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Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development

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Abstract

Several molecules of the Fibroblast growth factor family have been implicated in the development of the vertebrate brain, but the effectors of these molecules remain largely unknown. Here we study Erm and Pea3, two ETS domain transcription factors, and show that their expression correlates closely with the domains of *fgf8* and *fgf3* expression. In situ hybridization analysis in wild-type and *acerebellar* (*ace*) mutant embryos defective for *fgf8* demonstrates a requirement of Fgf8 for normal expression levels of *erm* and *pea3* transcripts in and close to various domains of Fgf8 action, including the prospective midbrain–hindbrain region, the somites, the neural crest, the forebrain, and developing eyes. Morpholino-oligomer-assisted gene knock-down experiments targeted against *fgf8* and *fgf3* suggest that Fgf3 and Fgf8 are co-regulators of these genes in the early forebrain anlage. Furthermore, inhibition of Fgf signaling by overexpression of *sprouty4* or application of the Fgf inhibitor SU5402 leads to a loss of all *erm* and *pea3* expression domains. Conversely, ectopically provided *fgf3* mRNA or implanted beads coated with Fgf8 elicit ectopic transcription of *erm* and *pea3*. Both activation and loss of transcripts can be observed within short time frames. We conclude that both the transcriptional onset and maintenance of these factors are tightly coupled to Fgf signaling and propose that *erm* and *pea3* transcription is a direct readout of cells to Fgf levels. Given the knowledge that has accumulated on the posttranslational control of ETS domain factors and their combinatorial interactions with other transcription factors, we suggest that the close coupling of *erm* and *pea3* transcription to Fgf signaling might serve to integrate Fgf signaling with other signals to establish refined patterns in embryonic development.

Keywords: ETS genes; *erm*; *pea3*; *fgf3*; *fgf8*; *sprouty*; *no isthmus*; *acerebellar*; Zebrafish; Cell signaling; Central nervous system; Midbrain–hindbrain boundary; Forebrain

1. Introduction

Molecules of the Fibroblast growth factor (Fgf) family play crucial roles in patterning the vertebrate embryo (Martin et al., 1998; Ornitz and Itoh, 2001). During regionalization of the neural plate Fgf8, in particular, acts as a potent organizing molecule, affecting cellular events as diverse as cellular identity, cell survival, proliferation, and axonal navigation (Crossley et al., 1996; Lee et al., 1997; Reifers et al., 1998, 2000b; Meyers et al., 1998; Martinez et al., 1999; Picker et al., 1999; Shanmugalingam et al., 2000; Xu et al., 2000). To understand how these different responses are elicited, and how they lead to proper organization of the midbrain–hindbrain (MH) region, it is important to gain insight into the events downstream of Fgf receptor activity in the brain anlage of vertebrate embryos.

Pattern formation in the MH region occurs in several discrete steps. Initially, the interface between the expression domains of two homeodomain transcription factors of the Gbx ond Otx class positions the MH organizing center in the anterior neural plate already during gastrulation stages. Subsequently, several signaling pathways employing *wnt1*, *pax2.1*, and *fgf8* are initiated in discrete expression domains around the position of the incipient MH organizer. While expression of these genes is initiated independently of each other, their interplay is essential to produce and/or to maintain the MH organizer. Once generated, the organizer controls cell-type specificity in the surrounding neural plate through secreting patterning molecules like Fgf8, Fgf17, and Wnt1 (reviewed in Joyner et al., 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Nakamura, 2001).

It is currently not understood how the various signaling factors interact to specify distinct gene expression domains in the MH domain, and how the various signaling events are integrated and relayed. In this paper, we study the relationship between Fgf signaling and the Ets family transcription factors Erm and Pea3 as putative downstream targets. ETS

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domain proteins are a family of transcription factors that have been implicated in a variety of transcriptional regulatory events controlling normal and abnormal cell behavior, including proliferation, differentiation, and migration (reviewed in Wasylyk et al., 1998). These proteins share an 85 amino acid winged-helix–loop–helix domain (the Erythroblastoma Twenty-Six (ETS) domain) with which they bind to DNA as monomers. Characteristically, ETS domain factors are involved in transcriptional control mechanisms that specify diverse sets of gene expressions as they are needed for tissue patterning and lineage commitment, e.g. the commitment of lymphoid cells to the T cell lineage (Anderson et al., 1999). A recurring theme in ETS domain protein function is the ability of these factors to form heterocomplexes with other types of transcription factors. Examples include the interaction of TCF-like ETS proteins as Elk-1/SAP-1 with serum response factor on the *c-fos* promoter (Dalton and Treisman, 1992), the association of Ets-1 with the bZIP protein MafB to regulate transcription of the *transferrin receptor* gene (Sieweke et al., 1996), the interaction of Ets-1 with the POU Homeodomain of Pit-1 (Bradford et al., 2000 and references therein) and the recruitment of Ets-1, Net, or Elk-1 to the *mb-1* promoter by the paired homeodomain protein Pax5 (Fitzsimmons et al., 1996).

An additional feature of ETS domain proteins is their posttranslational modification by cell signaling pathways. Most prominently, the Ras–Raf–mitogen activated protein kinase (MAPK) pathway has been demonstrated to target family members of the ETS, YAN, ELF, ERF, TCF, and PEA3 classes of ETS domain proteins (see Wasylyk et al., 1998 and references therein). In *Drosophila*, as an example, specification of R7 photoreceptor development requires activation of the Ras-MAP kinase pathways by the SEVENLESS receptor tyrosine kinase. Two antagonistic ETS proteins serve as targets of MAP kinases in this system: PointedP2, which becomes activated, and YAN, which loses its R7 cell fate inhibitory function upon phosphorylation (O'Neill et al., 1994). Similarly, this system seems to have been employed in ventral ectoderm patterning (reviewed in Schweitzer and Shilo, 1997).

In this study, we focus on the transcriptional control of two vertebrate ETS domain factors, erm (ETS related molecule, Monte et al., 1994) and pea3 (Polyoma enhancer activator 3, Xin et al., 1992; Higashino et al., 1993) in early zebrafish development. Zebrafish Erm and Pea3 (Brown et al., 1998; Münchberg et al., 1999) constitute the only known zebrafish members of the Pea3 subgroup of ETS domain factors which in addition includes ER81 (Janknecht, 1996). ER81 has been proposed to act downstream of eFgf signaling in the gastrulating *Xenopus laevis* embryo (Münchberg and Steinbeisser, 1999) and to interfere with activin signaling when overexpressed in animal caps (Chen et al., 1999). Pea3 and ER81 have been characterized in chicken and mice as factors involved in the definition of motor neuron pools and corresponding sensory afferents (Lin et al., 1998, reviewed in Ghosh and Kolodkin, 1998). Loss of function studies with ER81 support that this subdivision is functionally relevant for the establishment of sensory-motor circuits at the limb level (Arber et al., 2000). Furthermore, in vitro studies have provided evidence that Pea3 can be activated through phosphorylation by MAP kinases of both the extracellular signal-related kinase (ERK) and the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (O'Hagan et al., 1996), and that Erm is targeted by MAP kinase and protein kinase A signaling (Janknecht et al., 1996), thus supporting their roles as nuclear effectors of extracellular signaling.

