Fusicoccin signaling reveals 14-3-3 protein function as a novel step in left-right patterning during amphibian embryogenesis

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Summary

To gain insight into the molecular mechanisms underlying the control of morphogenetic signals by H^+ flux during embryogenesis, we tested Fusicoccin-A (FC), a compound produced by the fungus *Fusicoccum amygdali* Del. In plant cells, FC complexes with 14-3-3 proteins to activate H^+ pumping across the plasma membrane. It has long been thought that FC acts on higher plants only; here, we show that exposing frog embryos to FC during early development specifically results in randomization of the asymmetry of the left-right (LR) axis (heterotaxia). Biochemical and molecular-genetic evidence is presented that 14-3-3-family proteins are an obligate component of *Xenopus* FC receptors and that perturbation of 14-3-3 protein function results in heterotaxia. The subcellular localization of 14-3-3 mRNAs and proteins reveals novel cytoplasmic destinations, and a left-right asymmetry at the first cell division. Using gain-of-function and loss-of-function experiments, we show that 14-3-3E protein is likely to be an endogenous and extremely early aspect of LR patterning. These data highlight a striking conservation of signaling pathways across kingdoms, suggest common mechanisms of polarity establishment between *C. elegans* and vertebrate embryos, and uncover a novel entry point into the pathway of left-right asymmetry determination.

Key words: Left-right asymmetry, 14-3-3 protein, Fusicoccin, *Xenopus*

Introduction

The role of ion flux in controlling morphogenetic events during embryonic development and regeneration is becoming increasingly recognized as an important component alongside well-studied biochemical factors (Borgens et al., 1989; Nuccitelli, 1992; Robinson and Messerli, 1996; Altizer et al., 2001; Levin, 2003a). In particular, H^+ flux and the resulting pH gradients are known to be crucial in embryonic development and neoplastic progression (Perona and Serrano, 1988; Grandin and Charbonneau, 1989; Martinez-Zaguilan and Gillies, 1992; Uzman et al., 1998; Levin et al., 2002). As part of our effort to understand the contribution of endogenous ion flows and gradients to patterning (Cheng et al., 2002; Rutenberg et al., 2002; Levin et al., 2002), we have begun to characterize signaling pathways that control proton flux across cell membranes.

Fusicoccin (FC) is a metabolite of the plant pathogen *Fusicoccum amygdali* Del. The toxin is remarkably effective in stimulating a number of physiological processes in higher plants, including stomatal opening, cellular respiration and radicle emergence from seed embryos (Marre, 1979; Kinoshita and Shimazaki, 1999). FC binds with high affinity to a receptor that is formed from an interaction of proteins from the 14-3-3 family with the plasma membrane H^+-ATPase (Korthout and De Boer, 1994; Olsson et al., 1998; Palmgren, 2001). 14-3-3 proteins are a family of acidic, soluble proteins with a number of key signaling roles in cell cycle, apoptosis and prion diseases (Baldin, 2000; Fu et al., 2000). Binding of 14-3-3 to the C-terminal (regulatory) domain of the H^+ pump releases its auto-inhibitory action (Baunsgaard et al., 1998; Palmgren, 2001), which results in increased H^+-extrusion along with changes in cytoplasmic pH (Felle et al., 1986). Despite the considerable structural conservation of 14-3-3 proteins from plants and animals (Fu et al., 2000; Sehnke et al., 2002), and the large number of 14-3-3 partners identified thus far in both kingdoms (Van Hemert et al., 2001), the presence of FC binding sites in organisms other than higher plants has never been demonstrated (Meyer et al., 1993).

Modulation of H^+ currents is a hallmark of FC action; pH is a key regulator of many cellular parameters, such as gap-junctional communication between cells, which in turn is known to control a number of physiological processes (Levin, 2001). These considerations prompted us to investigate whether FC might be able to perturb embryonic patterning in a tractable vertebrate embryonic model, the frog *Xenopus laevis*, and thus be a useful tool to shed light on endogenous morphogenetic mechanisms. Interestingly, we found that exposure to FC had one major effect on embryogenesis: randomization of the left-right (LR) axis. The consistent asymmetry in the morphogenesis and placement of the heart,

gut, brain and other asymmetric organs in normal individuals is very strongly conserved across evolution (Neville, 1976). Deviations from the normal pattern of asymmetry (*situs solitus*) have significant medical implications for human patients with laterality defects (Kosaki and Casey, 1998), and this fascinating biological problem is now beginning to be understood in molecular detail (Burdine and Schier, 2000; Mercola and Levin, 2001; Yost, 2001). However, very early LR patterning events are poorly understood and considerable debate exists as to how early embryos of various species can align the LR axis. Our characterization of FC signaling reveals that 14-3-3E protein is asymmetrically localized in *Xenopus* at the first cell cleavage and represents a novel, very early step in the LR pathway.

Materials and methods

Assaying embryonic situs

Embryos were anesthetized by Tricaine, and scored at stage 45 by non-invasive light microscopy for the situs of the heart, gut and gall-bladder. Data was analyzed using the χ^2 test with Yates correction (for higher stringency).

Homogenization of embryos for biochemical analysis

Stage 5/6 or stage 10 embryos (5 g) were ground under liquid nitrogen in a mortar until a fine powder was obtained. 2 ml of homogenization buffer [20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 1 mM PMSF and 1 complete protease inhibitor tablet (Roche Biochemicals) per 50 ml buffer] was then added and the suspension ground until liquid. All subsequent steps were carried out at 4°C. The suspension was centrifuged for 10 minutes at 2500 ***g*** and the pellet (which consists mainly of residual yolk protein) was discarded. Subsequently, the supernatant was centrifuged at 10,000 ***g*** for 15 minutes and the pellet discarded. Finally, the supernatant was centrifuged for 60 minutes at 100,000 ***g*** and the resulting pellet (microsomal fraction) and supernatant (cytoplasmic fraction) collected.

Fusicoccin binding assay

Binding assays were performed in glass tubes in 20 mM Mes-Tris (pH 6.5), 1 mM $CaCl_2$, 5 mM $MgSO_4$, 2.3 mM DTT, 1 mM PMSF and 5×10^{-8} M [^{3}H]9′-nor-fusicoccin-8′-alcohol at 30°C. The final incubation volume was 0.9 ml and reactions were incubated for 3 hours. The incubation was started by the addition of 4 mg microsomal or cytoplasmic protein extract. The reaction was terminated by the addition of 1 ml ice-cold wash buffer (25 mM glycine-KOH, pH 9.5) to the tubes and adding this, together with another 4 ml of wash buffer, on a Millipore filtration manifold. Rapid filtration was performed through polyethyleneimine (1% w/w) pre-treated Whatman GF/B filters, which were washed with 5 ml distilled water and 5 ml wash buffer just before filtration. The filters were then washed twice with 5 ml of wash buffer. The whole procedure of filtering and washing took about 30 seconds. The filters were then transferred to 5 ml Pharmacia Optiphase HiSafe 3 cocktail. After 24 hours extraction, the radioactivity was measured for 5 minutes with a LKB Wallac Rackbeta 1219 liquid scintillation counter. Non-specific binding was measured in saturation experiments by the inclusion of 10 μM unlabelled FC. The binding data of the saturation and competition experiments were analyzed with Sigmaplot 5.0. P-peptide was used at a final concentration of 1 μM. Further deviations from the protocol are mentioned in the text and figures.

