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A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo

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Abstract

During early vertebrate development Fibroblast Growth Factor (FGF) signalling is required for multiple activities including specification of mesodermal, neural and heart tissue, as well as gastrulation movements and regulation of differentiation and pattern onset in the extending body axis. A current challenge is to understand how FGF signalling generates such diverse outcomes. A key FGF downstream pathway is the Ras-MAPK/Erk1/2 cascade, which culminates in the phosphorylation of target proteins, such as the Ets family of transcription factors. To begin to assess specificity downstream of FGF in the chick embryo we have characterised the patterns of *Fgfr1–4* expression and Erk1/2 activation, as well as expression of the Erk1/2 specific phosphatase, *Mkp3* and of three Ets factor genes (*Erm, Pea3* and *Er81*) from early blastula to the 10 somite stage. We identify new sites of *Fgfr* expression and show that nearly all regions of Erk1/2 activity are within *Fgfr* expression domains and require FGF signalling. Differences in intensity, duration, distribution and sub-cellular localisation of activated Erk1/2 are observed in distinct cell populations within the embryo and during wound healing. With few exceptions, a tight correspondence between Erk1/2 activation and *Mkp3* expression is found, while specific combinations of Ets factors are associated with distinct regions of Erk1/2 activation. These findings provide a comprehensive spatial and temporal map of FGF/Erk1/2 activity during early chick development and identify region and tissue specific differences in expression of *Fgfrs* as well as Erk1/2 phosphorylation and transcriptional targets which help to define response specificity.

Keywords: FGF; ERK1/2; Erk1/2; Chick embryo; Mkp3; Ets genes; Syn-expression

Introduction

Signalling via Fibroblast Growth Factors (FGFs) is one of the most important activities in the early vertebrate embryo as its loss leads to early embryonic lethality (reviewed by Bottcher and Niehrs, 2005). In particular, FGFs are required across species for induction of mesodermal and neural tissue as well as maintenance and patterning of the endoderm (e.g. Amaya et al., 1991; Arman et al., 1998; Bottcher and Niehrs, 2005; Delaune et al., 2005; Deng et al., 1997; Feldman et al., 1995; Slack et al., 1996; Storey et al., 1998; Streit et al., 2000; Sun et al., 1999; Wilson et al., 2000). FGF activity also mediates morphogenetic movements underlying gastrulation (Ciruna and Rossant, 2001; Nutt et al., 2001; Sivak et al., 2005; Yang et al., 2002). Later FGF signals provided by lateral endoderm then specify cardiac mesoderm (Alsan and Schultheiss, 2002). FGF signalling is required for the generation of all caudal tissues (Amaya et al., 1991; Griffin et al., 1995) and also plays a key role in regulating their differentiation. Declining levels of FGF signalling in the extending body axis create a differentiation wavefront which determines onset of neuronal differentiation and patterning in newly generated spinal cord (Diez del Corral et al., 2002; Diez del Corral et al., 2003) and also positions somite boundaries and is thus a crucial part of the mechanism underlying segmentation of the vertebrate body (Dubrulle et al., 2001; reviewed in Diez del Corral and Storey, 2004). FGF signalling additionally directs rostro-caudal character of newly generated body tissue via regulation of caudal Hox gene expression (Bel-Vialar et al., 2002; Dasen et al., 2003; Liu et al., 2001) and functions as a cell survival and patterning signal in the developing rostral nervous system (e.g. Chi et al., 2003; Lee et al., 1997; Storm et al., 2003).

Obviously, these very diverse outcomes are context dependent, with FGF signalling acting in a cellular environment

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defined by previous as well as current signalling activities. Specificity downstream of FGF signalling is provided at multiple levels within the pathway, beginning with specific binding affinities of FGF ligands (1–23) (Ornitz and Itoh, 2001) for their tyrosine kinase receptors (*Fgfr1–4* and their isoforms (Ornitz et al., 1996)). These can determine signalling strength and which downstream signal transduction pathways are activated (e.g. Umbhauer et al., 2000; Vainikka et al., 1994). The transduction of FGF signals during embryogenesis has been largely assessed with respect to three major pathways, PI3Kinase, PLCγ and Erk1/2/MAPK (reviewed by Bottcher and Niehrs, 2005) and it is clear that these pathways can work in concert to mediate cell fate decisions, for example, PI3K and Erk1/2 synergise to promote mesoderm induction in the frog (Carballada et al., 2001) and to coordinate cell behaviour with assignment of cell fate (Sivak et al., 2005). It is striking, however, that FGF signalling is responsible for nearly all Erk1/2 activity in early frog, fish and mouse embryos (Christen and Slack, 1999; Corson et al., 2003; Tsang et al., 2004) and in this paper we focus on FGF/Erk1/2 signalling activity during early development of the chick embryo.

On ligand binding Fgfrs dimerise and undergo auto-phosphorylation and activation of intra-cellular tyrosine kinase domains. This in turn leads to tyrosine phosphorylation of docking proteins FRS2α/β, which recruit the adaptor protein Grb2 and the protein tyrosine phosphatase Shp2 resulting in formation of multiple Grb2/Sos complexes and subsequent activation of the Ras/MAP kinase signalling pathway. This final signalling cascade involves sequential phopsphorylation and activation of MAPKKK (Raf), MAPKK (Mek) and MAPK(Erk1/2) (reviewed in Eswarakumar et al., 2005). The cellular response to Erk1/2 signalling is determined by the strength, duration, pattern and location of Erk1/2 activity. In the early frog embryo, high levels of Erk1/2 signalling promote a mesodermal cell fate while lower levels elicit an early neural cell state, suggesting that specific threshold levels of Erk1/2 signalling determine cellular response in this context (Delaune et al., 2005; reviewed by Stern, 2005). Another way in which Erk1/2 signalling can bring about distinct outcomes is if cells experience sustained or transient Erk1/2 activation. Strikingly, both of these signalling modes have been shown in vitro to drive differentiation or proliferation depending on cellular context and this emphasises the importance of context in determining the cellular response to Erk1/2 signalling (Marshall, 1995). It may also be possible for Erk1/2 signalling to adopt a more dynamic activity pattern, such as oscillations, which could be created by the action of feedback antagonists (Bhalla et al., 2002; Heinrich et al., 2002). Indeed, the duration of Erk1/2 signalling can determine whether activated Erk1/2 translocates to the nucleus or remains cytoplasmic (Marshall, 1995) and hence, whether it interacts directly with transcription factor targets.

A growing number of proteins have been shown to modulate Erk1/2 signalling, including: Spred, FRS2α, Sproutys, Sef, FLRTs and Mkp3 (Bottcher et al., 2004; Brunet et al., 1999; Camps et al., 1998; Casci et al., 1999; Furthauer et al., 2001, 2002; Groom et al., 1996; Hanafusa et al., 2002; Kramer et al., 1999; Lax et al., 2002; Reich et al., 1999; Sivak et al., 2005; Torii et al., 2004; Tsang et al., 2002; Wakioka et al., 2001). Expression of many of these genes has been reported to be induced by FGF signalling in embryos (*Sproutys 1,2,4, Sef, FLRT*s and *Mkp3*) (Bottcher et al., 2004; Eblaghie et al., 2003; Furthauer et al., 2001, 2002; Kawakami et al., 2003; Minowada et al., 1999; Tsang et al., 2002) and these genes have therefore been included in the FGF syn-expression group (reviewed by Niehrs and Meinhardt, 2002; Tsang and Dawid, 2004). Expression of *Sproutys 1,2*, *FLRT* and *Mkp3* are also known to be Erk1/2 dependent and so work as feedback antagonists of such signalling. These antagonists act at different levels in the pathway and in different sub-cellular compartments to modulate its activity. In some instances this feedback can determine sub-cellular localisation of Erk1/2 activity, e.g. Sef; (Torii et al., 2004) and help to separate cellular responses to FGF signalling, e.g. Sprouty and Spred (Sivak et al., 2005).

The FGF syn-expression group also includes members of the Ets family of transcription factors, *Pea3* and *Erm* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) and *Er81* (Munchberg and Steinbeisser, 1999), which constitute the Pea3 sub-group of Ets genes (De Launoit et al., 1997; Sharrocks, 2001). These factors are not only transcriptional targets of FGF signalling, but are also phosphorylated and thereby activated by Erk1/2 (Janknecht, 1996; Janknecht et al., 1996; O'Hagan et al., 1996). It has yet to be established whether other Ets genes, such as Ets1 and Elk1, which are also Erk1/2 substrates are regulated by FGF/Erk1/2 signalling in the embryo (Edmunds and Mahadevan, 2004; Sharrocks, 2001). Expression patterns of Pea3 family Ets genes have been described in the early embryos of fish, frog and mouse embryos (Chen et al., 1999; Chotteau-Lelievre et al., 2001; Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Strikingly, in the Zebrafish embryo *Pea3* and *Erm* are induced in a nested pattern by a local source of Fgf8 (Roehl and Nusslein-Volhard, 2001). This suggests that expression of these Ets genes depends on threshold levels of FGF/Erk1/2 signalling and that different combinations of Ets genes then help to mediate distinct cellular responses.

Here we present a detailed spatial and temporal map of the expression patterns of key components of the FGF signalling pathway and of MAPK/Erk1/2 activity in the early chick embryo from prestreak to the 10 somite stage, when the basic body plan is established. We determine whether FGF signalling is required for activation of Erk1/2 and the expression of *Mkp3* (the phosphatase also known as *Pyst1* or in human *Dusp6*, which specifically dephosphorylates Erk1/2) throughout the 10 somite embryo. We also assess activation of Erk1/2 and *Mkp3* following wounding of the embryonic epiblast. The location and levels of *Fgfrs1–4*, *Mkp3* expression and pErk1/2 activity in the embryo are compared with the expression of three key Ets genes (*Erm*, *Pea3* and *Er81*). Together these data help to define the repertoire of FGF pathway genes associated with specific sites of Erk1/2 activity and provide the foundation for a developmental atlas of FGF/Erk1/2 syn-expression groups in the early chick embryo.

