Electroporation was then carried out using two electrodes
FGF Signaling and Pyst1/MKP3

Though the biochemical basis of these inhibitory activities is not yet clear. In contrast, PYST1/MKP3 is a known specific inhibitor of downstream of FGF receptor activation. Previously identified inhibitory factors Sef [7–9], Spred [10], and Sprouty [6] (but see [14]) downstream of FGF receptor activation. Previously identified inhibition motif [16, 34]. As with Sef and Sprouty, it is expressed down-stream 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 (follow-ing EC culture methods) were electroporated in a custom built chamber (a kind gift of Ivor Mason) and exposed to 2 pulses of 50 ms using an Intracel Intracept TSS10 pulse generator.

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

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