Chromogenic in situ hybridization

In situ hybridization was performed according to a standard protocol (Harland, 1991). Briefly, *Xenopus* embryos were collected and fixed in MEMFA. Prior to in situ hybridization, embryos were washed in PBS+0.1% Tween-20 and then transferred to methanol through a 25%/50%/75% series. Probes for in situ hybridization were generated in vitro from linearized templates using DIG-labeling mix (Roche). Chromogenic reaction times were optimized for signal: background ratio.

Drug exposure

FC was obtained from Professor G. S. Muromtsev (Moscow), and tested using the lettuce seed germination assay (Lado et al., 1974) before use with embryos, as the FC obtained from Sigma was found to be of variable quality. FC was used at 50 μM in 0.1×MMR medium in systemic application experiments. Consistent with previous biochemical studies, which found optimal FC binding to receptors at lower pH (Drabkin et al., 1997), we observed that the maximal effect was achieved with FC dissolved in 0.1×MMR at pH<6 (embryos raised in medium with pH in the range 5 to 8 exhibit the normal control levels of heterotaxia: <1%, n=180).

Microinjection

For microinjection, peptides and mRNA were dissolved in water and injected into embryos in 3% Ficoll using standard methods (100 millisecond pulses in each injected cell with borosilicate glass needles calibrated for a bubble pressure of 55-62 kPa in water). Peptide stock was 2.6 mM in water; approximately 2.7 nL were injected into each cell. After 30 minutes, embryos were washed in 0.75×MMR for 30 minutes and cultured in 0.1×MMR until desired stages.

Immunohistochemistry

Embryos were fixed overnight in MEMFA and stored at 4°C in PBTr (1×PBS + 0.1% Triton X-100). They were embedded in gelatin/albumin medium and sectioned at 40 μm on a Leica vibratome. The sections were washed three times in PBTr, blocked with 10% goat serum and incubated with primary antibody at 1:200 in PBTr overnight. They were then washed six times with PBTr and incubated with an alkaline-phosphatase-conjugated secondary antibody overnight. After six washes in PBTr, detection was carried out using NBT and BCIP (X-Phos). Detection times were optimized for signal:noise ratio.

Results

Fusicoccin exposure induces heterotaxia

To examine the effects of FC on vertebrate development, *Xenopus* embryos were cultured in medium containing FC from fertilization to stage 14 (a time period long prior to the morphogenesis of asymmetric organs) and examined at stage 43. Development was apparently normal, with the exception of heterotaxia (Fig. 1; Table 1A). FC exposure induced a 25% rate of reversals in any one of the three organs evaluated: the heart, gut and gall-bladder, on a background of 1% heterotaxia observed in control (χ^2=81.2, $P<2\cdot10^{-19}$). Analysis of the phenotype induced by FC reveals that it is a loss of concordance between the three organs (heterotaxia), not an isomerism or pure situs inversus (Table 1B; examples of heterotaxic embryos are shown in Fig. 1). Overall embryonic development (including the dorsoanterior index) and morphogenesis of individual organs was normal, suggesting that ectopic FC signaling has a specific effect on the fundamental orientation or interpretation of LR cues. Microinjection of FC into the egg immediately after fertilization had the same effect (data not shown).

An endogenous FC receptor exists in frog embryos

In view of this dramatic effect of FC on development, we

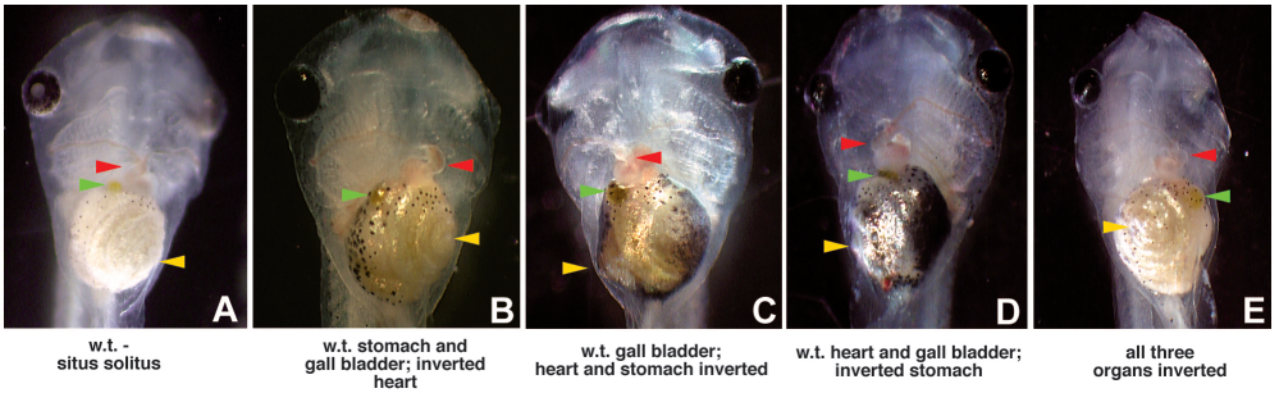

Fig. 1. Fusicoccin (FC) induces heterotaxia. Systemic exposure of *Xenopus* embryos to FC between fertilization and stage 14, or microinjection of FC into dorsal blastomeres at the four-cell stage, resulted in heterotaxia relative to control embryos (A). Exposed embryos exhibited cardiac inversions (B), heart and gut inversions (C), gut inversions (D), and complete mirror image inversions (E). All embryos are shown in ventral views (thus the embryo's right is the reader's left), with anterior toward the top. Red, yellow and green arrows indicate the asymmetry of the heart, gut and gall bladder, respectively.

addressed the question of whether an FC-receptor protein, or complex, is present in *Xenopus* embryos. Binding of the radiolabelled 9′-nor-fusicoccin-8′-alcohol probe ([^{3}H]FC) to proteins extracted from stage 5/6 embryos, was compared with binding characteristics of the classical higher plant FC receptor localized to plasma membranes. Intriguingly, unlike in plant cells, the embryonic FC-binding sites were not detected in the microsomal fraction, but instead are cytoplasmic (Fig. 2A). Because non-specific binding of [^{3}H]FC to proteins in the oocyte extract was very low (cf. Fig. 2B), further purification at this stage was not necessary for the binding studies. From the saturation binding curves (shown in Fig. 2A), an equilibrium dissociation constant (K_D) and the total number of receptors (B_{max}) can be estimated. The properties of the barley root plasma membrane receptor (K_D 1.50±0.11 nM, B_{max} 94.7±1.5 pmol.mg^{-1} protein) are in good agreement with many surveys of plant FC receptors (Abramycheva et al., 1991). The embryo receptor falls into the category of medium-high affinity with a K_D of 27.8±6.9 nM and a B_{max} of 25.1±3.4 pmol.mg^{-1} protein.

Table 1. Fusicoccin effects on *Xenopus* embryos

A Fusicoccin induces laterality defects in *Xenopus* embryos

	Control embryos	Fusicoccin-exposed
Situs solitus (wild type)	99% (330)	75% (76)
Laterality defects	1% (4)	25% (25)
Total	334	101

Fusicoccin-exposed embryos were significantly different from controls (χ^2=81.2, P<2·10^{-19}).