Materials and methods

Whole mount in situ hybridisation

Whole mount in situ hybridisation was carried out using standard techniques and a subset of embryos was subsequently photographed, embedded in agar and cryo-sectioned at 15 μm. Plasmids were kindly provided for *Fgfr1* (Cek1), *Fgfr2* (Cek3) *Fgfr3* (Cek2) by E. Pasquale (Patstone et al., 1993), quail sequence for *FREK/Fgfr4* by C. Marcelle (Marcelle et al., 1994), *Mkp3* by S. Keyse (Eblaghie et al., 2003) and Ets genes *Pea3, Erm* and *Er81* by J. Lin and T. Jessell (Lin et al., 1998). Sense probe controls for *Mkp3, Erm, Pea3, Er81* in the early embryo (HH10) are presented in supplementary Figures S1 and S2.

Immunocytochemistry

Embryos were fixed rapidly in ovo with cold 4% paraformaldehyde, dissected and then pinned out in fix for 1–2 h at 4°C, washed in PBS (Phosphate Buffered Saline) and dehydrated to 100% methanol and stored overnight at −20°C. Embryos were then rehydrated to PBS, treated with 3% H_2O_2/PBS 2 h (RT), washed in PBS/0.5% Tween20, incubated in block (PBS, 0.5% T20 (PBST), 5% heat inactivated goat serum and 2% BSA) overnight and subsequently incubated in rabbit antibody against dual phosphorylated (dp)Erk1/2 (1:50) (Cell Signalling Tech; as in Corson et al., 2003) for 2–4 days at 4°C. Embryos were then washed in PBST and re-blocked (30 min) and incubated with a biotinylated anti-rabbit antibody (1:1000) (Jackson) overnight at 4°C. This was followed by extensive PBST washes, re-incubation in block (as above) and incubation in StreptAvidin–HRP conjugate (1:50) (Becton Dickinson) or StreptAvidin-Cy3 (1:4000) (Jackson) overnight at 4°C. Following extensive PBS washes some embryos then underwent a standard DAB (diaminobenzidine tetrahydrochloride) reaction to reveal HRP labelled cells. Nuclei were visualised in Cy3-labelled embryos with DAPI. Embryos were photographed in wholemount and cryo-sectioned at 15 μm using standard procedures and then photographed again, using a Lieca DM compound microscope with a NikonD1 camera.

This antibody against dpErk1/2 has been used by a number of groups to detect activated Erk1/2 (see (Corson et al., 2003)). To confirm that our protocol also specifically detected dpErk1/2 we ran a series of no primary controls ($n=7$ runs with 2–6 embryos each) in parallel with embryos exposed to the primary antibody and although a diffuse brown background was observed following exposure to DAB, we did not detect cellular labelling in the absence of the primary antibody (see Supplementary Figure S1).

Treatment with inhibitors

Hamburger and Hamilton (HH)(Hamburger and Hamilton, 1951) stage 10 embryos were prepared on Isopore membrane 1.2 μm filters (Millipore RTTP01300) and maintained in separate wells in a 4 well plate (Nunc 176740) in Leibovitz's L15 medium supplemented with 5% calf serum and then treated with DMSO, 60 μM SU5402 or 20 μM PD184352 for 2 h at 37°C in 5% CO_2. Embryos were then fixed and processed for insitu hybridisation or immunocytochemistry as described above.

Western blots

Stage 10 embryos were grown on filters and exposed to inhibitors (as described above) and these were then cut from the extra-embryonic membrane and lysed on ice. Three embryos were pooled in 50 μl lysis buffer, homogenised and 10 μg lysate run on a polyacrylamide gel, transferred to a PVDF membrane and incubated with antibodies to phospho-ERK 1/2 antibody (Cell Signalling) (1:1000) or MKP3 (gift of S. Keyse; 1:350) or for total loading, α-Tubulin (Abcam). Detection was performed using HRP-conjugated secondary antibodies and enhanced chemi-luminescence.

Wounding

Embryos of HH3 were placed in EC culture and a small wound was made in the embryonic epiblast parallel with the primitive streak with a dissecting pin. Embryos were then incubated for 2 h, fixed and processed as above for detection of dpErk1/2 or *Mkp3*.

Results

Analysis of Fgfr expression patterns

Although expression of *Fgfrs1–3* has been described previously from late primitive streak stages (Walshe and Mason, 2000) we wished to establish which *Fgf* receptors are present earlier prior to primitive streak formation and neural induction. We also wanted to characterise the expression pattern of *Fgfr4* (previously reported for Hamburger and Hamilton (HH)(Hamburger and Hamilton, 1951) stages 2–6 and HH14–26, using radioactive insitu methods (Marcelle et al., 1994) and to compare this with that of *Fgfrs1–3* during the critical period when the body plan is being established from Eyal-Giladi (EG)(Eyal-Giladi and Kochav, 1975) stage X to HH10.

Fgfrs at pre- and early primitive streak stages (EG, X- HH3+)

In prestreak embryos of EG stage X and early streak (HH2) *Fgfr1* and *Fgfr4* are expressed in similar domains throughout the epiblast at low levels, while neither *Fgfr2* nor *Fgfr3* are detected at these early stages (Figs. 1A–D_1). By HH3 *Fgfr1*, *Fgfr2* and *Fgfr4* are present in the neural plate (Figs. 1E–H_2). *Fgfr1* is detected throughout this tissue and is strikingly absent from the primitive streak (Figs. 1E–E_2). In contrast, *Fgfr4* expression is refined to the rostral-most embryonic epiblast, including the rostral edge of the neural plate (Figs. 1H–H_2). *Fgfr4* is also detected very weakly in the anterior primitive streak/node, as reported previously (Marcelle et al., 1994). Expression of *Fgfr2* is present weakly within the neural plate and is also detected in the underlying hypoblast (Figs. 1F, F_1). *Fgfr3* transcripts are still not detected at HH3 (Fig. 1G).

Fgfrs at late primitive streak/gastrula stage (HH4+–5)

By HH4+/5 when the primitive streak is maximally extended and prechordal mesoderm/notochord is just emerging at its tip, all four receptors are expressed (Figs. 1I–L_2). *Fgfr1* appears highest in the neural plate and this domain is now expanded caudally into epiblast adjacent to the primitive streak, while a reduced level of transcripts is detected at the midline above the emerging prechordal mesoderm/notochord. *Fgfr1* is the first and only receptor to be expressed within the primitive streak and at these stages is present only in the epiblast layer of this tissue (Figs. 1I–I_2). Strong expression of *Fgfr2* is now also observed throughout the neural plate and (like *Fgfr1*) is detected weakly at the ventral midline (Figs. 1J–J_2). *Fgfr1* and *Fgfr2* also appear asymmetric in the epiblast of the node at this stage (Figs. 1I_2, J_2). *Fgfr2* continues to be expressed in the spreading hypoblast and nicely marks this cell population as it is displaced to the periphery of the embryo by emerging endoderm and mesoderm (Fig. 1J). *Fgfr3* is first detected at this time, appearing diffusely in the neural plate (Figs. 1K–K_2). At this stage *Fgfr4* is further refined to the rostral lateral edges of the neural plate (Figs. 1L–L_2).