A brief report on the distribution of *erm* and *pea3* transcripts in zebrafish has appeared previously (Münchberg et al., 1999), but both the establishment and maintenance of their expression pattern as well as the possible function of these genes have remained unclear. Here we demonstrate that *erm* and *pea3* are expressed in close proximity to centers of Fgf signaling throughout embryogenesis and that they are highly susceptible to interference with intracellular Fgf signaling. Specifically, lack of *fgf8* gene function in the *acerebellar* (*ace*) mutant or morpholino-antisense 'knock-down' experiments affecting *fgf8* and *fgf3* translation suggests that Fgf8 and Fgf3 are two regulators of the transcription of *erm* and *pea3* in specific expression domains, including the MH region and the forebrain. Finally, RNA misexpression and bead implantation experiments show that both Fgf3 and Fgf8 are able to ectopically activate *erm* and *pea3* transcription with fast kinetics. We conclude that *erm* and *pea3* transcription is tightly linked to Fgf signaling and may provide a direct intracellular readout for Fgfs.

2. Results

2.1. pea3 and erm are co-expressed with fgfs during early embryonic development

In order to compare the transcriptional regulation of *erm* and *pea3* to sites of Fgf signaling, we performed whole-mount in situ hybridizations (ISHs) with antisense probes of full-length *erm* and *pea3* as well as *fgf8* and *fgf3*, two *fgf* genes that are expressed during early zebrafish development (Kiefer et al., 1996; Fürthauer et al., 1997; Reifers et al., 1998 and this paper). Analysis of *erm* and *pea3* expression reveals a strong correlation with the expression domains of both *fgf8* and *fgf3*, except for early cleavage stages, when *erm* and *pea3* RNAs are provided maternally and show global distribution throughout the embryo (Fig. 1 and data not shown). Transcripts become progressively restricted to the margin of the pregastrula, where *fgf8* and *fgf3* are also expressed. With the beginning of gastrulation, expression of *fgf8*, *erm*, and *pea3* diminishes ventrally, but stays strong dorsally, at the site of the embryonic shield (Fig. 1A). At 70% of epiboly, *fgf3* expression can be detected in the ante-

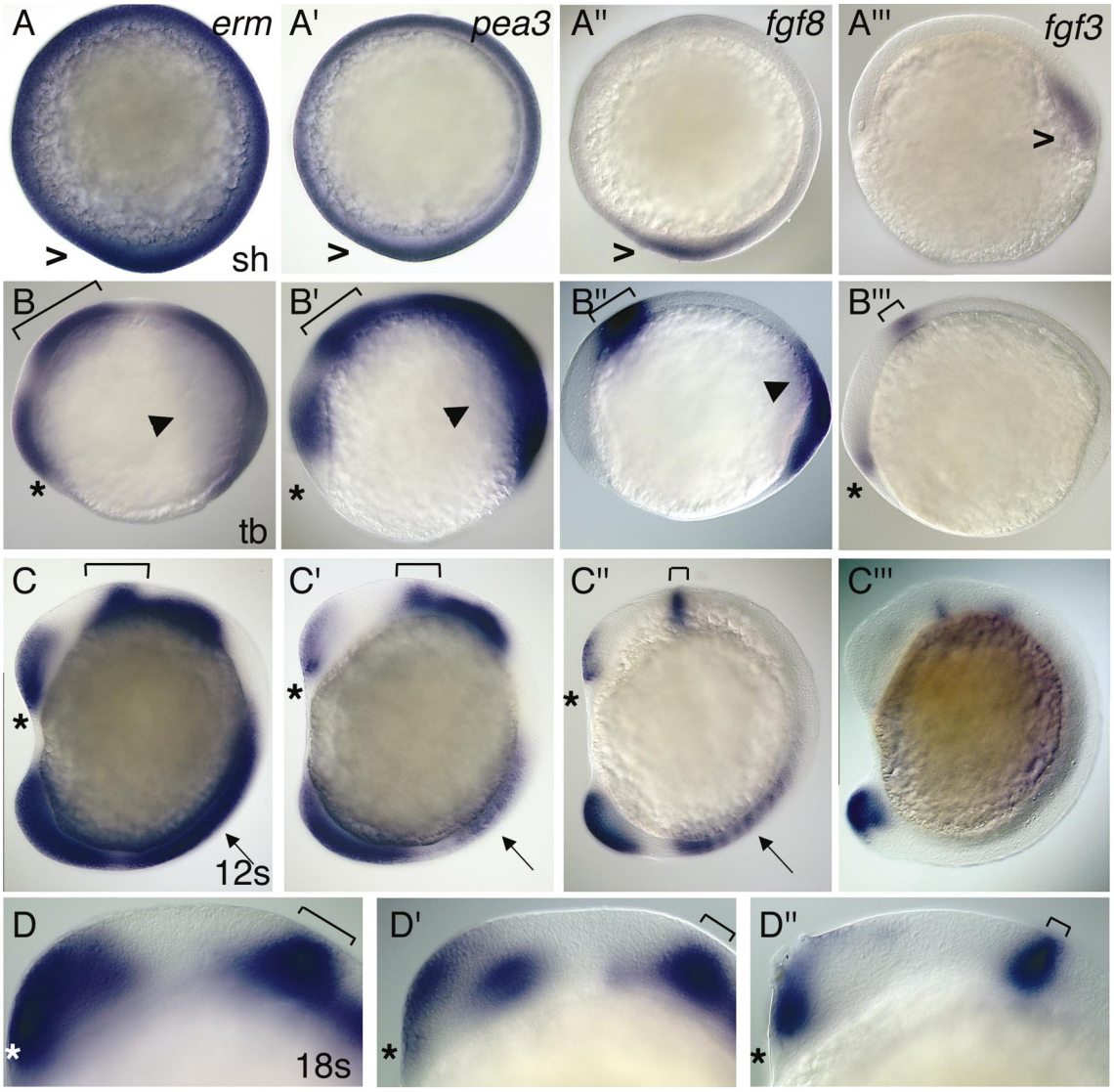

Fig. 1. Comparison of *erm* and *pea3* expression with the expression domains of *fgf8* and *fgf3*. Digoxigenin-labeled antisense probes of full-length *erm* (panels A–D), *pea3* (panels A′–D′), *fgf8* (A″–D″), and *fgf3* (A‴–C‴) were used for whole-mount ISHs of wild-type embryos of different stages of development as indicated (sh, shield stage; tb, tailbud; 12 and 18 s, 12 and 18 somite stages, respectively). (A–A″) Animal pole views with the shield to the bottom. (A‴) Lateral view, shield to the right. (B–B‴, C–C‴, D–D″) Lateral views, anterior to the left. Indicated are the expression in the shield (>), the margin of the ventrolateral expression domains during gastrulation (arrowheads), the expression in the forebrain (asterisks), at the level of the posterior midbrain/anterior hindbrain (brackets) and the somites (arrows). Prominent staining for *erm* and *pea3* at the hindbrain level in panels (C) and (C′) stems mostly from placodal expression and is paralleled by expression of both *fgf8* and *fgf3*.