B Phenotypic distribution of the effect of FC on laterality

Organs affected	Incidence in FC-exposed embryos
Heart alone	40% (10)
Gut alone	4% (1)
Gall bladder alone	0% (0)
Heart and gut	8% (2)
Heart and gall bladder	4% (1)
Gut and gall bladder	12% (3)
Heart, gut and gall bladder	32% (8)
Total affected embryos	25

To test whether the radioligand used in the saturation binding experiment, [^{3}H]FC, has the same affinity for the receptor as FC, a competitive binding experiment was carried out with a fixed concentration of [^{3}H]FC and increasing amounts of unlabelled FC (Fig. 2B). For the barley root plasma membrane and embryo cytoplasmic fraction the IC_{50} values were 1.30±0.12 nM and 660±25 nM, respectively. The values for K_D and IC_{50} are related by the following expression: K_D=IC_{50} – [Radioligand]. Whereas the K_D values calculated with these two methods for the plant receptor are in good agreement, the values obtained for the embryo receptor differ by a factor of 24. We conclude that the plant receptor does not differentiate between the [^{3}H]FC probe and unlabelled FC, whereas the embryo receptor is strongly sensitive to the chemical modifications, which were necessary to synthesize [^{3}H] FC from FC. Structure-activity relationships for the plant FC receptor show that FC derivatives usually have lower affinities for the receptor than [^{3}H]FC, except for FC itself (Ballio et al., 1981). Having obtained evidence for an endogenous target of FC signaling in *Xenopus*, we sought to further characterize the mechanism involved (Olsson et al., 1998).

14-3-3-blocking peptide disrupts FC binding in vitro and randomizes laterality in embryos

To test the hypothesis that 14-3-3 proteins participate in the formation of the *Xenopus* FC receptor, as they do in plants, we took advantage of one of the 14-3-3 hallmarks: the formation of a tight complex with phosphorylated ligands containing either of the two sequence motifs R[S/Ar]XS^{P}XP or RX[S/Ar]XS^{P}XP, where Ar denotes aromatic residues and S^P denotes phosphorylated Ser/Thr (Yaffe et al., 1997). The snug fit of such ligands into the highly conserved amphipathic groove of the 14-3-3 protein specifically and effectively disrupts the interaction between 14-3-3 and partners, in vivo as well as in vitro (Ballio et al., 1981; Yaffe et al., 1997; Booij et al., 1999; Bunney et al., 2001). We used a phosphopeptide modeled on the phosphorylated hinge 1 region of nitrate reductase (NR), which contains a 'strong' 14-3-3 interaction motif (RSXS^{P}XP) that is also found in many mammalian 14-

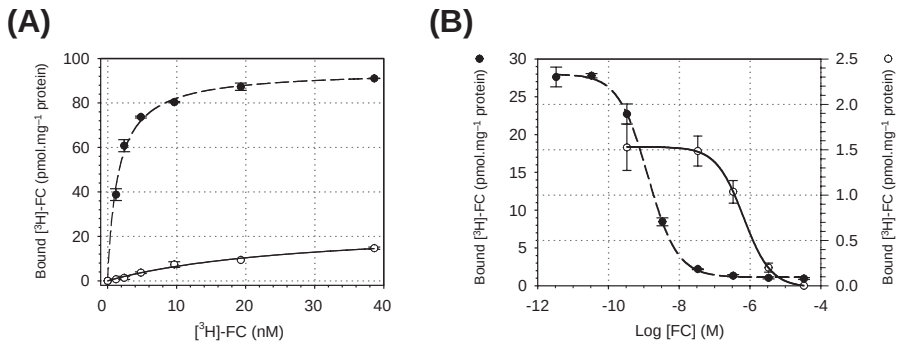

Fig. 2. Biochemistry of the FC receptor in *Xenopus* tissue. *Xenopus laevis* embryos possess a Fusicoccin-binding site whose properties differ from that of the classical higher plant Fusicoccin receptor. (A) Saturation analysis of specific binding of [^{3}H]9′-nor-fusicoccin-8′-alcohol to plasma membranes of barley roots (black circle) and embryo cytoplasmic protein extract (white circle). Data was fitted using the equation describing a rectangular hyperbola. The resulting coefficients gave rise to values for the K_D and B_{max}, which are referred to in the main text. The data are expressed as the means±s.e.m. of a representative experiment. (B) Competitive binding experiments with [^{3}H]9′-nor-fusicoccin-8′-alcohol and unlabelled Fusicoccin A. Barley root plasma membranes were incubated with 1 nM radioligand (black circle) and embryo cytoplasmic protein extract was incubated with 4 nM radioligand (white circle). The data was fitted using the equation describing a sigmoidal curve of 3 parameters (Sigmaplot, SPSS scientific).

3-3 partners (e.g. Raf-kinase, Cdc25, PKC and BAD) (Yaffe et al., 1997), and binds to a domain fully conserved in *Xenopus* 14-3-3 proteins (Rittinger et al., 1999; Aitken et al., 2002; Wurtele et al., 2003). Addition of this phosphorylated NR peptide (1 μM) to the FC-binding assay (using 4 nM radioligand) resulted in a 75% reduction in FC-binding to the oocyte receptor as compared with the control (bound [^{3}H]FC was, respectively, 1.010±0.024 and 0.260±0.013 pmol.mg^{-1} protein). In light of the specificity and effectiveness of this peptide (Bunney et al., 2001), these data suggest that 14-3-3 proteins are essential components of the embryo FC receptor.

On the basis of the FC-induced heterotaxia and the involvement of 14-3-3 proteins in in vitro FC-binding, we formulated the hypothesis that endogenous 14-3-3 proteins are instrumental in correct LR asymmetry. We tested this model by complementing the FC gain-of-function data with a competitive loss-of-function experiment using the same NR-peptide as was utilized in the FC-binding assay. Microinjection of the NR-peptide (which is expected to act as a dominant negative by interfering with FC/14-3-3 complex formation) into embryos at the one-cell stage resulted in a 31% incidence of heterotaxia (Table 2; χ^2=58.27, P<2.2·10^{-14}). By contrast, injections of a control peptide (which is identical in amino acid sequence, except that the serine in the binding motif is not phosphorylated) did not induce heterotaxia. These results are consistent with the hypothesis that 14-3-3 proteins mediate the randomizing effect of FC, and suggest the possibility that 14-3-3 proteins are endogenously involved in the patterning of the LR axis.

Table 2. 14-3-3 peptide injections induce heterotaxia

	Control peptide-injected embryos	Phospho-peptide-injected embryos
Situs solitus (wild type)	99% (199)	69% (64)
Laterality defects	1% (2)	31% (29)
Total	201	93

Phospho-peptide-injected embryos were significantly different from controls (χ^2=58.27, P<2.2·10^{-14})

14-3-3 protein function is upstream of the left-sided signaling factor *XNR1* in the LR pathway

To test the idea that 14-3-3 signaling was involved in LR patterning, we performed an overexpression experiment, reasoning that the regulatory functions of 14-3-3 proteins, especially those that depend on specific spatio-temporal patterns of 14-3-3 function, would be overwhelmed by ubiquitous overexpression. The ability to misexpress individual members of the 14-3-3 family also allowed us to address differential roles for these proteins. The data are summarized in Table 3. Embryos injected with mRNA encoding β-gal exhibited the normal 1% incidence of laterality defects. Embryos injected at the one-cell satge (immediately after embryonic turning) with approximately 300 pg of mRNA encoding the 14-3-3Z subtype exhibited a 6% incidence of heterotaxia. By contrast, one-cell injections of equal amounts of mRNA encoding 14-3-3E protein induced a 30% incidence of heterotaxia. These data are consistent with 14-3-3E proteins, rather than the 14-3-3Z subtype, being involved in the LR pathway.