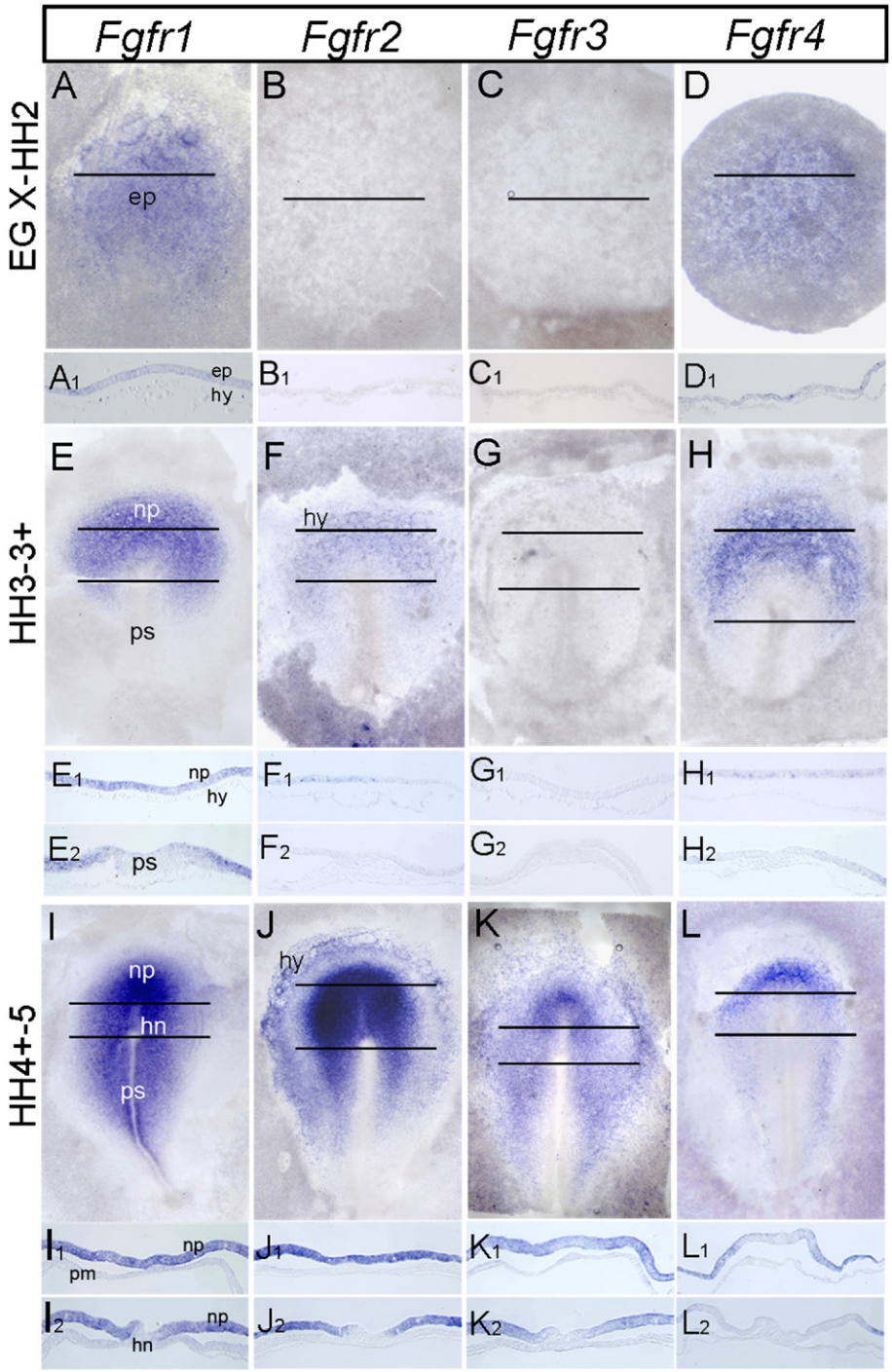

Fig. 1. Expression patterns of *Fgfrs1–4* from pre to late primitive streak stages. Embryos at EG, prestreak stage X express *Fgfr1* and *Fgfr4* in the epiblast layer: (A) *Fgfr1*; (B) *Fgfr2*; (C) *Fgfr3*; (D) *Fgfr4*. By HH stage 3–3+embryos express *Fgfrs1, 2 and 4*: (E) *Fgfr1*; (F) *Fgfr2*; (G) *Fgfr3*; (H) *Fgfr4*. At HH4+–5 all four *Fgfrs* are now expressed; (I) *Fgfr1* is now present in the primitive streak as well as neural plate; (J) *Fgfr2* in present in hypoblast and neural plate; (K) *Fgfr3* and (L) *Fgfr4*, are detected in neural plate and lateral epiblast. Black lines indicate level of transverse sections (TS) presented below each embryo. TSs are through the neural plate and node. ep=epiblast; hy=hypoblast; np=neural plate; ps=primitive streak; hn=Hensen's node; pm=paraxial mesoderm.

Fgfrs at headfold/early segmentation (HH6–7)

At these early somite stages *Fgfr1* is detected throughout the neural plate (with the exception of the ventral midline underlain by notochord), the primitive streak, including Hensen's node and the emerging caudal paraxial mesoderm (Figs. 2A–A_2). In contrast, *Fgfr2* expression is restricted to the neural plate rostral to the primitive streak and is weakly detected in forming precardiac mesoderm/endoderm (Figs. 2B–B_2). *Fgfr3* is similarly localised in the neuroepithelium, but is additionally detected in segmenting paraxial mesoderm (Figs. 2C–C_2). *Fgfr2* and *r3* are also detected in the displaced hypoblast in

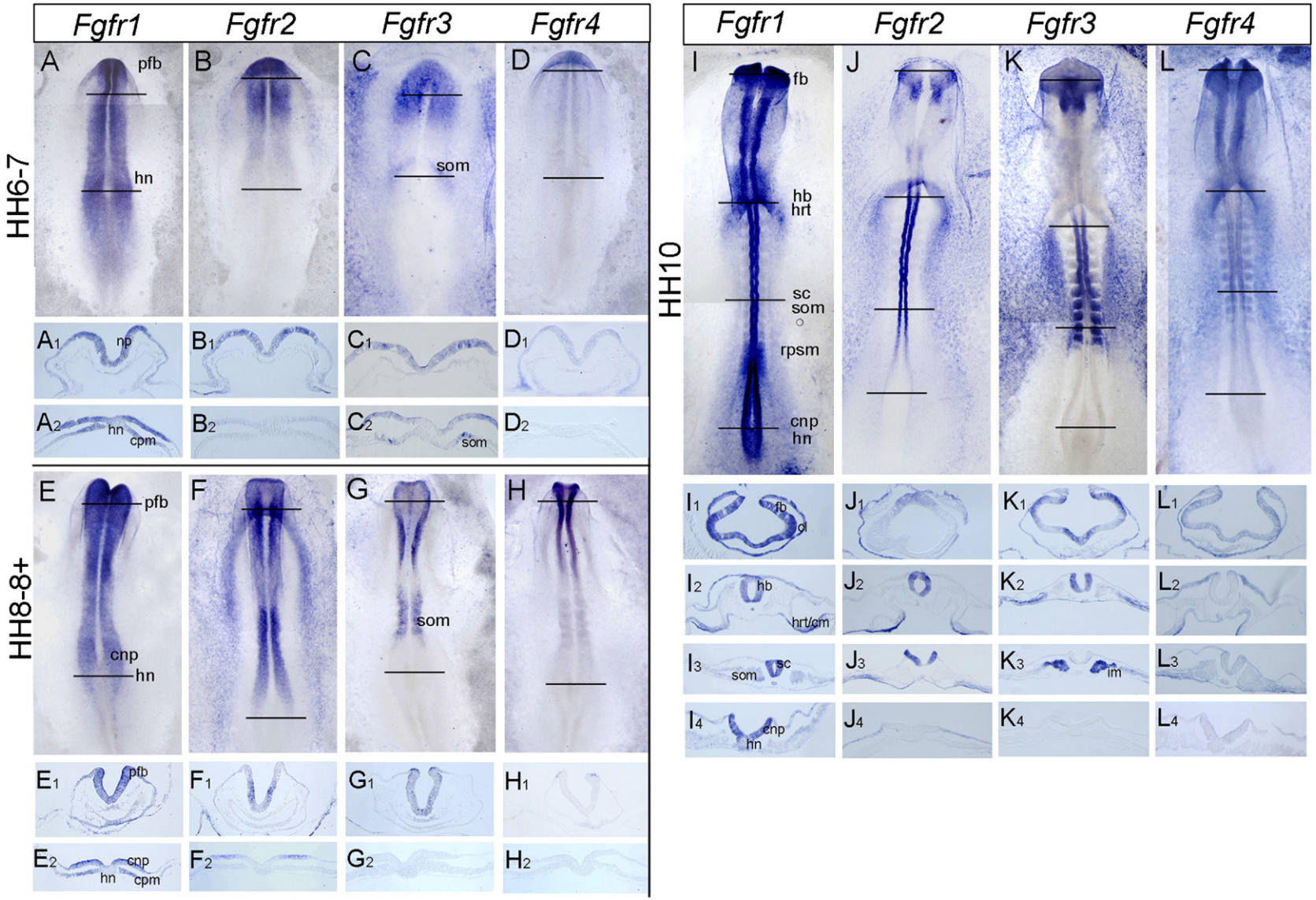

Fig. 2. Expression patterns of *Fgfrs1–4* from headfold to 10 somite stages. At HH6–7 all four *Fgfrs* are expressed (A) *Fgfr1*; (B) *Fgfr2*; (C) *Fgfr3*; (D) *Fgfr4*. At HH8–8+ (E) *Fgfr1*; (F) *Fgfr2*; (G) *Fgfr3*; (H) *Fgfr4*. At HH10–11 (I) *Fgfr1*; (J) *Fgfr2*; (K) *Fgfr3*; (L) *Fgfr4* with TSs at HH6–7 and HH8–8+ are through rostral neural plate and the node; TSs at HH10 are through forebrain/rostral neural tube, hindbrain/heart, spinal cord/somites and node. Abbreviations as in Fig. 1 and cpm=caudal paraxial mesoderm; pfb=presumptive forebrain; som=somite; fb=forebrain; hb=hindbrain; hrt=heart; sc=spinal cord; rpsm=rostral presomitic mesoderm; ol=optic lobe; im=intermediate mesoderm.

extra-embryonic regions. *Fgfr4* transcripts are detected at low levels in neural plate rostral to the primitive streak and at even weaker levels in forming somites and precardiac mesoderm/endoderm (Figs. 2D–D_2).

Fgfrs at early neurulation (HH8–8+)

At HH8–8+*Fgfr1* continues to be expressed throughout the neural tube, but is detected at different levels in distinct regions. High *Fgfr1* levels are present in the presumptive forebrain to hindbrain (Fig. 2E), but lower levels are detected in the neural tube flanked by somites and these rise again slightly in more caudal neural tissue (Fig. 2E). *Fgfr1* transcripts also continue to be absent from the ventral midline (Fig. 2E). At this stage *Fgfr1* expression is detected throughout primitive streak, in the emerging paraxial mesoderm and at low levels in precardiac mesoderm/endoderm (Figs. 2E–E_2). In contrast, *Fgfr2* expression is more restricted within the nervous system; it is detected in an intermediate region of the dorso-ventral axis of the presumptive caudal forebrain and midbrain (Figs. 2F, F_1), is weakly detected in the future rostral hindbrain, then appears in the neural tube flanked by somites, fading caudally into the caudal neural plate (Figs. 2F–F_2). *Fgfr2* also persists in precardiac mesoderm/endoderm and displaced hypoblast (Figs. 2F, F_1). *Fgfr3* is detected dorsally within the presumptive forebrain–hindbrain and unlike the other *Fgfrs* is now expressed in the ventral midline of the neural tube from the forebrain to midbrain (Figs. 2G–G_1). *Fgfr3* is also uniquely expressed in somites as well as in the adjacent neural tube at this stage (Fig. 2G). Finally *Fgfr4*, is present in the dorsal neural tube from forebrain to hindbrain regions of the CNS and is also still detected weakly in the somites (Figs. 2H–H_2).

Fgfrs at 10 somite stage (HH10)

Fgfr1 continues to be expressed throughout the neural tube and regressing primitive streak. It is also detected in the precardiac mesoderm and associated endoderm and at this stage additional domains are now apparent in the ectoderm at the level of the future otic vesicle, rostral presomitic mesoderm and lateral plate mesoderm (Figs. 2I–I_4). Within the neural tube *Fgfr2* is detected in the caudal forebrain and then in rostral hindbrain and again in somite flanked neural tube (Figs. 2J–J_2). It is also present in precardiac mesoderm and strongly expressed in the associated endoderm (Figs. 2J, J_2, J_3), but is absent from the caudal neural plate (Fig. 2J_4). *Fgfr3* is expressed like *r2* in the caudal forebrain, then fades caudally, and appears again in neural tube flanked by somites 1–4 but is then absent from more caudal neural tissue (Figs. 2K, K_1–K_4). In contrast, the most recently formed somites strongly express *Fgfr3*, as does the adjacent intermediate mesoderm (Figs. 2K, K_3). *Fgfr2* and *r3* transcripts also continue to be expressed in extra-embryonic tissues (Figs. 2J, K). *Fgfr4* is detected strongly in the rostral forebrain and extends caudally within the neuroepithelium to hindbrain levels. It is also expressed at low levels in precardiac mesoderm/endoderm, in ectoderm in the region of the future otic vesicle and in recently formed somites (Figs. 2L, L_1–L_4).