rior region of the prospective neural plate, while *fgf8* starts to be expressed in the prospective midbrain/anterior hindbrain region (Reifers et al., 1998). Anterior *fgf3* expression recedes to a telencephalic domain until gastrula stage. At 80–90% of epiboly, *erm* and *pea3* transcripts accumulate at similar levels as *fgf3* and *fgf8* in the prospective fore- and MH, respectively, becoming refined until tailbud stage to a horseshoe-shaped domain around the prospective forebrain, to the territory covering posterior midbrain and anterior hindbrain, the lateral ectoderm at the MH level, the trunk as well as the tailbud (Fig. 1B). At the onset of somitogenesis, *fgf8* becomes similarly expressed in the prospective forebrain (Reifers et al., 1998). Transcripts of *erm* and *pea3* are present in the prospective telencephalon during segmentation stages, with a slightly stronger expression at the basal side (Fig. 1C,D). In addition, *erm* and *pea3* expression persists in the MH boundary (MHB), the hindbrain and the adjacent ectoderm as well as in the eye anlage and in the somites, which are all regions of *fgf8* localization (Fig. 1; Reifers et al., 1998 and data not shown). Expression of *fgf3* is also found in the tailbud, the MHB, in part of the hindbrain, and the presumptive otic placode (Fig. 1C).

Characteristically, the expression domains of *erm* and *pea3* are broader and less confined than the expression domains of either *fgf8* or *fgf3*. This is for example evident during gastrulation stages, when both *erm* and *pea3* expres-

sion extend further ventrolaterally than *fgf8* (Fig. 1B). Similarly, the forebrain expression domains of *fgf3* and *fgf8* are narrow and precise, while the expression of *erm* and *pea3* are more extensive (Fig. 1B–D). In addition to spatial distribution of transcripts, expression dynamics of these factors seems to be temporally prefigured by expression changes of *fgf8* or *fgf3*. This can be observed in the refinement of expression patterns in the anterior neural plate during late gastrulation: *erm* and *pea3* transcripts are progressively accumulating around the emanating expression domains of *fgf3* and *fgf8* in the prospective forebrain and MH regions, respectively, while they are at the same time lost from interspatial domains (Fig. 1B and data not shown).

Finally, when *erm* and *pea3* expression are compared to each other, *erm* transcripts appear spatially less restricted than *pea3* transcripts. Thus, during gastrulation, *erm* expands further ventrolaterally than *pea3* (arrowheads in Fig. 1B). At tailbud stage, different expression domains of *pea3* within the prospective MH region and the adjacent tissues can be clearly distinguished, while *erm* expression