Interestingly, injections of *14-3-3E* mRNA at the two-cell or four-cell stages each resulted in only a 7% incidence of heterotaxia. (Table 3). The more than fourfold stronger effect of injections at the one-cell stage compared with injections

Table 3. *14-3-3* mRNA misexpression at the one-cell stage induces heterotaxia

	Control (β-gal) at one-cell stage	*14-3-3Z* mRNA at one-cell stage	*14-3-3E* mRNA at one-cell stage	*14-3-3E* mRNA at two-cell stage	*14-3-3E* mRNA at four-cell stage
Situs solitus (wild type)	99% (151)	97% (91)	70% (81)	93% (127)	93% (143)
Heterotaxia	1% (1)	3% (3)	30% (35)	7% (10)	7% (10)
Total	152	94	116	137	153

All results were significantly different from controls: *14-3-3Z* mRNA at one-cell stage, χ^2=1.02, P<0.32; *14-3-3E* mRNA at one-cell stage, χ^2=46.78 P<7.95·10^{-12}; *14-3-3E* mRNA at two-cell stage, χ^2=6.96 P<0.008; and *14-3-3E* mRNA at four-cell stage, χ^2=5.98 P<0.02.

occurring after the first cleavage (about 1.5 hours later) suggest that 14-3-3E proteins function during very early LR patterning steps. To confirm this hypothesis, we investigated whether 14-3-3E activity was upstream of the earliest known asymmetric gene in *Xenopus*: the left-sided signaling factor *XNR1* (Lowe et al., 1996; Lohr et al., 1998). Embryos receiving injections of *14-3-3E* mRNA at the one-cell stage were fixed at stage 22 and processed for in situ hybridization with an *XNR1* probe. The results are summarized in Table 4. Control embryos (injected with lacZ mRNA) exhibited the correct left-sided expression in 94% of cases (Fig. 3A,B), the remaining 6% showed no expression [a known phenomenon in wild-type unperturbed *Xenopus* embryos (Lohr et al., 1998)]. By contrast, embryos injected with *14-3-3E* mRNA exhibited 30% bilateral expression of the left-sided marker (Fig. 3C,D), as well as 12% right-sided expression and 23% absent expression (χ^2=50.6, P<1.2·10^{-12}). These data show that the randomizing effects of overexpression of 14-3-3 protein act upstream of the earliest known *Xenopus* asymmetric marker.

14-3-3E protein is asymmetrically localized in very early frog embryos

Having determined that alterations of 14-3-3E protein activity can disrupt LR patterning during very early stages of development, we sought to further characterize the endogenous 14-3-3-dependent mechanism by investigating the embryonic expression of members of the *Xenopus* 14-3-3 family. We first examined localization of 14-3-3 protein by immunohistochemistry on sections of embryos (Fig. 4) using well-characterized isoform-specific antibodies (Kumagai et al., 1998). Both 14-3-3E and 14-3-3Z signals were detected, demonstrating that these proteins exist endogenously from the earliest stages of embryonic development in *Xenopus*.

Maternal 14-3-3Z protein was localized throughout the vegetal 75% of the unfertilized egg (Fig. 4A). During the first few cleavages, protein signal was very weak or not detectable (Fig. 4B,C). By gastrulation, zygotic 14-3-3Z protein could be detected in the endodermal yolk mass (Fig. 4D). Consistent with the low effectiveness of 14-3-3Z misexpression in randomizing the LR axis, these localization patterns exhibited no significant LR asymmetries.

The localization of 14-3-3E protein was markedly different from 14-3-3Z (demonstrating lack of cross-over of the two antibodies). Prior to fertilization, 14-3-3E was localized as a fairly tight spot in the center of the egg (Fig. 4E). Strikingly, after the first cleavage, 14-3-3E was localized to only one of the two blastomeres (Fig. 4F). This pattern continues at the second cell cleavage, when dorsoventral (DV) pigment differences allow DV and LR orientation of the embryo, showing that localization is present in the right blastomeres (Fig. 4G). This asymmetric localization of 14-3-3E protein strongly supports the hypothesised endogenous role for 14-3-3E in LR asymmetry, and is the earliest LR-asymmetric molecular localization described in any species to date. By gastrulation, zygotic 14-3-3E is detected in the endodermal yolk mass, in a domain that is more restricted than that of 14-3-3Z.

Table 4. *14-3-3E* mRNA misexpression randomizes laterality of *XNR-1* expression

	Control	*14-3-3E* injected
Left side	94% (64)	35% (26)
Right side	0% (0)	12% (9)
Bilateral	0% (0)	30% (22)
None	6% (4)	23% (17)
Total	68	74
% incorrect	6%	65%

14-3-3E injected embryos were statistically different from controls (χ^2=50.6, P<1.2·10^{-12}).

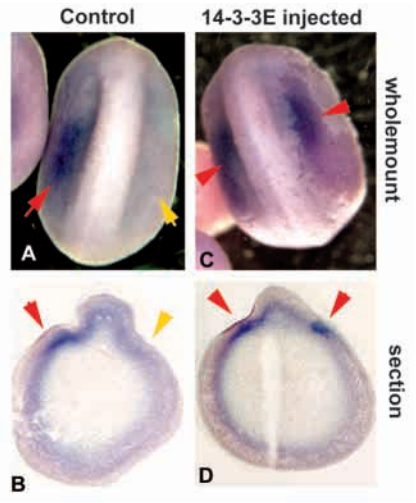

Fig. 3. 14-3-3 protein misexpression randomizes the localization of *XNR1* expression. Control embryos examined for the expression of *XNR1* by in situ hybridization exhibit the normal left-sided signal (whole mount in A; section in B). By contrast, embryos receiving injections of *14-3-3E* mRNA at the one-cell stage often exhibit bilateral *XNR1* expression (whole mount in C; section in D). Red arrowheads indicate expression; yellow arrowheads indicate lack of expression.

In light of the asymmetry of 14-3-3E localization in the first two cleavages, we next examined the results of the 14-3-3E overexpression. In embryos that were injected with *14-3-3E* mRNA (using the same conditions as in the above experiments that induced subsequent heterotaxia), very high levels of 14-3-3E protein were detected throughout each blastomere during the two- and four-cell stages (Fig. 4I,J). These results confirmed that the injections of *14-3-3E* mRNA indeed resulted in high levels of protein that was present ubiquitously, and also served as a cross-check for the fact that the 14-3-3E antibody does recognize 14-3-3E.