Analysis of patterns of Erk1/2 activation and Mkp3 expression

FGF signalling can lead to phosphorylation and activation of Erk1/2 in many contexts. We have demonstrated previously that exposure to FGF stimulates Erk1/2 activity in the chick neural plate (Eblaghie et al., 2003). Here, we therefore next wished to assess the changing patterns of Erk1/2 activation during development and to correlate these with expression profiles of *Fgfr*s 1–4. This was carried out using an antibody that recognises the dual phosphorylated form of Erk1/2. FGF induced Erk1/2 signalling is required for expression of *MAP-Kinase phosphatase3* (*Mkp3*) in both chick neural plate and limb bud mesenchyme (Eblaghie et al., 2003, and see Kawakami et al., 2003; Tsang et al., 2004 Smith et al., 2006). *Mkp3* specifically dephosphorylates and inactivates Erk1/2 (Brunet et al., 1999; Camps et al., 2000; Groom et al., 1996) and its dependence on FGF/Erk1/2 signalling constitutes a negative feedback loop that serves to modulate Erk1/2 signalling. In general, the pattern of *Mkp3* expression shows a striking coincidence with regions of Erk1/2 activation during these early stages of development and we therefore present data for these two components of the Erk1/2 pathway together.

Erk1/2 activation and Mkp3 at pre- and early primitive streak stages (EG, X-HH3)

At prestreak stages dpErk1/2 and *Mkp3* are detected at low levels in the epiblast (Figs. 3A, B). DpErk1/2 is, however, strongly detected in nuclei as well as the cytoplasm of cells at the edge of the spreading blastodisc (Figs. 3C, D, E–E_2). *Mkp3* transcripts are also detected at low levels in a discrete domain at the edge of the blastodisc (Fig. 3F). However, whether these nuclear dpErk1/2 possessing cells co-express *Mkp3*, has yet to be determined. At early streak stages (HH3) low level dpErk1/2 is now detected across the neural plate, with cells displaying varying levels of activity suggestive of dynamic signalling. The dpErk1/2 is also detected in the epiblast layer of the early primitive streak (Figs. 3G, G_1–G_3) where mitotic cells revealed by DAPI staining have elevated levels (Figs. 3H–H_2). In contrast, *Mkp3* is restricted to the forming neural plate and transcripts are strikingly absent from the primitive streak (Figs. 3I, I_1, I_2, I_3). This is one of only a few embryonic tissues in which we do not find a correspondence between Erk1/2 activity and *Mkp3* expression.

It has been previously shown that wounding leads to activation of Erk1/2 in frog and mouse embryos (Christen and Slack, 1999; Corson et al., 2003). Some data in the frog shows that this Erk1/2 activity is FGF independent (Christen and Slack, 1999) and so we wished to ascertain whether *Mkp3* expression, which is FGF dependent, is associated with wounding in the chick epiblast. Erk1/2 activation in response to wounding is detected in the nucleus (Corson et al., 2003) and so this approach might also help to confirm that our detection methods are able to reveal nuclear labelling. Wounding the epiblast at the lateral edge of the neural plate at HH3 led to local accumulation of dpErk1/2 in both nucleus and cytoplasm, without concomitant induction of *Mkp3* (5/5 cases) (Figs. 3J, K,

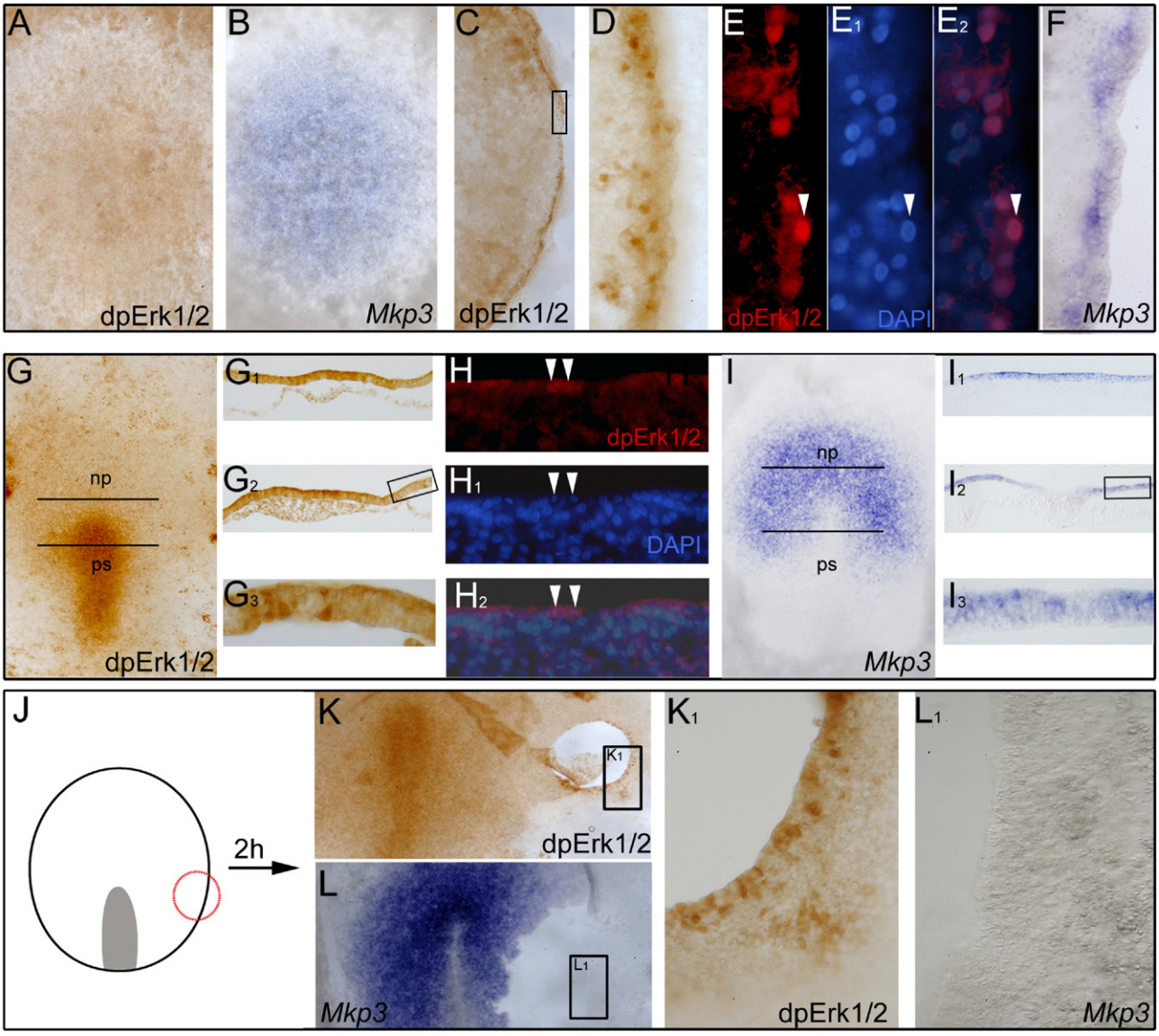

Fig. 3. Erk1/2 activity and *Mkp3* expression patterns in pre- and early primitive streak embryos and during wound healing. (A), DpErk1/2 and (B), *Mkp3* are detected at low levels in the prestreak epiblast. DpErk1/2 is located in the nucleus and cytoplasm of cells at the edge of spreading blastodisc (C, D, E, E_1) (DAPI) and (E_2) (DpErk/DAPI merged) (black outlined box in panel C indicates panel D, white arrowheads indicate nuclear DpErk) along with (F), *Mkp3*. DpErk1/2 is detected in the presumptive neural plate and primitive streak at HH3, (G), and TSs (G_1, G_2, G_3, H, H_1)(DAPI), (H_2) (DpErk/DAPI merged), white arrowheads indicate dpErk1/2 positive cells with DAPI labelled mitotic figures, black outlined box in panel G_2 indicates panel G_3. *Mkp3* at HH3 (I) and TSs (I_1,I_2, I_3) black outlined box in panel I_2 indicates panel I_3. Location of wound (red) in HH3 embryo (J). DpErk1/2, (K, K_1) and *Mkp3* (L, L_1) following wounding. Abbreviations as in Fig. 1.

K_1, L, L_1). Interestingly, dpErk1/2 is only detected around the lateral edge of the wound and is absent on the side abutted by *Mkp3* expressing neural plate (Figs. 3K_1, L_1).

Erk1/2 activation and Mkp3 at late primitive streak/gastrula stage (HH4+)

By HH4+ a gradient of Erk1/2 activation is apparent across the neural plate; high levels are detected at the ventral midline and node and decline laterally (Figs. 4A, A_1–A_4). A similar pattern of *Mkp3* expression is seen in the neural plate at this stage (Figs. 4B, B_1–B_4). DpErk1/2 and *Mkp3* are now also both present at low levels in the epiblast layer of the primitive streak (Figs. 4A_3, A_4; B_3, B_4).

Erk1/2 activation and Mkp3 at headfold stages (HH5–6)

By HH5–6 dpErk1/2 activation and *Mkp3* levels decline in the neural plate, but high levels remain at the ventral midline (Figs. 4C, C_1, C_2; D, D_1–D_4). Laterally both are detected in the precardiac mesoderm and associated underlying endoderm (Figs. 4C_2, D_2). High levels of dpErk1/2 and *Mkp3* are now detected in the node, primitive streak and its flanking epiblast. At these stages dpErk1/2 and *Mkp3* still exhibit left–right asymmetry in the node, with high levels on the right hand side. Within the primitive streak dpErk1/2 and *Mkp3* are clearly present in epiblast cells but *Mkp3* transcripts are additionally detected in emerging paraxial mesoderm, which exhibits little Erk1/2 activity (Figs. 4C_3, C_4).