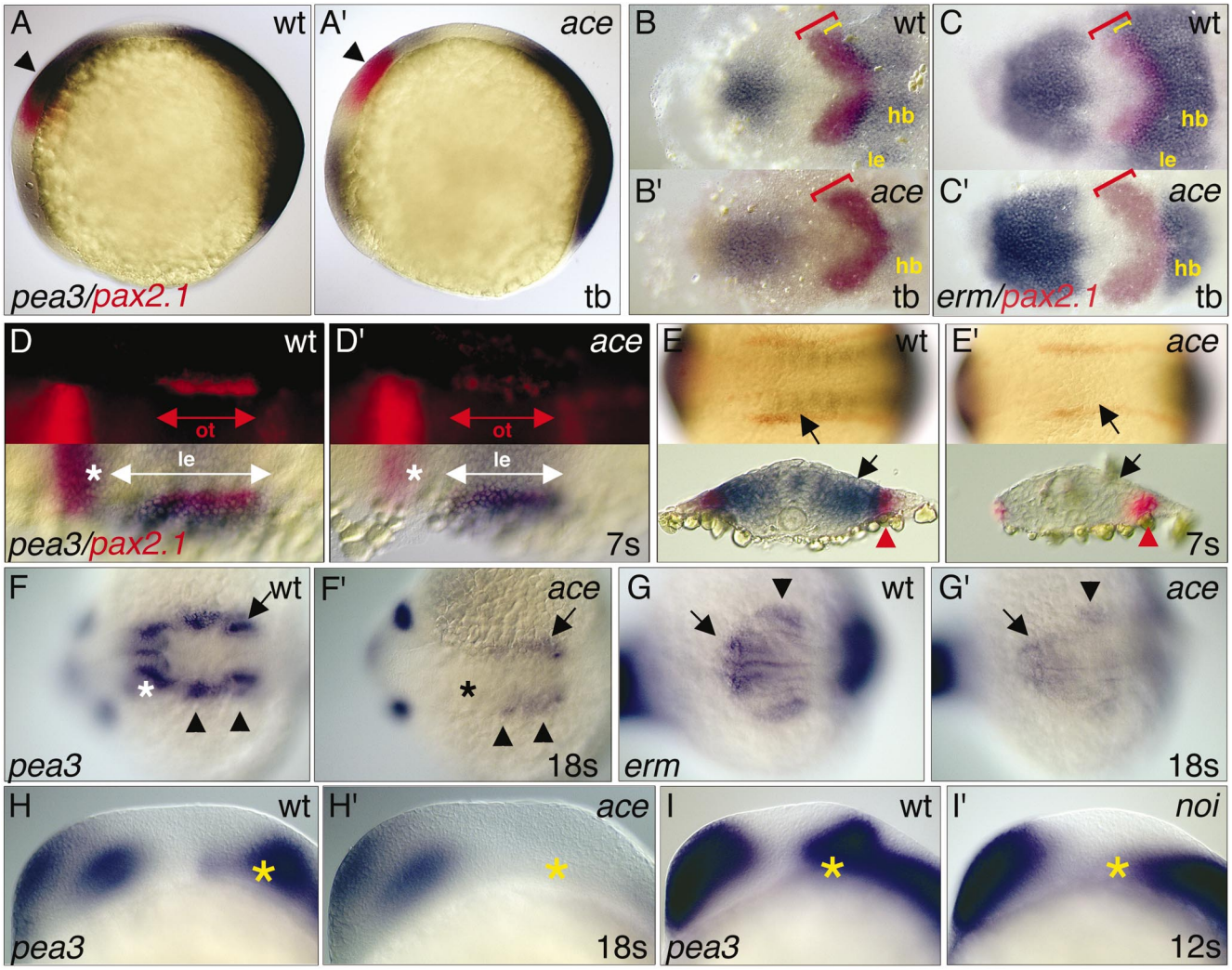

Fig. 2. Embryos lacking *fgf8* gene function display a loss in specific *erm* and *pea3* expression domains. Whole-mount ISHs were performed with digoxigenin-labeled antisense probes of *erm* (panels C and G) or *pea3* (all other panels), both detected in blue, in combination with a fluorescein-labeled antisense probe of *pax2.1* (panels A–E′), detected in red. Expression is compared between wild-type (panels A–I) and homozygous *ace* embryos (panels A′–H′) or *noi* embryos (I′) of the indicated stages. (A, H, I) Lateral views. (B–F) Dorsal views. (G) Frontal view. (A, A′) Arrowheads point at the overlap between *pea3* and *pax2.1*. (B–C′) Red brackets indicate the width of the *pax2.1* domain. (hb) Medial hindbrain domain and its residual expression in *ace* embryos. Yellow brackets indicate the overlap of *pea3* or *erm* expression with *pax2.1* expression; (le) lateral expression domain. (D, D′) Upper and lower halves correspond to dark field/fluorescence and bright field images, respectively, of the same specimens. Asterisks mark expression of *pea3* at the MHB. Arrows mark the antero-posterior extent of the lateral *pea3* expression domain (le, white) and the *pax2.1* expression domain demarcating the otic placode (ot, red). (E, E′) Cross-sections from equivalent specimens at the level of the first somite are given at the lower half of each panel. Arrows point at somitic expression. Red arrowheads mark *pax2.1* expression in the presumptive pronephros, where *pea3* seems to co-localize. (F, F′) MHB expression is marked with an asterisk. Arrowheads point at the neural crest adjacent to the hindbrain. The arrow marks expression in the otic vesicle. (G, G′) Arrowheads point at the expression in the optic vesicle. The arrow points at expression in the telencephalon and olfactory placode. (H, H′) *pea3* fails to be reexpressed at the MHB (asterisk) at later stages (I, I′). After early segmentation stages, MHB expression (asterisk) is progressively affected in *noi* mutant embryos, correlating with the loss of *fgf8* from this domain.

is found in a more uniform domain that seems to contain all expression domains of *pea3* (Fig. 2B,C; further discussed below). Similar observations can be made at other stages and expression sites, e.g. at the 12 somite stage, where corresponding correlations are found in the forebrain domain, the MHB, the somites, and the tailbud domain (Fig. 1C), suggesting that *erm* and *pea3* follow different kinetics or respond to different thresholds of the same signal.

2.2. Normal expression levels of erm and pea3 depend on fgf8 function in many expression domains

Spatial co-expression has been suggested to be a characteristic feature of functionally linked genes (Niehrs and Pollet, 1999). The observed co-expression of *erm*, *pea3*, *fgf8*, and *fgf3* prompted us to study the possibility of a functional link between the transcription of these ETS factors and Fgf signaling under conditions of altered Fgf signaling in the embryo. First, we investigated the expression of these factors in *ace*^{ti282a} mutants which carry a putative null allele of the *fgf8* gene (Reifers et al., 1998). *Fgf8* function has been implicated in various aspects of embryonic development. Homozygous *ace* embryos display defects in proper MH organizer maintenance (Reifers et al., 1998) and row 1 organizer function (Shanmugalingam et al., 2000), in tectum patterning (Picker et al., 1999) and specification of cerebellar fate (Reifers et al., 1998 and unpublished observations), as well as in forebrain patterning including commissure formation (Shanmugalingam et al., 2000). Additionally, defects can be found in somitogenesis (Brand et al., 1996), heart development (Reifers et al., 2000b), inner ear development (Adamska et al., 2000; S. Léger and M. Brand, manuscript in preparation) and proper development of neural crest derivatives (H. Grandel, personal communication).

We found that in homozygous *ace* embryos, both *erm* and *pea3* expression levels are reduced or completely lost in various expression domains. Differences in the expression pattern of these genes became apparent during late gastrulation. At tailbud stage, the presumptive forebrain region and the trunk/tailbud expression are unaffected by the *ace* mutation (Fig. 2A). However, a striking difference between *ace* embryos and wild-type siblings could be observed at the posterior midbrain/anterior hindbrain level (Fig. 2A–C). Wild-type expression at this location comprises at least three distinct domains: a wing-like expression domain overlapping partially with the expression domain of *pax2.1*, a medial domain in the prospective ventral hindbrain and two longitudinal stripes in the adjacent ectoderm that contain the presumptive otic placode in their posterior part (Fig. 2B,C). Intriguingly, both the wing-shaped domain and the lateral stripes are absent in *ace* embryos at this stage, while the medial hindbrain domain is strongly reduced (Fig. 2B′,C′). During later development, MHB expression is not regained in *ace* (Fig. 2D,F,I).

In contrast to the situation in *ace* mutants, the onset of expression of both *erm* and *pea3* is normal (data not shown) in homozygous *noi*^{tu29a} embryos which lack the *pax2.1* gene function (Lun and Brand, 1998). However, in *no isthmus* (*noi*) mutants, a downregulation of both *erm* and *pea3* can be observed at the MH junction from the 8 somite stage onwards, and this loss persists to later stages of development (Fig. 2H and data not shown). Taking into account that *fgf8* transcription becomes dependent on functional Pax2.1 at that time point (Lun and Brand, 1998), this finding can be reconciled with the concept that *fgf8* is the primary regulator of *erm* and *pea3* in the MH region. While MHB expression of *erm* and *pea3* is lost in *ace* mutants, these embryos start to re-express both genes in the ectodermal domain adjacent to the MH territory during early somitogenesis. A double-staining with a probe for *pax2.1* reveals that in wild-type embryos (Fig. 2D) this lateral expression extends anteriorly to the level of the MHB and encloses the lateral *pax2.1* expression domain at the hindbrain level that marks the otic placode at this stage (S. Léger and M. Brand, unpublished observations). In *ace* embryos of the same stage (Fig. 2D′), the posterior part of this expression domain containing the otic placode is not affected, while the anterior part is missing (arrows in Fig. 2D,D′).

In addition to expression in the MH region, *erm* and *pea3* transcription is affected in various other tissues in which *ace/fgf8* is known to be involved. These include the somites where expression is completely missing (Fig. 2E). Residual expression can be found in the intermediate mesoderm in an overlap with *pax2.1* (red arrowhead in Fig. 2E′) at the level of the first somites, the presumptive pronephros. In addition, the developing eye, the forebrain, and the olfactory placodes show reduced staining (Fig. 2F) that could result from the expression of additional Fgfs in this region. At the 18 somite stage, a strong reduction in premigratory neural crest tissue can be observed (Fig. 2F′). This is in agreement with later defects in the cranial skeleton found in *ace* mutant fish (H. Grandel, unpublished).