Fusicoccin exposure abolishes the asymmetry in 14-3-3E localization

The fact that FC exposure randomizes asymmetry (Table 1) could be due to an alteration of 14-3-3 protein function or localization. We investigated the mechanism underlying the destabilizing effects of FC by examining the localization of 14-3-3E proteins in embryos exposed to levels of FC that cause heterotaxia. Control embryos at the two-cell stage exhibit the asymmetric pattern of 14-3-3E localization (Fig. 5A,B). Interestingly, embryos exposed to FC from fertilization showed bilaterally-symmetrical localization of 14-3-3E (Fig. 5C,D). These data demonstrate that ectopic early FC exposure can abolish the normal asymmetry in 14-3-3E protein localization.

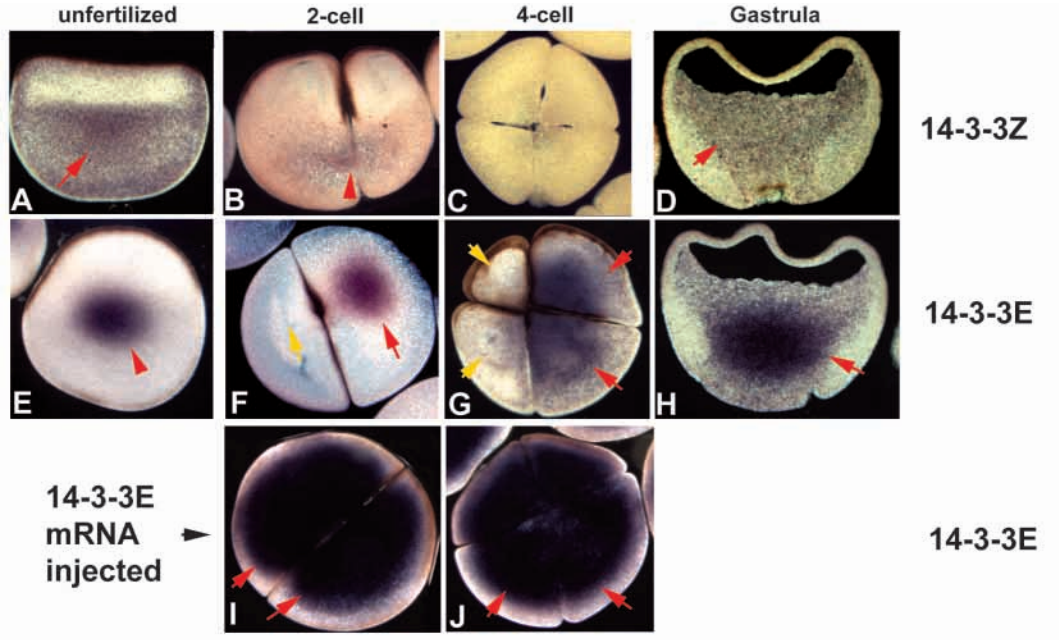

Fig. 4. Localization of 14-3-3 proteins during early development. Embryos were fixed, sectioned and processed for immunohistochemistry with 14-3-3 protein antibodies. (A-D) 14-3-3Z protein. (A) Unfertilized embryos sectioned parallel to the animal-vegetal (AV) axis display signal in the vegetal-most two-thirds of the embryo. (B) By the two-cell stage, the staining is much reduced in intensity. (C) By the four-cell stage, staining is almost completely absent. (D) At stage 10, weak staining is seen throughout the endodermal yolk mass of the gastrulating embryo. (E-J) 14-3-3E protein. (E) By contrast to 14-3-3Z, the 14-3-3E signal is seen in a coherent spot in the center of the unfertilized embryo. (F) By the two-cell stage, signal can be detected in only one of the two blastomeres. (G) At the four-cell stage, signal is seen in the right blastomeres. (H) During gastrulation, a strong signal is detected in the endodermal yolk mass. To check the mRNA construct as well as the antibody, embryos injected immediately after fertilization with 14-3-3E mRNA were processed for immunohistochemistry. A strong 14-3-3E signal can be detected throughout the embryo at the two- and four-cell stages (I,J). Red arrowheads indicate protein localization; yellow arrowheads indicate lack of signal.

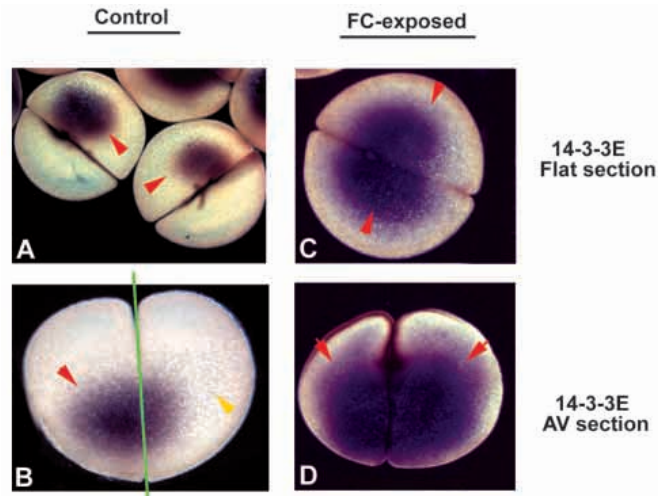

Fig. 5. Ectopic Fusicoccin abolishes the asymmetry of 14-3-3E protein localization. (A,B) Control embryos exhibit 14-3-3E protein localization in only one blastomere. (C,D) By contrast, exposure to Fusicoccin abolishes the asymmetry and results in localization in both blastomeres. Sections in panels A and C were taken perpendicular to the animal-vegetal (AV) axis. Sections in panels B and D were taken parallel to the AV axis. Red arrowheads indicate localization; yellow arrowheads indicate lack of signal. Green line in panel B indicates cleavage plane between the blastomeres.

14-3-3 mRNA localization in frog embryos

The embryonic expression of *14-3-3* mRNA has been described in *Xenopus*, but not at early stages (Wu and Muslin, 2002). The asymmetry in 14-3-3E protein localization led us to investigate whether this phenomenon was presaged by similar localization in the mRNA. We used whole-mount in situ hybridization to examine localization of maternal and zygotic mRNA for three 14-3-3 genes. At least three different *14-3-3* transcripts are present in early *Xenopus* embryos; representative data are shown in Fig. 6. Maternal mRNA for the 14-3-3T protein (Gawantka et al., 1998) is localized to the cortex of most blastomeres during the first few cleavages (Fig. 6A,B). Zygotic 14-3-3T is expressed in the roof of the blastocoel in blastula *Xenopus* embryos (data not shown), and is later expressed throughout the nervous system, including very strong expression in the head and somites, and a thin border at the caudal end of the embryo, up to the anus (Fig. 6C). Maternal mRNA for 14-3-3Z (Gawantka et al., 1998) is also located in the cortex of cleaving blastomeres in the first two cell cleavages (Fig. 6D). Interestingly, at the 16- and 32-cell stages, it is located in the animal half of vegetal cells (Fig. 6E), and in some blastomeres exhibits an unusual horseshoe pattern (Fig. 6F). No left-right asymmetries were detected. By contrast, *14-3-3E* mRNA exhibits an asymmetry at the first cell cleavage consistent with that seen in 14-3-3E proteins (Fig. 6G,H). We confirmed this pattern by sectioning (Fig. 6I,J). These data illustrate that 14-3-3 mRNAs are subject to

localization events resulting in spatially-complex geometric patterns.