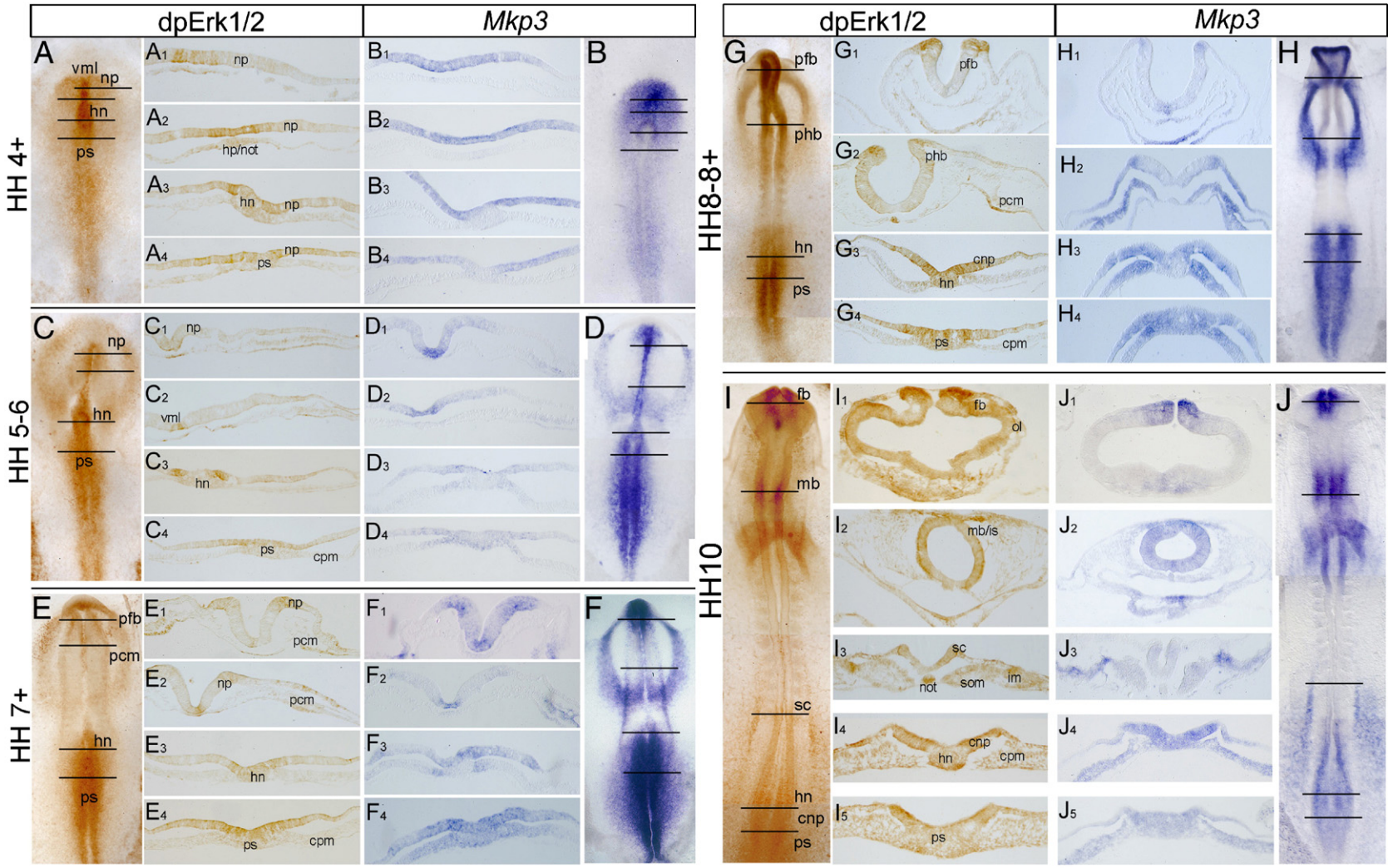

Fig. 4. Erk1/2 activity and *Mkp3* expression patterns from late primitive streak to 10 somite embryos. At HH4+ dpErk1/2, (A) and TSs (A_1) (rostral neural plate, laterally precardic mesoderm and endoderm), (A_2) (headprocess/notochord and neural plate), (A_3) (node), (A_4) (primitive streak, presomitic mesoderm) and *Mkp3*, (B) and TSs through the same region as in panel A, (B_1, B_2, B_3, B_4). At HH5–6 dpErk1/2, (C) and TSs (C_1, C_2, C_3, C_4) and *Mkp3*(D), and TSs (D_1, D_2, D_3, D_4) (TS levels as in panel A). At HH7+ dpErk1/2 (E), and TSs (E_1,E_2, E_3, E_4) and *Mkp3* (F), and TSs (F_1, F_2, F_3, F_4) (TS levels as in panel A). At HH8–8+ dpErk1/2 (G) and TSs (G_1) (forebrain), (G_2) (hindbrain, laterally precardic mesoderm and endoderm), (G_3) (node), (G4) (primitive streak, presomitic mesoderm) and *Mkp3*, (H), and TSs (H_1, H_2, H_3, H_4) (TS levels as in panel G). At HH9+10-, dpErk1/2 (I), and TSs (I_1) (forebrain), (I_2) (isthmus/midbrain/hindbrain border, heart), (I_3) (spinal cord, somites, intermediate mesoderm), (I_4) (node), (I_5) (primitive streak, caudal presomitic mesoderm) and *Mkp3* (J), and TSs (J_1, J_2, J_3, J_4, J_5) (TS levels as in panel I). Abbreviations as in Figs. 1 and 2 and hp/not=head process/notochord; vml=ventral midline; mb/is=midbrain/isthmus.

Erk1/2 activation and Mkp3 at early somite stages (HH7+)

At HH7+ dpErk1/2 and *Mkp3* are now strongly detected in the dorsal neural folds of the prospective forebrain, in the ventral midline/floorplate throughout the neural axis and in precardiac mesoderm and endoderm (Figs. 4E, F; E_1, F_1). *Mkp3* is also expressed at high levels in the prospective caudal hindbrain, while dpErk1/2 is weakly detected here and at the lateral edges of the neural plate (Figs. 4E, F; E_2, F_2). Both dpErk1/2 and *Mkp3* are detected asymmetrically in the node (Figs. 4E, F; E_3, F_3). Within the primitive streak, dpErk1/2 is detected most robustly in the epiblast layer, while strong *Mkp3* expression persists in the emerging presomitic mesoderm (Figs. 4E, F; E_4, F_4).

Erk1/2 activation and Mkp3 in early neurulation stages (HH8–8+)

By HH8 the prospective forebrain has extended rostrally and its lateral border is no longer underlain by precardiac mesoderm/associated endoderm (Figs. 4G, H). At this stage dpErk1/2 and *Mkp3* are both detected in the forebrain, dorsally and at the ventral midline, as well as in the underlying foregut (Figs. 4G_1, H_1). At the level of the hindbrain *Mkp3* and dpErk1/2 are present in the neuroepithelium, the notochord, the caudally displaced precardiac mesoderm, the first formed somite(s) and the adjacent ectoderm (Figs. 4G_2, H_2). A gap in Erk1/2 activity and *Mkp3* expression then appears in the embryo caudal to this level and both are then detected again in the caudally extending body axis in the primitive streak, adjacent epiblast/caudal neural plate and caudal paraxial mesoderm (Figs. 4G, $G_{3,4}$; H, $H_{3,4}$).

Erk1/2 activation and Mkp3 at the 10 somite stage (HH10)

Many of the domains of activated Erk1/2 and *Mkp3* described above have been refined by the 10 somite stage and some new sites of detection are apparent (Figs. 4I, J). DpErk1/2 and *Mkp3* are both found in the rostral forebrain (Figs. 4I_1, 4J_1). DpErk1/2 is also present in a dorsal to ventral gradient in the forming isthmus at the junction of the midbrain/hindbrain (4I_2, 4J_2). *Mkp3* appears more uniform in this region, but this may reflect a requirement for a more precise comparison in the same embryo (Figs. 4I_2, J_2). DpErk1/2 and *Mkp3* are both expressed at a low level in the vicinity of caudal hindbrain; a region overlying the developing heart in which they are also detected (Figs. 4I, J; I_2, J_2). DpErk1/2 and *Mkp3* continue to be conspicuously absent from the body axis at the level of the somites, but are detected caudal to this in the open neural folds, intermediate mesoderm, caudal notochord, caudal neural plate and primitive streak (Figs. 4I_3–I_5 and 4J_3–J_5). Caudal paraxial mesoderm emerging from the streak still has lower levels of dpErk1/2 than *Mkp3* (Figs. 4I_5, J_5). Low level dpErk1/2 and *Mkp3* are also now apparent in medial rostral presomitic mesoderm and in lateral plate mesoderm at the level of the future forelimb bud (Figs. 4I, I_5; J, J_5).

FGF signalling is required for most Erk1/2 activity and Mkp3 expression in the early chick embryo

To assess whether dpErk1/2 and *Mkp3* in embryonic tissue reflect activation via the FGF signalling pathway we cultured HH10 embryos in the presence of the FGF receptor inhibitor SU5402 for 2 h and compared both dpErk1/2 and *Mkp3* levels with control DMSO only treated embryos. In all cases ($n=5$) we found a reduction in dpErk1/2 in key sites, including dorsal forebrain, isthmus, developing heart and all caudal tissues in SU5402 exposed embryos compared with untreated controls ($n=5$)(Figs. 5A, B). A similar attenuation was also observed in *Mkp3* expression ($n=9$ cases) compared with DMSO only treated control embryos, although some transcripts are still detected in lateral plate mesoderm which expresses very high levels of *Mkp3* ($n=7$; Figs. 5C, D).