2.3. Onset and maintenance of erm and pea3 transcription are dependent on Fgf signaling throughout the embryo

Several *erm* and *pea3* expression domains are affected in *ace* embryos, but others are unchanged compared to wild-type embryos, including expression in the gastrulating embryo, the early forebrain expression, and expression at the hindbrain level, while others are only mildly affected by the *ace* mutation. In order to test whether other Fgfs are involved in the establishment or maintenance of the persisting expression domains, we chose two ways of experimentally interfering with all Fgf signaling in the embryo. First, we injected embryos with different doses of *sprouty4* mRNA and analyzed the expression of *erm* and *pea3* at the gastrula stage. *sprouty4* is a zebrafish homologue of the *Drosophila* gene *sprouty* and is a putative intracellular inhibitor of tyrosine kinase signaling (Hacohen et al., 1998; Casci et al., 1999; Fürthauer et al., 2001). The unilateral injection of

high doses (150 pg) of *sprouty4* lead to no apparent morphological aberrations at the gastrula stage (not shown). As to the expression of *erm* and *pea3*, low doses (75 pg) of misexpressed *sprouty4* did not cause a clear phenotype ($n = 40$). In contrast, high doses (150 pg) lead to unilateral absence of *erm* or *pea3* in one-third of the embryos ($n = 3/9$ and 3/11, respectively), while the non-injected side appears not to be affected (Fig. 3A,B). In the remaining cases, only patches of reduced staining or no reduction could be observed. In either case, reduction of *erm* or *pea3* staining was always limited to cells that were derived from the injected blastomere, as indicated by the detection of the injection marker beta-Galactosidase. This argues in favor of a cell-autonomous effect of *sprouty4*, as would be inferred from its mode of action in *Drosophila* (Casci et al., 1999).

As a second approach that would allow us to resolve the temporal aspects of Fgf dependence, we selectively applied a pharmacological inhibitor of Fgf signaling, SU5402, at various stages of development. SU5402 has been shown to block the kinase domain of Fgf receptor 1, thereby preventing its activating function (Mohammadi et al., 1997). Since this domain is identical in all four Fgf receptors, SU5402 can be used as a general inhibitor of Fgf signaling and phenocopy aspects of the *ace* mutant phenotype (Reifers et al., 2000b and data not shown). To follow the requirement of Fgf activity for gene expression over a longer period of development, we applied SU5402 in individual experiments during the following intervals: (i) 30–60% epiboly, (ii) 60% epiboly to tailbud, (iii) tailbud to 7 somite stage, (iv) 7–12 somite stage, and (v) 12–18 somite stage. At all experimental stages, we found that after inhibitor treatments, *erm* and *pea3* transcripts were hardly detectable or completely absent from the inhibited embryos, while non-treated siblings displayed wild-type expression (Fig. 3C–F′ and data not shown). This finding indicates that FGF signaling is necessary for both onset and maintenance of *erm* and *pea3* transcription throughout embryogenesis.

2.4. Both Fgf8 and Fgf3 can elicit ectopic erm and pea3 transcription

The Fgf interference experiments suggest that Fgf signaling is necessary for the proper onset and maintenance of *erm* and *pea3* transcription. In order to investigate whether Fgf is also sufficient to activate transcription of these factors, we performed two types of gain-of-function experiments. First, in order to test the ability of *fgf3* to elicit *erm* or *pea3* transcription, we injected 75–150 pg *fgf3*-RNA in one of the two blastomeres and analyzed gene expression in the gastrula. We find that this injection leads to a global overexpression of both *erm* and *pea3* throughout the embryo on both the injected and the non-injected sites, suggesting that *fgf3* is sufficient to drive the expression of these genes during gastru-

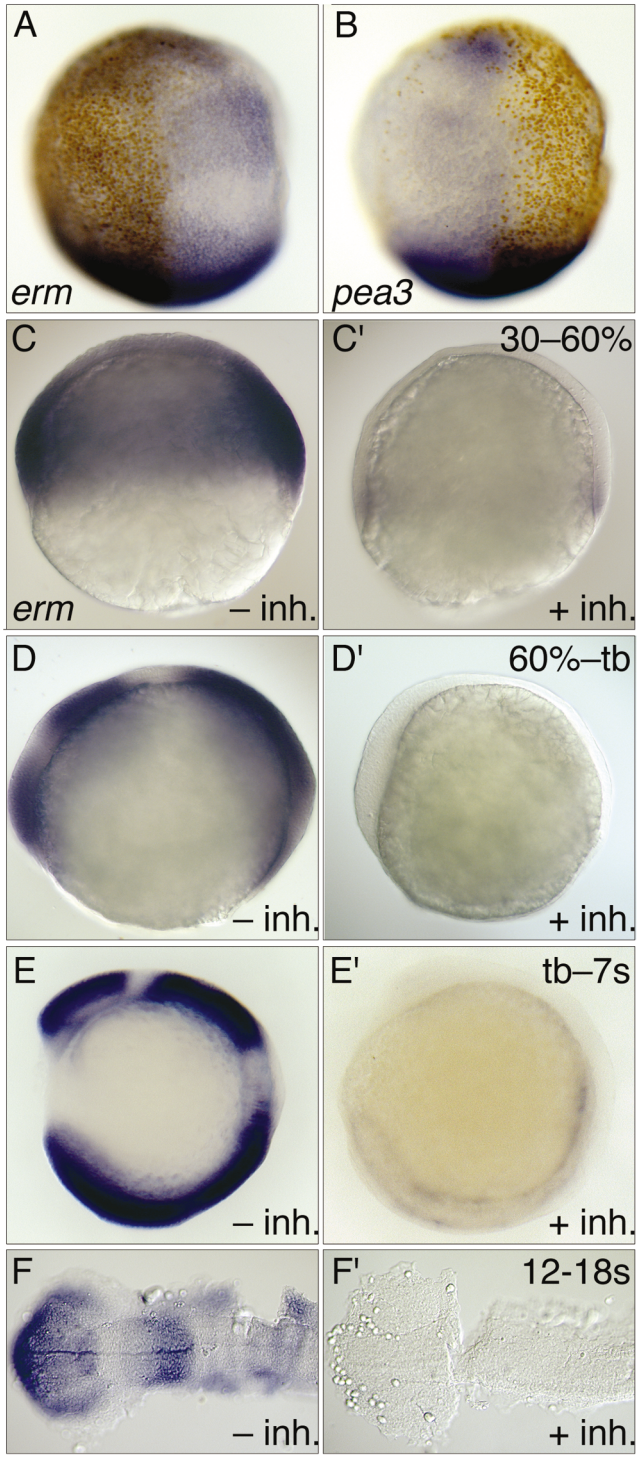

Fig. 3. FGF signaling is required to maintain expression of *erm* and *pea3*. Whole-mount ISHs detecting *erm* (panels A, C–F′) or *pea3* (panel B) in blue. (A, B) Dorsal views, anterior up; (C–E′) lateral views, anterior to the left; (F, F′) dorsal views on flat-mounted embryos, anterior to the left. (A, B) One of the two blastomeres was injected with *sprouty4* mRNA and *lacZ* mRNA as injection marker. The progeny of the injected blastomere was detected by staining against beta-Galactosidase protein (in brown). Staining reveals that *erm* (A) or *pea3* (B) are downregulated in the injected side in a cell-autonomous fashion. (C–F′) Detection of *erm* RNA in embryos treated with the FGF inhibitor SU5402 (C′–F′) compared to non-treated controls (C–F). Treatment was performed between the stages indicated in the right panels.

lation. As a positive control, we tested for the induction of *sprouty4*, a potential target of Fgf signaling (Fürthauer et al., 2001). All injected embryos displayed global induction of *sprouty4* in the same manner as *erm* or *pea3* expression (data not shown). Phenotypically, we noticed that *fgf3* injections also caused a weak dorsalization of the embryos (Fig. 4A′), similar to what was described after *fgf8* misexpression (Fürthauer et al., 1997; Reifers et al., 1998).