Discussion

Despite the plethora of 14-3-3 proteins found in a wide number of species (Ferl et al., 2002; Tzivion and Avruch, 2002), the presence of FC binding sites in organisms other than higher plants has so far not been demonstrated (Meyer et al., 1993), although cotylenin A, a compound structurally related to FC (and an effective competitor with FC for binding to its receptor) has been shown to affect differentiation in murine (M1) and human myeloid leukemia cells (Yamada et al., 2001). We found a remarkably specific developmental effect of Fusicoccin exposure in vertebrate embryos: disruption of left-right asymmetry. The effect is not confined to the laterality of any one organ; it is not a loss of asymmetry, nor a full reversal, but rather the randomization of the heart, gut and gall bladder, all of which normally exhibit strongly-conserved left-right asymmetry (Table 1, Fig. 1). The incidence of heterotaxia reported in the various experiments must be interpreted bearing in mind that if all three organs are fully randomized, the maximum incidence of heterotaxia is <88% (as in fully randomized embryos all three organs will occasionally be in their correct locations simply by chance and thus scored as situs solitus). Together with the low incidence of laterality defects in control embryos and the lack of confounding general defects arising from FC exposure, statistical analysis of the randomizing effects of FC (and several other manipulations described below) clearly suggests a novel LR patterning element related to FC signaling.

Elements of Fusicoccin signaling are shown to be conserved to vertebrates. This conclusion is confirmed by the biochemical data demonstrating the existence of a FC receptor in *Xenopus* embryos (Fig. 2). The existence of an FC receptor complex suggests the possibility that endogenous FC-like compounds exist in vertebrates and remain to be characterized. This new aspect of FC action also warrants further study into possible subtle teratological effects of certain food products in the light of reports that plants may produce FC-like molecules (Muromtsev et al., 1994) (A.H.D.B. and M. Wang, unpublished).

Interestingly, the *Xenopus* FC receptor is cytoplasmic, which is consistent with the localization patterns of 14-3-3 proteins we described (e.g. Fig. 4A,G). There may well be other differences between the receptor as it exists in plant compared with in animal cells. The ability of the dominant-negative NR peptide to compete with in vitro FC binding to 14-3-3 (Fig. 2), and to induce heterotaxia in embryos (Table 2), suggests that 14-3-3 proteins are components of the endogenous FC receptor in *Xenopus*. 14-3-3 proteins have known roles in key processes, such as the cell cycle, apoptosis and prion diseases, and are thus crucial biomedical targets (Baldin, 2000; Fu et al., 2000; Van Hemert et al., 2001; Baxter et al., 2002). Higher doses of the NR peptide induced non-specific teratogenic defects in embryos (e.g. exogastrulation; data not shown), which is consistent with known important later roles for various 14-3-3 proteins in *Xenopus* development (Wu and Muslin, 2002). The amount of 14-3-3-blocking peptide used in our studies specifically induced heterotaxia. Left-right patterning is thus a new role for this important and versatile family of signaling proteins.

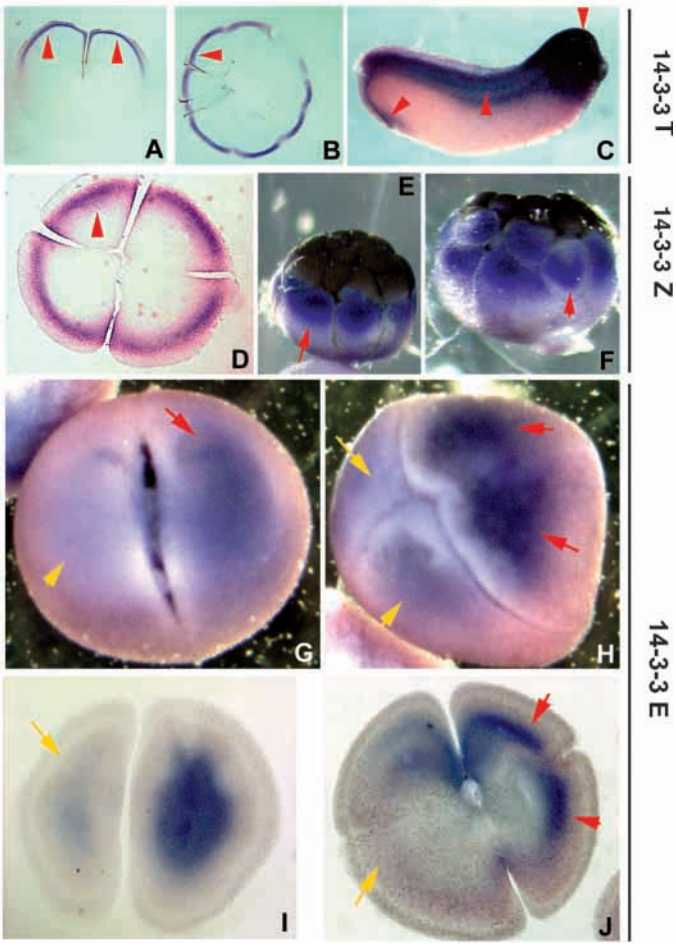

Fig. 6. mRNA localization of 14-3-3 family during early development. A representative set of embryonic stages are shown for each mRNA. Maternal mRNA for 14-3-3 isoform T is localized to the animal pole of the unfertilized egg (data not shown), and to embryonic blastomeres at the two-cell stage (A; parallel to AV axis). (B) This pattern is observed in most blastomeres during the first few cleavages (perpendicular to the AV axis). The mRNA is located near the cell cortex. (C) 14-3-3T is later expressed throughout the nervous system, including very strong expression in the head and somites, and a thin border at the caudal end of the embryo, up to the developing anus. (D) 14-3-3 isoform Z is detected in the cortex of cleaving cells (four-cell stage embryo sectioned perpendicular to AV axis). During subsequent cleavages, transcripts are also detected in the animal half of vegetal cells (E) and in an unusual horseshoe pattern in cleavage-stage embryos (F). *14-3-3E* mRNA is present throughout the animal pole of the unfertilized egg (data not shown). It becomes localized to one of the two blastomeres at the first cell division (G). This pattern is maintained at the four-cell stage (H). Sections of G and H, shown in I and J, respectively, confirm the pattern. Note that *14-3-3E* mRNA at the two-cell stage is located in the central cytoplasm and not in the cell cortex (compare panel I with panels D and B). Red arrowheads indicate presence of mRNA; yellow arrowheads indicate lack of signal.