We also assessed levels of Erk activity and MKP3 expression in whole stage 10 chick embryos by Western blot. This shows that total Erk activity is reduced when FGF signalling is blocked for 2 h, while MKP3 levels decline dramatically (Fig. 5E). This difference likely reflects Erk stimulation associated with an FGF independent wounding response (Christen and Slack, 1999) induced when the embryo is cut out or detached from the vitelline membrane in culture filter. When Erk signalling is blocked with the MEK inhibitor PD184352, both Erk activity and MKP3 levels are lost (Fig. 5E). Overall these findings indicate that activation of the Erk1/2 signalling pathway in the early chick is, as in the mouse embryo (Corson et al., 2003), largely FGF dependent and in most tissues confirm the regulatory relationship between FGF/Erk1/2 signalling and *Mkp3* expression (Eblaghie et al., 2003; Kawakami et al., 2003; Smith et al., 2006; Tsang et al., 2004).

Analysis of Ets factor expression patterns

To reveal diversity in response to Erk1/2 signalling at different times and in different tissues we next examined the repertoire of Pea3 family Ets factors expressed during early development.

Ets gene expression at pre- and early primitive streak stages (EG, X- HH3+)

Only *Pea3* expression is detected in prestreak embryos, where it is localised in the epiblast layer (Figs. 6A–C_1). Once the streak has formed however, *Erm* and *Pea3* are broadly expressed in the epiblast which includes the neural plate (Figs. 6D, D_1; E, E_1). They are also detected in the primitive streak (Figs. 6D_2, E_2), where they overlap with *Er81* expression (Figs. 6F, F_2).

Ets gene expression at late primitive streak/gastrula stage (HH4+–5)

All three Ets genes are expressed at HH4+–5; *Erm* and *Pea3* are expressed throughout the neural plate and primitive streak and while *Er81* is restricted to the primitive streak (Figs. 6G, H,

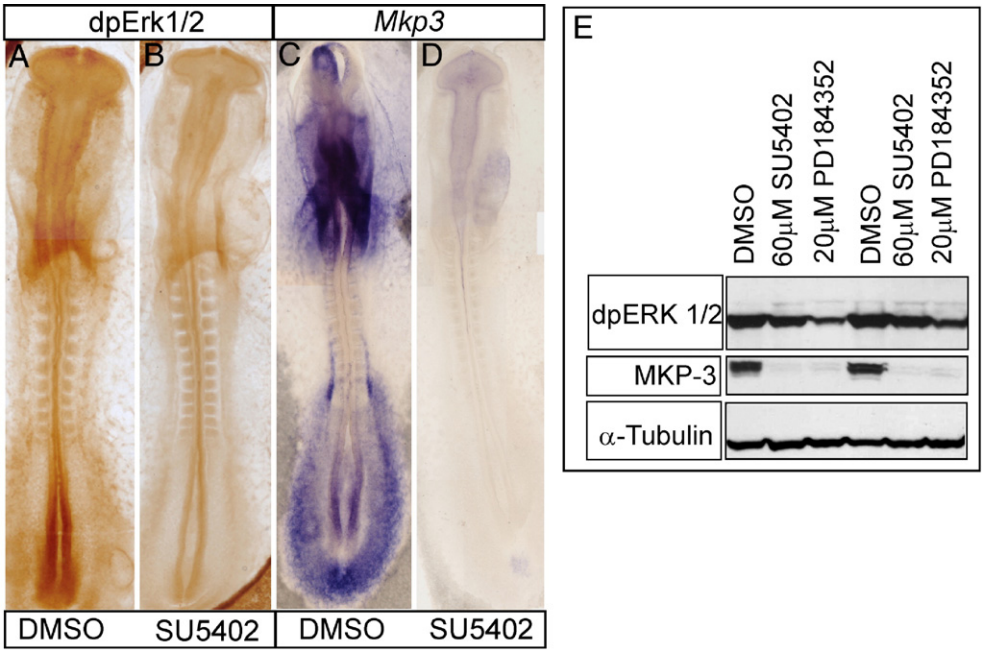

Fig. 5. FGF signalling is required for Erk1/2 activity and *Mkp3* expression throughout the 10 somite embryo. DpErk1/2 in embryos exposed to control DMSO (A) and the FGF receptor antagonist SU5402 (B). *Mkp3* in embryos exposed to control DMSO (C) and SU5402 (D). Western blots of whole HH10 embryos exposed to DMSO, SU5402, PD184352, (E). Three separate experiments were carried out for western blotting, two of which are shown here.

I; G_1, H_1, I_1). *Erm* appears absent/weakly expressed in the node (Figs. 6G, G_2), however, this region strongly expresses *Pea3* and *Er81* (Figs. 6H_2, I_2).

Ets gene expression at headfold/early segmentation (HH6–7)

As the notochord emerges from the primitive streak *Erm*, the most widely expressed of these Ets genes, is found throughout the neural plate with the exception of intermediate regions of the rostral neural plate (Figs. 6J, J_1). In contrast, *Pea3* expression is lost from this tissue except in the ventral midline (floor plate and head process/notochord) (Figs. 6K, K_1). *Pea3* is also now present in the prospective caudal hindbrain, somites, caudal neural plate, precardiac mesoderm, emerging axial and caudal paraxial mesoderm and the primitive streak (Fig. 6K, K_1, K_2). *Er81* expression is confined to the primitive streak, where its levels of expression in the node are asymmetric (Figs. 6L, L_1, L_2).

Ets gene expression at early neurulation stages (HH8–8+)

Erm and *Pea3* are both expressed in the dorsal forebrain and the prospective caudal hindbrain and precardiac mesoderm (Figs. 6M, N; M_1, N_1), but *Erm* expression is present throughout the neural axis to the level of the somites. Strikingly, both *Erm* and *Pea3* are then absent from neural tube flanked by somites, although *Pea3* continues to be expressed in somites (Figs. 6M, N). In caudal regions both *Erm* and *Pea3* are expressed in the open neural folds, caudal neural plate and primitive streak (Figs. 6M, N; M_2, N_2). Strikingly, both these genes are also detected in the paraxial mesoderm but *Pea3* is expressed more strongly than *Erm*, while *Er81* expression is confined to the caudal neural plate and primitive streak (Figs. 6O, O_1, O_2).

Ets gene expression at 10 somite stage (HH10)

Erm continues to be expressed throughout the neural tube rostral to the forming somites and in cardiac mesoderm (Figs. 6P, P_1, P_2). It is additionally detected in ectoderm at the level of the future otic vesicle (Fig. 6P_2) and at low level in recently formed somites and intermediate mesoderm (Figs. 6P, P_3). *Erm* is then expressed extensively in caudal tissues, throughout presomitic mesoderm, caudal neural tissue and the primitive streak (Figs. 6P, P_3, P_4). *Pea3* is present in rostral forebrain, isthmus region of the midbrain/hindbrain, caudal hindbrain and adjacent ectoderm as well as precardiac mesoderm, somites and the intermediate mesoderm (Figs. 6Q, Q_{1-3}). *Pea3* is further detected in rostral and caudal presomitic mesoderm as well as caudal neural tissue and the primitive streak (Figs. 6Q, Q_4). *Er81* is weakly expressed in the dorsal forebrain (Figs. 6R, R_1) and is detected in precardiac mesoderm (Fig. 6R, R_2), lateral plate mesoderm, caudal neural plate and the primitive streak (Figs. 6R, R_{3-4}).

Discussion

Key sites of Erk1/2 activation are characterised by distinct repertoires of Fgfrs, Mkp3 and Ets factor expression

The repertoire of *Fgfr*, *Mkp3* and *Ets* factor expression patterns in key regions of Erk1/2 activation are summarised in Fig. 7. These observations provide insight into the involvement of Erk1/2 activity in early FGF mediated developmental