Because global Fgf overexpression might induce *erm* and *pea3* indirectly, we next tested whether Fgf8 is directly able to elicit *pea3* transcription after implantation of Fgf8-coated heparin beads. We implanted beads into shield-stage embryos from heterozygous *ace* intercrosses and analyzed gene expression in the gastrula. In half of the analyzed cases ($n = 8/17$), we find that *pea3* is transcriptionally activated as a local response to the beads (see Fig. 4D,D′). In the remaining cases, *pea3* was induced all over the embryo (not shown), probably reflecting a higher protein dosage due to variation in the coating and/or the release of Fgf8. In agreement with this interpretation, all these embryos had an elongated and dorsalized appearance, while the embryos that showed local responses had a normal shape.

We were not able to distinguish *ace* from wild-type embryos in the globally affected cases. However, at least one of the embryos displaying a local response to the bead implantation could be clearly assigned to be *ace* homozygous on the basis of its characteristic loss of endogenous expression domains in the non-implanted side (Fig. 4E). This case is indicative of the fact that endogenous *fgf8* gene function is not needed to mediate the local response observed in the bead implantation experiments. In summary, we conclude that Fgf8 and Fgf3 can elicit *pea3* transcription outside its endogenous domains, and that presence of Fgf signals is the main factor causing its expression.

2.5. Fgf8 and Fgf3 may act to co-regulate early forebrain expression of pea3

From the inhibition results we concluded that an Fgf signal is responsible for the proper onset and maintenance

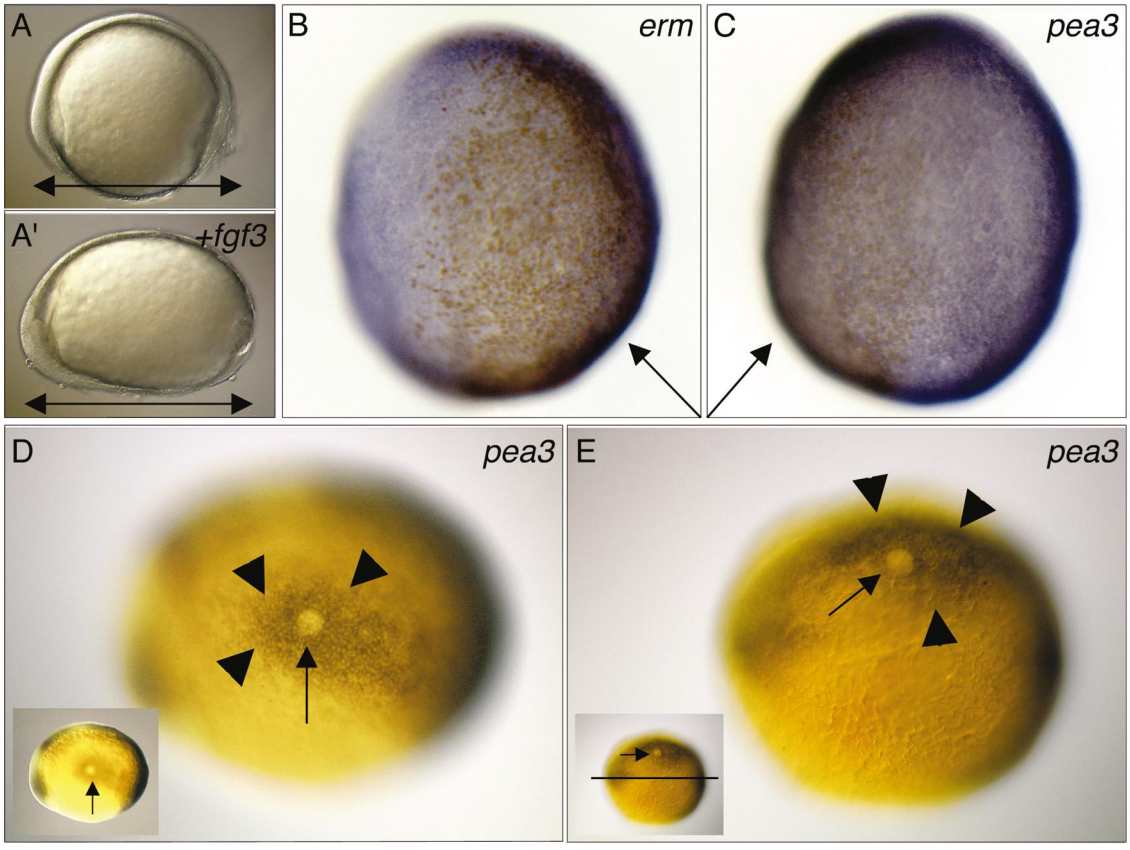

Fig. 4. Fgf8 and *fgf3* are able to elicit ectopic expression of *erm* and *pea3*. (A, A′, B, C) Misexpression of *fgf3* RNA in one half of embryos results in global overexpression of both *erm* and *pea3*. Morphologically, injected embryos display a slightly elongated and dorsalized phenotype (A′) compared to non-injected controls (A). Arrows indicate the elongation of the embryos. (B, C) Whole-mount ISHs with probes for *erm* (B) and *pea3* (C) reveal that these genes are globally overexpressed at gastrula stage upon injection of *fgf3* RNA. The injected side is visualized by the detection of beta-Galactosidase (in brown, marked with arrows). Overexpression can be seen throughout the embryo. (D, E) Fgf8-coated beads were implanted into shield-stage embryos that were subsequently fixed at gastrula stage and stained for *pea3* RNA. Bead locations are indicated with arrows. Ectopically induced *pea3* transcripts are marked with arrowheads. (D) Lateral view, anterior to the left. The bead is located at the ventrolateral side, as indicated in the insert. (E) Dorsal view, anterior to the left. The bead is located at the right side of the embryo (the dashed line in the insert marks the midline).

of the putative forebrain expression domain of *erm* and *pea3*. The analysis of the *ace* mutant and the co-expression analysis showed that the Fgf8 gene is not the primary regulator of these domains in the pregastrula. Therefore, we investigated the role of *fgf3* which is expressed at the right place and time and has previously been suggested to contribute to forebrain patterning (Shanmugalingam et al., 2000). We aimed at analyzing the expression of the ETS factors after depleting the embryo of Fgf3. Since no *fgf3* mutant has been reported in zebrafish to date, we chose a recently developed gene 'knock-down' strategy (Nasevicius and Ekker, 2000), by injecting morpholino-antisense-oligonucleotides directed against the translational start site of *fgf3* (fgf3-MO) or *fgf8* (fgf8-MO) into the yolk cell of two to four cell stage embryos and fixing them at tailbud stage for whole-mount ISH with the *pea3* probe. To assess the efficiency of the procedure, we let part of the embryos develop to 28 h post fertilization (hpf) and analyzed their overall morphology.