The NR peptide targets the binding of all 14-3-3 proteins (it does not distinguish between the isoforms). By contrast, misexpression of specific mRNAs allowed us to address differential activities of the various 14-3-3 family members. Injection of *14-3-3E* mRNA at the one-cell stage causes

ubiquitous expression of high levels of 14-3-3E protein at the two- and four-cell stages (Fig. 4I,J), confirming the specificity of our mRNA and antibody reagents, and demonstrating that high levels of 14-3-3E protein can saturate and overwhelm the normal 14-3-3E protein localization machinery (Fig. 4F,G). Such overexpression of 14-3-3E, but not of 14-3-3Z, causes heterotaxia (Table 3). These data demonstrate that different 14-3-3 isoforms may have distinct developmental roles, and that the E isoform but not the Z can interact with mechanisms involved in LR asymmetry. The misexpression experiments further allowed us to probe the timing of 14-3-3E involvement in LR asymmetry. 14-3-3E function is upstream of the expression of *XNR1*, as its localization is randomized by 14-3-3E overexpression (Table 4). Moreover, the fact that injections after the first cell division have a far smaller effect on LR asymmetry than do injections immediately after fertilization suggests that processes occurring during the first 1.5 hours after fertilization involve 14-3-3E. As, like most exogenous mRNAs, *14-3-3E* mRNA is efficiently translated within about an hour of microinjection (Fig. 4I,J), it is likely that 14-3-3E-mediated LR patterning mechanisms function during the first two cell divisions. This is consistent with a lack of laterality phenotypes reported from loss-of-function studies that introduced 14-3-3-blocking reagents after the first cleavage (Wu and Muslin, 2002).

The proposed LR pathway function of 14-3-3E protein during the first two cell cleavages is consistent with the endogenous protein localization data. Maternal protein for both 14-3-3E and 14-3-3Z exists in unfertilized eggs, and exhibits different localization patterns, suggesting that 14-3-3 family proteins interact with different components of the subcellular localization machinery and, thus, can be targeted to different locales within the blastomeres (Muslin and Xing, 2000). Whereas 14-3-3Z is expressed at a low level and exhibits no significant asymmetries, 14-3-3E is asymmetric at the first two cell cleavages (Fig. 4F,G). Although the maternal mRNA for 14-3-3E is also asymmetric at these stages (Fig. 6G-J), the asymmetries in protein and mRNA expression overlap temporally. The 2-cell stage lasts about 20 minutes at room temperature; this time period is probably too brief to allow significant levels of protein to be translated from the mRNA and correctly localized. Thus, the mRNA asymmetry is unlikely, by itself, to account for the observed protein localization. The asymmetric protein localization is probably caused by movement of maternal protein, which exists even prior to fertilization. The reason for asymmetrically localizing both mRNA and protein is unknown, but may represent redundancy in as-yet-uncharacterized later roles that depend on mRNA localization.

In *Xenopus*, the first cell cleavage bisects the dorsal and ventral progenitor blastomeres (which become obvious when pigment differences enable the identification of dorsal versus ventral blastomeres at the second cell division). When one of two blastomeres is injected with any reagent, the other blastomere gives rise to the contralateral side of the embryo and thus serves as a convenient internal control in many studies (e.g. Warner et al., 1984; Harvey and Melton, 1988; Vize et al., 1991; Louie et al., 2000). In our experience with microinjections of lineage markers, the first cleavage plane corresponds very well to the LR midline of the tadpole. Whether the first cell division truly gives rise to left and right blastomeres has been questioned (Danilchik and Black, 1988). The plane of first cleavage can be experimentally repositioned independently of the plane of bilateral symmetry by lateral compression of the uncleaved egg (Black and Vincent, 1988). However, the first cell division in unperturbed embryos appears normally to occur along the LR midline in most *Xenopus* embryos (Klein, 1987; Masho, 1990), and, in *Triturus*, separation of blastomeres at the two-cell stage results in randomization of one of the twins (Ludwig, 1932), thus supporting the view that the first cleavage results in left and right blastomeres in some amphibians.

The identification of LR asymmetry in 14-3-3E protein localization at the first cell cleavage is of significance for several reasons. First, this is the earliest LR-asymmetric molecular localization shown to date in any species. Second, this new entry point into the LR pathway can be used to address upstream mechanisms of LR asymmetry. These data constrain the first step of asymmetry to about the 1.5 hours following fertilization; by utilizing the reagents described in this study, we are currently characterizing the machinery that establishes asymmetric 14-3-3E protein localization, in efforts to identify the molecular nature of ‘Step 1’ of LR asymmetry.

The specific mechanisms underlying the asymmetric localization of 14-3-3E protein and mRNA are unknown. They may involve differential degradation, anchoring or directed transport by motor proteins. We favor the latter possibility because of a number of studies linking motor protein function with LR asymmetry (Supp et al., 1997; Takeda et al., 1999; Vogan and Tabin, 1999; Hirokawa, 2000b; Brueckner, 2001; Levin, 2003b). Animal-vegetal asymmetries in mRNA localization have been well-studied in *Xenopus* (Deshler et al., 1997; Mowry and Cote, 1999). Although LR-asymmetric mRNA localization has recently been found (Levin et al., 2002), the observed localization for 14-3-3E protein demonstrates the existence of previously uncharacterized localization mechanisms. Moreover, aside from the LR-relevant asymmetry, the complex subcellular localization seen for the various members of the 14-3-3 family (Fig. 6D-F) shows that novel patterns of mRNA destinations exist in early embryonic cells (Oleynikov and Singer, 1998). 14-3-3 proteins are known to participate in directed intracellular localization events in a number of species (Brunet et al., 2002). The mechanisms that direct messenger molecules to spatially-complex locales within cells, as well as the developmental significance of these patterns in the large blastomeres of early embryos, remain to be characterized.

The asymmetry in 14-3-3E localization provided a way to probe the mechanism of FC action on LR patterning. The simplest prediction would have been that the randomization of laterality by FC would be due to an alteration of 14-3-3 protein binding to its target. Surprisingly, we found that FC exposure abolished the asymmetry of 14-3-3E protein localization. Taken together, all these data suggest the following model of this new aspect of LR asymmetry. We propose (Fig. 7A) that the 14-3-3E protein is normally asymmetrically localized at the two-cell stage and provides differential signaling to the left and right sides, which eventually feeds into the asymmetric gene cascade (Levin and Nascone, 1997; Levin, 2003c). Overexpression of 14-3-3E protein prior to the first cell division overwhelms the endogenous localization machinery and 14-3-3E accumulates in both blastomeres, thus providing