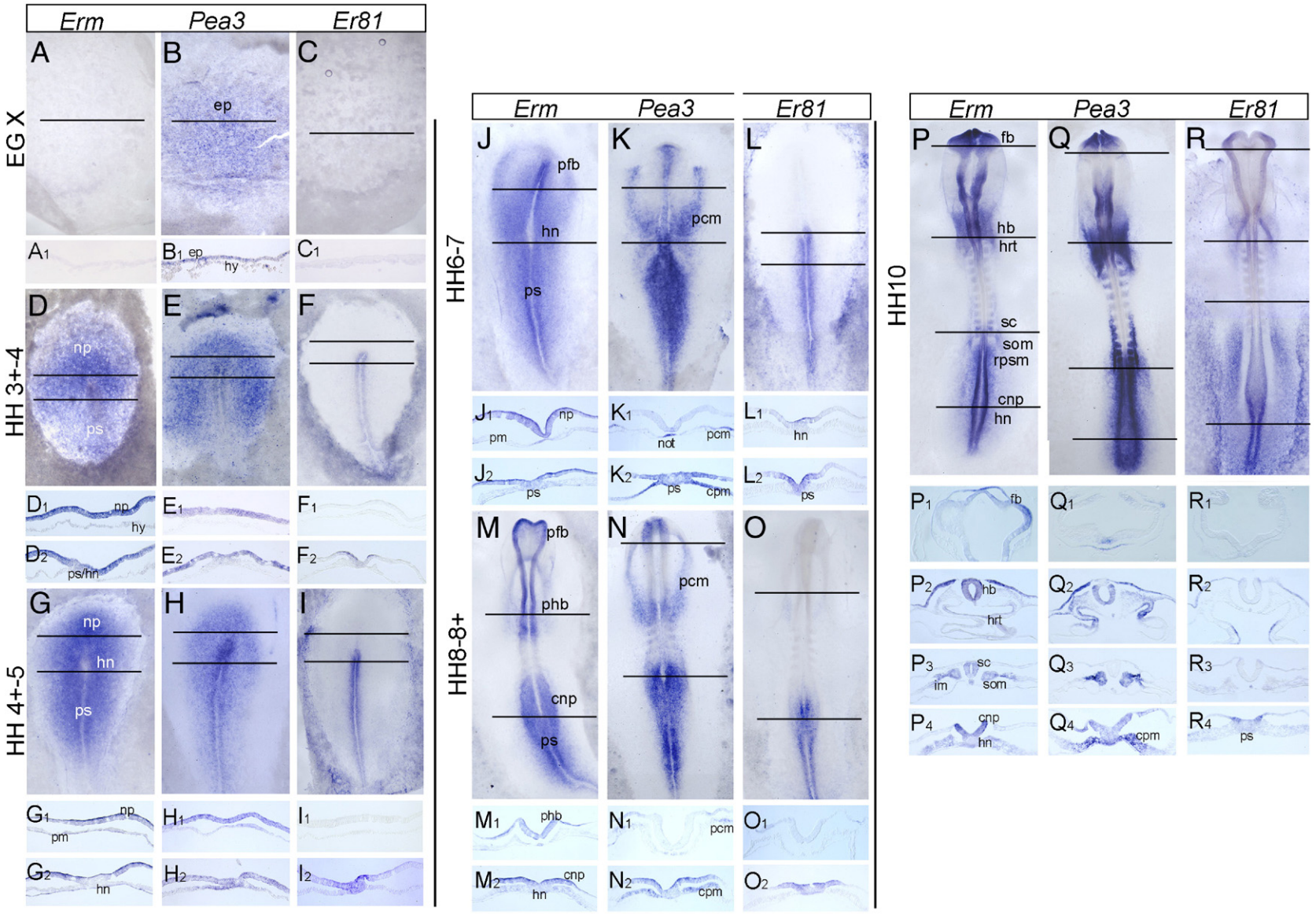

Fig. 6. Ets transcription factor expression patterns from prestreak to 10 somite embryos. Expression patterns of the Ets factors (A) *Erm*, (B) *Pea3* and (C) *Er81* at prestreak stages (all TSs through epiblast and hypoblast layers). At HH3+–4 *Erm* (D) (TSs through (D_1) neural plate and (D_2) node), *Pea3* (E), (E_1, E_2) (TSs as in panel D) and *Er81*(F, F_1, F_2) (TSs as in panel D) are all expressed. At HH4+–5 the nested expression of these Ets genes is particularly apparent, *Erm*, (G, G_1, G_2); *Pea3*, (H, H_1, H_2), and *Er81*, (I, I_1, I_2). At HH6–7, *Erm*, (J, J_1, J_2) *Pea3*, (K, K_1, K_2), and *Er81*, (L, L_1, L_2) (all TSs as in panel D). At HH8–8+, *Erm* (M) (TSs through (M_1) hindbrain and (M_2) primitive streak/caudal neural plate; *Pea3* (N) (TSs through (N_1) forebrain, precardic mesoderm and (N_2) and primitive streak/caudal neural plate and *Er81* (O) (TSs (O_1) and (O_2) as in panel M). At HH10 *Erm* (P), TSs through (P_1) (forebrain), (P_2) (hindbrain, heart), (P_3) (spinal cord, somites, intermediate mesoderm), (P_4) (node); *Pea3* (O, O_1, O_2, O_3, O_4) (TS levels as in pane P) and *Er81*, (R, R_1, R_2, R_3, R_4) (primitive streak). Abbreviations as in Figs. 1 and 2.

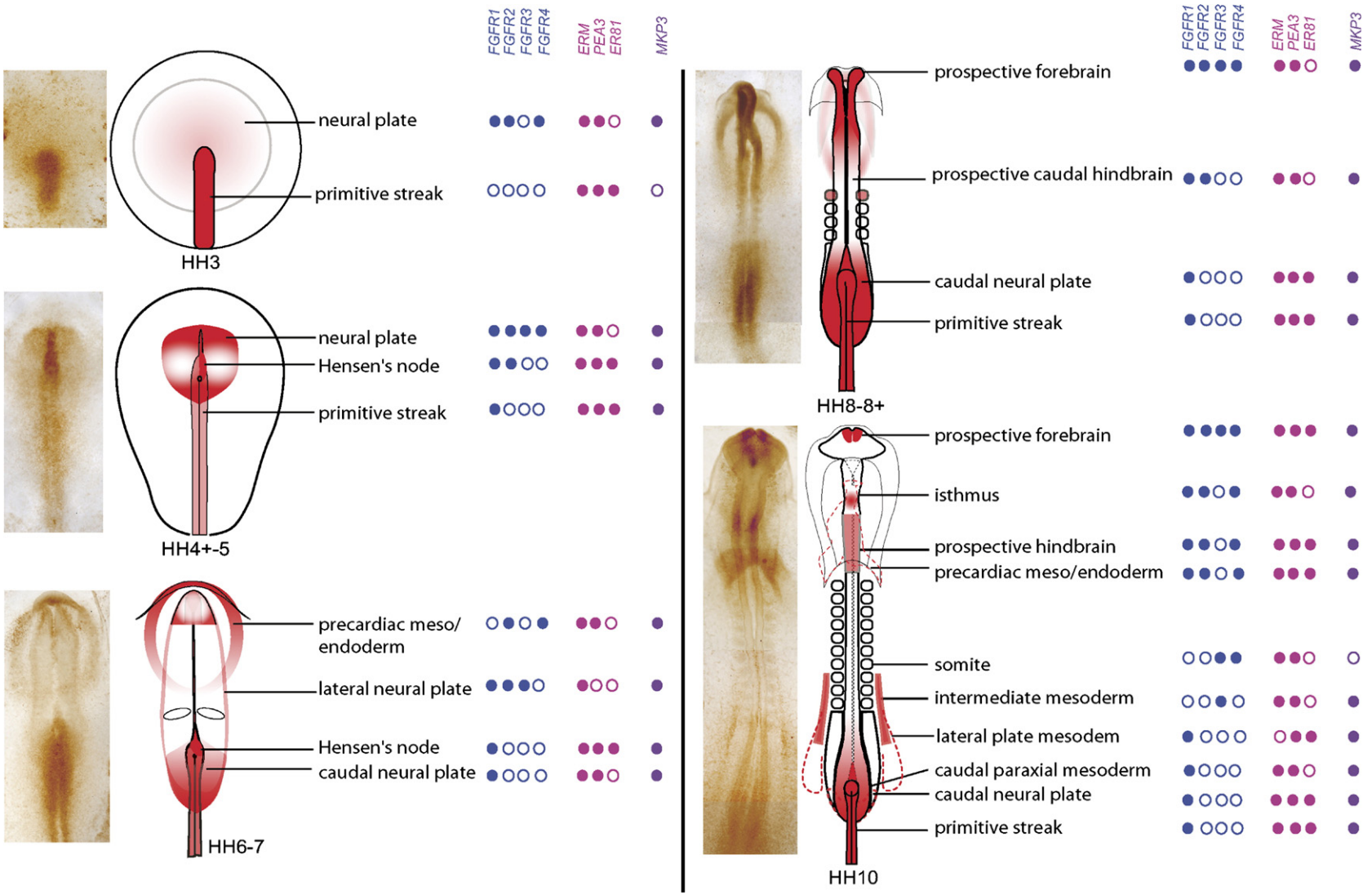

Fig. 7. Atlas of FGF/Erk1/2 activity and *Fgfr*, *Ets* factor and *Mkp3* expression repertoires from early primitive streak to the 10 somite embryo. Summary of the main sites of Erk1/2 activity (DAB/brown labelling in embryos, red in schematics) at key stages with associated repertoires of *Fgfrs*, *Ets* and *Mkp3* expression.

processes and give an indication of how the context of Erk1/2 activation varies during the generation of different tissues. Here we discuss these findings with respect to some key developmental events.

Primitive streak cells give rise to the endodermal and mesodermal layers of the embryo. A number of FGFs are expressed in and around the early primitive streak (FGF2, *Fgf3; Fgf4; Fgf8*) (Mahmood et al., 1995; Riese et al., 1995; Shamim and Mason, 1999; Streit et al., 2000) and see (Karabagli et al., 2002) and its formation depends on a combination of FGF, Nodal and Chordin signalling (Bertocchini et al., 2004). Strikingly, although dpErk1/2 and all three Ets factors are detected in the early primitive streak we find that this is not accompanied by expression of *Fgfrs* or *Mkp3*. This surprising finding suggests that very low level *Fgfr* expression, an unknown *Fgfr* or another pathway is responsible for this early site of Erk activity. By late primitive streak stages *Fgfr1* and *Mkp3* are now both present in this tissue and their appearance coincides with the onset of cell movement through the primitive streak as gastrulation commences.

Neural induction is initiated by FGF signalling in the chick prior to gastrulation (Streit et al., 2000; Wilson et al., 2000) and the detection of dpErk1/2 in the epiblast layer of prestreak embryos where *Fgfrs 1* and *4*, *Mkp3* and *Pea3* are also expressed, corresponds well with induction by FGF8 presented by the underlying hypoblast (reviewed in Stern, 2005). By early streak stages dpErk1/2 levels have increased in the neural plate which now additionally expresses *Fgfr2* and *Erm*. At this time dpErk1/2 and *Fgfr2* are also detected in the hypoblast underlying the neural plate. Our data reveal that the neural plate is a major site of dpErk1/2 activity. This then becomes patterned as the axial mesoderm emerges beneath the neural plate; dpErk1/2, *Mkp3, Pea3* and *Erm* are now most strongly detected in ventral and lateral (future dorsal) regions and at low level in presumptive hindbrain region of the neural plate. As development proceeds Erk activity becomes increasingly restricted within the neuroepithelium with key sites in the dorsal forebrain (with *Fgfrs1,4, Mkp3, Erm* and *Pea3*) and isthmus region of the midbrain/hindbrain (with *Fgfr1, 2*, (weakly *r4*) *Mkp3, Erm* and *Pea3*). Importantly, cells in the caudal neural plate which progressively give rise to the caudal hindbrain and spinal cord also continue to experience Erk1/2 activity and express *Fgfr1, Mkp3*, *Erm*, *Pea3*, and *Er81*.