Injection of fgf8-MO results in a morphological phenotype that is strikingly similar to the phenotype displayed by *ace* homozygous mutants (Araki and Brand, 2001). In our experiments, 100% ($n = 33/33$) of the 28 hpf larvae displayed a lack of the isthmus and cerebellum and a reduced ear vesicle, both indicative of the genetic *ace* mutant phenotype, while injection of a standard control morpholino never resulted in such a phenotype (Fig. 5B,D, and data not shown). In further agreement with the genetic *ace* mutation, we observe a similar reduction of the *pea3* expression domain at the MH level: staining in the expression domains overlapping with the *pax2.1* domain as well as the ectodermal lateral domain including the otic vesicle were absent, while the hindbrain expression was diminished to a medial region. The forebrain expression in these embryos appeared to be normal or slightly reduced (not shown).

When we additionally added fgf3-MO to the injection solutions, 12.5% ($n = 3/24$) of the fgf3-MO/fgf8-MO-double-injected embryos displayed a complete loss of *pea3* forebrain expression (Fig. 5E); 87.5% ($n = 21/24$) displayed a reduced forebrain expression compared to control embryos. This indicates a potential contribution of Fgf3 expression to the anteriormost expression domain of *pea3*. To determine whether the observed effect on the forebrain expression of *pea3* was specific to the inhibition of *fgf3* translation, we looked for morphological defects at 28 hpf that could be due to a loss of Fgf3 protein. Although a zebrafish mutant for *fgf3* has not been reported yet, genetic studies in mice (reviewed in Torres and Giraldez, 1998) and zebrafish (S. Léger and M. Brand, unpublished observations) make it likely that *fgf3* synergizes with *fgf8* and contributes to inner ear development. Using the ear vesicle as a readout for the efficiency of our interference with *fgf3* translation, we observed that fgf8-MO/fgf3-MO injected embryos have smaller ear vesicles than displayed in the *ace* mutants (compare Fig. 5F,D) with a high consistency ($n = 25/26$). From these experiments, we conclude that Fgf3 and Fgf8 co-operate in regulating *erm* and *pea3* expression in the forebrain.

3. Discussion

We demonstrate that transcription of the ETS factors *erm* and *pea3* is tightly linked with active Fgf signaling during gastrulation and somitogenesis stages of zebrafish embryos: (1) *erm* and *pea3* are expressed in close proximity to centers of Fgf signaling throughout embryogenesis; (2) *erm* and *pea3* transcript levels are selectively reduced in various regions of Fgf8 activity in *acerebellar* (*ace*)/*fgf8* mutants; (3) general interference with Fgf signaling by overexpression of *sprouty4* or application of the FgfR inhibitor SU5402 leads to the complete absence of *erm* and *pea3* transcripts in the affected tissues, an effect that can be observed throughout embryogenesis with fast kinetics; (4) Fgf3 and Fgf8 ectopically elicit *erm* and *pea3* transcription in overexpression or bead implantation experiments; and (5) *pea3* can be selectively reduced in the early forebrain anlage by co-inhibition of *fgf8* and *fgf3* translation through antisense-morpholino oligomers. These findings lead us to conclude that *erm* and *pea3* transcription is tightly linked to Fgf signaling and may provide a direct intracellular readout for active Fgf signaling.

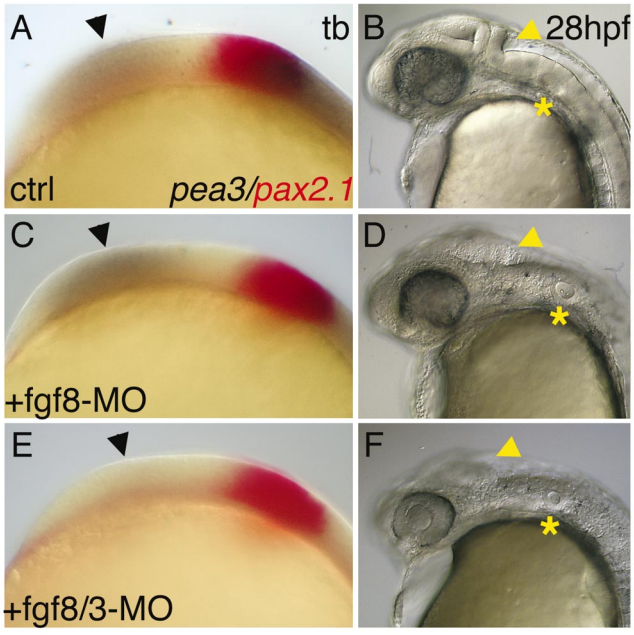

Fig. 5. Early forebrain expression of *pea3* is co-regulated by Fgf8 and Fgf3. Embryos were injected with morpholino-antisense oligos directed against translational start sites of *fgf8* (fgf8-MO) or *fgf3* (fgf3-MO) to block their translation. Analysis was performed at gastrula stage by ISHs for *pea3* (detected in blue) and *pax2.1* (detected in red; A, C, E) or at 28 hpf for morphological changes (B, D, F). (C, D) Embryos injected with fgf8-MO. (E, F) Embryos co-injected with fgf8-MO and fgf3-MO. (C, E) Forebrain expression is marked with an arrow and compared to control embryos (B). Morphological phenotype at 28 hpf demonstrating the effects of gene-knock-down on MHB (arrowhead) and ear (asterisk).

3.1. erm and pea3 are transcriptionally regulated by both Fgf8 and Fgf3 signaling

Redundancy is a general problem encountered in Fgf signaling. The identification of target genes of Fgf signaling therefore may serve two needs: they facilitate the analysis of signaling events, and they may be important to understand how different Fgf signals are integrated on the cellular level. Several lines of evidence lead us to conclude that at least two Fgf signals – Fgf8 and Fgf3 – are involved in regulating the transcription of *erm* and *pea3*. The first line of evidence is the apparent co-expression of *erm* and *pea3* expression with expression sites of *fgf8* and *fgf3*, including the blastoderm margin of the pregastrula embryo, the germ ring and shield, the axial mesoderm of the gastrulating embryo as well as the early expression domain of *fgf3* in the presumptive forebrain and the wing-shaped expression of *fgf8* in the future midbrain–anterior hindbrain region. Later stages show correlations in various expression domains, including the somites, the eye anlage, the ear placode, and the MHB.

Secondly, Fgfs are believed to exert their function over some distance by a diffusion mechanism that would also allow the creation of concentration gradients. In agreement with this, both our analysis of wildtype expression and our observations made upon misexpression of *fgf3* RNA or implantation of Fgf8-coated beads demonstrate that *erm* and *pea3* transcription is upregulated in the tissue surrounding local Fgf sources, in patterns that are conceivably more diffuse. Furthermore, there is a characteristic spatial and temporal correlation between the dynamics of *fgf* expression and the detectable mRNAs of Erm and Pea3, as would be expected if the two genes are inducible targets of Fgf signaling. Notably, *erm* expression generally appears to be less confined in its domains compared to *pea3* distribution. This could reflect a different sensitivity to Fgf signals, but might also be due to secondary mechanisms refining *pea3* expression to a smaller subdomain of cells. Either mechanism could have important consequences for the organization of downstream events.