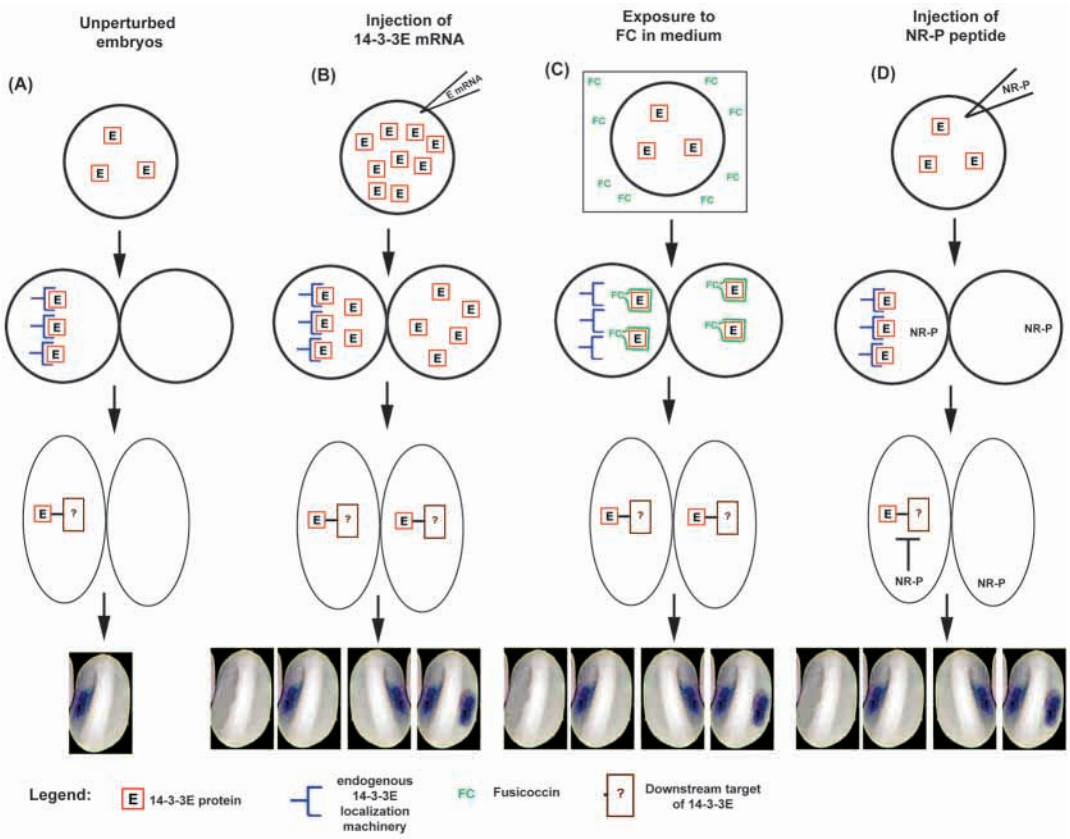

Fig. 7. A model of 14-3-3 signaling in LR asymmetry in normal and perturbed embryos. Our results suggest the following model. (A) In unmanipulated embryos, endogenous localization machinery ensures that only one cell of a two-cell embryo contains 14-3-3E protein. This protein interacts with an unknown target (see Discussion for probable candidates) whose activation on one side of the midline feeds into the pathway of asymmetric genes. (B) When 14-3-3E protein is misexpressed by the injection of *14-3-3E* mRNA immediately after fertilization, excess 14-3-3E protein overwhelms the localization machinery and is present in both cells at the first cleavage. This subsequently provides identical signal to the L and R sides, resulting in a randomization of asymmetry. (C) Exposure to FC in the medium abolishes the asymmetric localization of 14-3-3E (and induces heterotaxia as in B) by competing for its binding with the endogenous localization mechanism. (D) Injection of NR-P peptide abolishes the asymmetry by interfering with the one-sided binding of 14-3-3E to its downstream target.

equal signal to both lineages and randomizing asymmetry (Fig. 7B). FC exposure randomizes asymmetry by virtue of abolishing the differential 14-3-3E localization (Fig. 7C). This may be owing to competition of excess exogenous FC for 14-3-3 binding by whatever localization machinery maintains the tight asymmetric distribution of 14-3-3E protein. Such mechanisms may involve FC-like molecules, further suggesting that compounds related to FC occur endogenously in animal embryos. In this model, the dominant-negative NR peptide induces heterotaxia by virtue of interference with the binding of 14-3-3E to its downstream target (Fig. 7D).

What targets might be downstream of 14-3-3E signaling? The molecular mechanisms underlying LR patterning are now beginning to be understood (Levin and Mercola, 1998a; Burdine and Schier, 2000; Mercola and Levin, 2001; Yost, 2001) and include motor proteins, ion flux, gap junctions, and a variety of asymmetrically-expressed downstream transcription factors and signaling molecules. No previously-described LR patterning step in vertebrates is known to involve an FC-like molecule or 14-3-3 proteins. However, a number of possible mechanisms may be involved. Recent experiments have suggested that kinesin motor proteins are involved in LR patterning (Marszalek et al., 1999; Hirokawa, 2000a). As 14-3-3 proteins have been shown to affect the activity of kinesin (Dorner et al., 1999), it is possible that FC signaling affects LR asymmetry through modification of kinesin function.

Another possible model involves interactions between gap junctions and 14-3-3 proteins. Gap junctions composed of connexin proteins have been shown to be an important step in LR patterning in *Xenopus* (Levin and Mercola, 1998b) and chick (Levin and Mercola, 1999) embryos. Connexin proteins undergo serine phosphorylation, which affects their gating properties (Lampe et al., 2000), and are upregulated in certain tissues together with 14-3-3 proteins (Villaret et al., 2000). Connexin 43 binds directly to tubulin (Giepmans et al., 2001) and 14-3-3 might regulate its function in the same fashion that it regulates other tubulin-binding proteins such as CLIC4 (Suginta et al., 2001). Thus, perhaps 14-3-3 proteins bind connexins or a connexin-regulating protein in a phosphorylation dependent manner, thereby affecting LR asymmetry through modulation of the junctional flow that initiates at the fourth cell cleavage.

In our view, the most likely mechanism involves the regulation of ion flux across cell membranes. 14-3-3 proteins

are known to control a variety of H^+ pumps (Bunney et al., 2001; Bunney et al., 2002) and ion channels (De Boer, 2002). Proton flux may indirectly affect permeability states of connexin-based gap junctions through changes in cytosolic pH (Ek-Vitorin et al., 1996). Furthermore, we recently showed that K^+ and H^+ flux is asymmetric in early embryos and controls LR asymmetry (Levin et al., 2002). 14-3-3 proteins (including 14-3-3E) have recently been shown to be able to modulate K^+ currents in *Xenopus* oocytes (Chan et al., 2000; Benzing et al., 2002). In light of the differential LR subcellular localization of ion pumps, such as the H^+/K^+-ATPase (Levin et al., 2002), and of the ability of 14-3-3 proteins to control the localization of their binding partner (Muslin and Xing, 2000), we propose that 14-3-3E protein functions in the LR pathway by differentially regulating the endogenous activity and/or localization of LR-relevant ion channels or pumps on each side of the midline. Other (non-LR) roles for 14-3-3 proteins clearly exist in *Xenopus* (such as in mesoderm induction), and have been described in loss-of-function studies (Wu and Muslin, 2002).

Recently, it was reported that, in *Caenorhabditis elegans*, a 14-3-3 protein (PAR-5) is required for cellular asymmetry in the early embryo (Morton et al., 2002). PAR5 likewise functions in axial asymmetry in *Drosophila* (Benton et al., 2002). Analogously to the suggested role of the FC/14-3-3 receptor in *Xenopus*, PAR-5 in *C. elegans* acts at an early step in establishing polarity, but its precise role is unclear. It was suggested that PAR-5 binds to and blocks recruitment of one or more PAR proteins (notably PAR-2 and PAR-3) to the cell cortex (Morton et al., 2002). The involvement of 14-3-3 proteins in cellular asymmetry in early cleavages of both *C. elegans* and *Xenopus* is further evidence of a deep and fundamental underlying similarity in the mechanisms by which asymmetry and polarity, whether on the cellular level, or on the scale of the organism, is established. The finding that elements of FC signaling are conserved from plants to animals presents a new perspective from which to investigate novel aspects of large-scale morphogenetic control in vertebrates, and is likely to make manipulation of 14-3-3 protein signaling a powerful tool for addressing aspects of asymmetry at every scale.

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