The number of *Fgfrs* expressed in the neuroepithelium also increases during development and distinct regions within this tissue are characterised by specific combinations of these genes: for example, in contrast to the caudal neural plate (above), rostral-most neural plate (prospective forebrain) expresses all four *Fgfrs*. Furthermore, expression of additional *Fgfrs* reflects differentiation onset in the forming spinal cord at HH10. Here undifferentiated cells in the caudal neural plate express *Fgfr1*, but as cells leave this region and form the preneural tube (rostral to the node) they express *Fgfrs1* and *2*. At the level of the closed neural tube flanked by somites where neuronal differentiation and ventral patterning commence (Diez del Corral et al., 2002) *Fgfrs 1,2, 3* (and eventually r4) are now expressed.

The establishment of left–right body axis in the embryo commences in the node at HH5 (Levin et al., 1995). *Fgf8* and *Fgf4* are asymmetrically expressed in the node at this time (Boettger et al., 1999; Shamim and Mason, 1999) and *Fgf8* has been shown to be a key signalling molecule regulating left–right asymmetry, determining the turning of the embryo and positioning of the heart, gut and other visceral organs (Boettger et al., 1999). Here we note that dpErk1/2 activity and *Mkp3*, *Fgfr1, Pea3* and *Er81* are also asymmetrically localised in the node at these stages, indicating that Erk1/2 signalling is likely to mediate FGF8 activity in this context. Specification of cardiac mesoderm is also mediated by FGF8, which is expressed by endoderm underlying this presumptive cardiac tissue (Alsan and Schultheiss, 2002). Here we identify this precardiac mesoderm and associated endoderm as sites of active Erk signalling that express *Fgfrs 1*, *2* (and weakly *4*), *Mkp3, Erm, Pea3* and *Er81*.

The emergence of the paraxial mesoderm, which forms somites that give rise to skeletal muscle, dermis and vertebrae, commences at late primitive streak stages, at the time when *Fgfr1* is first detected in the streak. Although dpErk1/2 is detected in the primitive streak at all stages, only low levels were found in recently emerged caudal presomitic mesoderm, consistent with the presence of only degrading *Fgf8* transcripts in this region (Dubrulle and Pourquie, 2004). However, as this tissue expresses *Mkp3* which is dependent on Erk1/2 activity, it seems likely that such signalling is still active here. At HH10 there also appears to be a second peak of Erk activity and *Mkp3* expression in the rostral medial presomitic mesoderm which may be associated with the raised levels of *Fgfr1* in this tissue at these later stages. Presomitic tissue also expresses a specific combination of Ets factors; *Erm* is detected at low levels throughout the presomitic mesoderm, *Pea3* is expressed strongly as this tissue first emerges from the primitive streak, while *Er81* is confined to the epiblast layer of the streak. Importantly, Ets genes appear to be induced by different thresholds of FGF signalling in the early zebrafish ectoderm with *Erm* requiring a lower level than *Pea3* (Roehl and Nusslein-Volhard, 2001). This regulatory relationship correlates well with the expression patterns and levels of these Pea3 family Ets genes in the caudal presomitic mesoderm and also within the developing nervous system, where *Erm* is more widely expressed than *Pea3,* and *Er81* transcripts are found in a subset of regions with high Erk1/2 activity (see Fig. 7).

The relationship between FGF signalling and Erk1/2 activity

The detection pattern of dpErk1/2 coincides well with the location of cell populations likely to be exposed to known sources of FGF ligand and in particular *Fgf8* (Karabagli et al., 2002). We have shown previously that expression of *Mkp3* in the limb and early neural plate depends on FGF/Erk1/2 signalling (Eblaghie et al., 2003; Smith et al., 2006) and demonstrate here a striking localisation of *Mkp3* expression to nearly all sites of dpErk1/2 activity in the early embryo. The dependency of dpErk1/2 activation and *Mkp3* expression on FGF signalling is also demonstrated by their near complete loss

when we block FGF signalling in 10 somite stage embryos. The sites of FGF dependent Erk1/2 signalling identified here are the same as those characterised in the 10 somite mouse embryo (excluding the intermediate mesoderm and extra embryonic tissues (Corson et al., 2003), indicating that this is a conserved regulatory relationship.

As noted above, at very early stages there is an exception to this regulatory relationship. The primitive streak is exposed to FGFs, but does not express any of the *Fgfrs* examined in this study or the FGF/Erk1/2 dependent gene *Mkp3*. FGF dependent Erk1/2 activation is characteristic of early primitive streak equivalent tissue in Xenopus (Christen and Slack, 1999; Curran and Grainger, 2000; Schohl and Fagotto, 2002) and Zebrafish (Tsang et al., 2004) although little Erk1/2-activation is detected in the early mouse primitive streak (Corson et al., 2003). In the mouse, zebrafish and frog, *Mkp3* is, however, expressed in the primitive streak/equivalent tissue (Dickinson et al., 2002; Klock and Herrmann, 2002; Mason et al., 1996; Tsang et al., 2004). Although there must be differences in turn over rate of protein and mRNA these discrepancies may reflect species specific differences in the timing/extent of Erk1/2 activation in the early primitive streak and the repertoire of FGF induced Erk1/2 antagonists deployed in this tissue (Tsang and Dawid, 2004).

Conversely, there are regions of the embryo in which FGF receptors are expressed, but in which Erk1/2, Mkp3 and Ets genes are not detected. These include the caudal forebrain at HH10 and in particular, the neural tube flanked by somites, which expresses *Fgfrs1*, *2* and *3*, but lacks the latter set of genes indicative of dpErk1/2 activation at these early stages. Although other FGF downstream pathways could be active, this observation may support the generalisation that restriction of ligand, rather than receptor distribution controls pathway activation during early development.

Distribution and dynamics of Erk1/2 activity

Graded dpErk1/2 levels are detected across the neural plate with high medial levels falling away laterally at HH4+ and this is mirrored by *Mkp3* expression with both dpErk1/2 and *Mkp3* becoming restricted to lateral and medial regions of the neural plate by HH5–6. A similar gradient of activation is also apparent in the caudal neural plate, with cells close to the primitive streak possessing higher levels than more rostral tissue and this is again reflected in the levels of *Mkp3*. Although a gradient of dpErk1/2 is less apparent in the caudal paraxial mesoderm, cells in the dorsal primitive streak have higher levels of dpErk1/2, suggesting that activation declines as cells move through and emerge from the streak. A graded decline in Erk activity is also indicated by the gradual loss of *Mkp3* transcripts from caudal presomitic mesoderm and this is most evident at HH10. Finally, in the presumptive isthmus region at the midbrain/hindbrain boundary a gradient of dpErk1/2 is detected from dorsal to ventral.

There are also cell populations in the embryo which experience sustained dpErk1/2. These include the primitive streak, caudal neural plate, cardiac mesoderm and prospective dorsal forebrain. In contrast, some tissues clearly experience transient Erk1/2 activation; these include the early neural plate, which has broad low level dpErk1/2 that is lost (as noted above in a graded fashion) and cells that are displaced out of the caudal neural plate and primitive streak into the newly differentiating body axis. Sustained versus transient Erk activity has been shown to regulate proliferation versus differentiation in several contexts (Marshall, 1995). Interestingly, at these early stages of chick embryogenesis dpErk1/2 was only detected in known proliferating cell populations.

With the exception of the edge of the spreading blastodisc, the closing wound and the dividing cells in the primitive streak, all dpErk1/2 detected appeared to be localised in the cytoplasm. The detection of nuclear dp-Erk1/2 in some sites strongly suggests that this cytoplasmic labelling is an accurate indication of the sub-cellular location of Erk signalling in chick embryonic tissues. A similar observation has been made in the mouse embryo (Corson et al., 2003), where it is suggested that there may be low levels or fast turnover of nuclear dpErk1/2, mediated by the presence of MKP1 and 2 in the nucleus. Our data suggest that this may also be due to the presence of MKP3 at these sites as this cytoplasmically located phosphatase not only specifically binds to and inactivates dpErk1/2 (and can thereby set the level of Erk signalling) but can also anchor Erk2 in the cytoplasm and so regulate the sub-cellular localisation of Erk activity (Karlsson et al., 2004). This is consistent with the absence of *Mkp3* expression at the wound edge and the early streak where we detect nuclear as well as cytoplasmic dpErk1/2. Cell by cell analysis of Mkp3 and dpErk1/2 localisation in cells at the edge of the extra-embryonic epiblast is, however, required to determine whether this regulatory relationship holds in all cases. As noted by Corson and colleagues cytoplasmic Erk targets include p90Rsk which then translocates to the nucleus to regulate transcription of immediate early genes. Erk activity is also linked to chromatin remodelling, with nuclear Erk1/2 substrates, MSK1 and 2, phosphorylating nucleosomal proteins, while phosphorylation of the Ets factor Elk1 promotes association with histone acetyltransferases (HATs) (Edmunds and Mahadevan, 2004). Inhibition of Erk1/2 signalling also blocks cell migration in many contexts and Erk1/2 cytoplasmic phosphorylation targets include Integrin and Myosin light chain Kinase (MLCK), Calpain, FAK and Paxillin, which mediate membrane protrusions and focal adhesion dynamics/turn over (reviewed in Huang et al., 2004). Indeed, in the early chick embryo, Erk activity has recently been shown to regulate cell movement in the presomitic mesoderm (Delfini et al., 2005).

In conclusion, Erk1/2 activity can direct diverse cellular processes, including proliferation/differentiation and cell movements as well as specification of distinct cell fates and can act via a range of mechanisms to alter gene transcription (Dailey et al., 2005) in addition to regulation of the cytoskeleton. Here we define key sites of Erk1/2 activity in the early chick embryo and characterise them with respect to expression of *FGF receptors*, *Mkp3* and three Erk1/2 phosphorylation targets, *Erm, Pea3* and *Er81*. We demonstrate that *Mkp3* expression is a good read out of Erk1/2 activity in the early embryo and that almost all Erk1/2

activity and *Mkp3* expression depends on FGF signalling at these stages. Furthermore, the globally nested expression of the Pea3 family of transcription factors correlates well with levels of Erk1/2 activity. Together these data have allowed us to create a spatial and temporal map of FGF/Erk1/2 syn-expression groups, which can be used to help identify further members of the regulatory networks that provide specificity downstream of FGF/Erk1/2 signalling.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.10.014.

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