Thirdly, the conclusions from the expression analysis and the gain-of-function experiments are further supported by the selective interference with Fgf8 or Fgf3 protein levels, either by studying homozygous *ace/fgf8* mutant embryos or by selective knock-down of protein translation through morpholino-antisense oligomers. For *ace/fgf8* mutant or fgf8-MO injected embryos, the most striking phenotype in the change of expression pattern concerns the MH region. At tailbud stage, *ace* mutants lack most of *erm* and *pea3* expression in this territory, including the domain that overlaps with *pax2.1* expression at the presumptive MHB and most of the hindbrain expression, while a small hindbrain domain is persisting. MHB expression is never regained in *ace*, in agreement with the fact that *ace* embryos also fail to induce and maintain MHB expression of other *fgf* genes like *fgf17* that might be able to rescue target gene expression (Reifers et al., 2000a). Conversely, *erm* and *pea3* expression at the MHB level is retained at wild-type levels in *noi/pax2.1* mutant embryos up to early somitogenesis stages, but is progressively lost thereafter. This loss correlates with the progressive loss of *fgf8* expression from this region in *noi* mutants und therefore stresses the major role of Fgf8 for Fgf signaling in this territory.

At the gastrula stage, *erm* and *pea3* expression in the presumptive forebrain does not seem to be strongly affected in *ace* mutants. Our morpholino-antisense oligomer injections suggest that *fgf3* is a potential co-regulator of *fgf8* for expression of *pea3* in this domain. This is consistent with our finding that *fgf3* can activate *pea3* upon misexpression, and gets additional support from our co-expression analysis. In addition, this evidence is complementary to our previous finding that additional Fgf signaling acts redundantly with Fgf8 in early forebrain development (Shanmugalingam et al., 2000), and suggests that Fgf3 is one of the Fgfs involved.

Other sites in which *erm* and *pea3* expressions are affected in *ace* mutant embryos include the somites, the developing eye, and the forebrain. These findings are in good agreement with aspects of the mutant analysis and therefore will not be discussed in detail here. Interestingly, the loss of expression in the hindbrain-derived neural crest correlates with a loss of craniofacial skeleton phenotype displayed by *ace* mutants that is indicative of a role of *fgf8* in normal development of this tissue (H. Grandel, unpublished).

3.2. erm and pea3 transcription can be ectopically activated by Fgf8 orFgf3

One interesting question resulting from the finding that Fgf8 and Fgf3 are involved in regulating *erm* and *pea3* transcription is whether they are also sufficient to induce these factors ectopically. Indeed, both ectopically provided *fgf3* mRNA or implanted Fgf8 beads were able to elicit ectopic expression of *erm* and *pea3* in regions where it is not naturally expressed. In the case of *fgf3* overexpression in one half of the embryo, massive upregulation of *erm* and *pea3* transcripts could be detected both on the injected and the contralateral sides. In addition, ectopically provided Fgf8 on heparin-coated beads elicited a local response surrounding the bead. Since this local response could also be observed in homozygous *ace* embryos, endogenous Fgf8 is not required to mediate this inductive effect. While the qualitative result – the ectopic upregulation of target mRNA – is the same in both experiments, the apparent differences in the size of the affected area most likely result from the differences in the experimental conditions: RNA injection at the two-cell stage presumably results in Fgf3 expression prior to the shield stage, the time point at which the implantations were performed. In addition, the amount of protein secreted by the RNA-injected cells might differ from the protein amount coated on the beads. Notably, the bead implantation experiments also demonstrate that *pea3* can be detected within at most 4 h after application of Fgf8.

Potentially, this time span is even shorter, but has not yet been challenged experimentally.

3.3. General dependence of erm and pea3 transcription on Fgf signaling

In addition to the transcriptional activation of *erm* and *pea3* upon creation of ectopic Fgf8 or Fgf3 signaling domains, we find evidence that general interference with Fgf signaling correlates with an overall loss of transcripts of these genes. After *sprouty4* misexpression, we observed a complete loss of *erm* or *pea3* staining in 30% of the embryos injected with 150 pg *sprouty4* mRNA. The loss of transcripts was always confined to the progeny of the injected blastomere, as demonstrated by detection of the injection marker *lacZ*. The seemingly low penetrance of this phenotype is in agreement with the observation that *sprouty4* overexpression causes only transient effects in zebrafish (Fürthauer et al., 2001). In accordance with the mode of action that has been proposed for *Drosophila* photoreceptor differentiation (Casci et al., 1999), *sprouty4* thus seems to act as a cell-autonomous inhibitor of Fgf signaling.

While the *sprouty4* misexpression experiment does not distinguish between a loss of transcript and a failure to activate transcription, the selective application of the Fgf receptor inhibitor SU5402 at various time points during embryonic development provided evidence that interference with Fgf receptor activation reduces *erm* and *pea3* transcripts to a minimum level within 2.5 h or even faster after application. This loss of transcripts is observed even in later segmentation stages, suggesting that *erm* and *pea3* transcription remains tightly linked to Fgf signaling during embryonic development.

The finding that all expression domains of *erm* and *pea3* are susceptible to interference with Fgf signaling suggests that other Fgfs besides Fgf8 and Fgf3 are able to control transcription of these factors. In return, expression domains that are remaining in specific loss-of-function situations are thus likely reflecting redundant expression of other *fgf* genes. Thus, *ace* embryos display only a transient lack in their presumptive otic expression domain around tailbud stage, which presumably reflects Fgf activity at the hindbrain level in early somitogenesis. Moreover, *ace* embryos do not display defects in *erm* or *pea3* expression prior to the late gastrula stage, which is unlikely to be due to a rescue effect of maternal *fgf8* mRNA, since fgf8-MO injections – which should also inhibit translation of maternal mRNAs – do not create a different phenotype. Instead, *fgf3* and at least one other member of the *fgf* gene family are expressed in the germring and may mask the effects of early loss of *fgf8* function (I. Araki and M. Brand, unpublished).

4. Perspectives

Our results suggest that *erm* and *pea3* are two direct target genes activated by cells that are receiving an Fgf8 or Fgf3 signal as well as other, so far uncharacterized Fgf signals. Thus, these genes are good candidates for factors that will relay further cellular responses to Fgf signaling and serve to integrate Fgf signaling with other signals. Indeed, both factors are very likely nuclear targets of Fgf signaling also on the protein level, as suggested by in vitro studies (Janknecht et al., 1996; O'Hagan et al., 1996; see also Wasylyk et al., 1998).

Further studies will thus focus more on the protein function of Erm and Pea3, taking into account the likely activation of these factors by various other signaling cascades (O'Hagan et al., 1996; O'Hagan and Hassell, 1998; Janknecht et al., 1996) and the possibility of molecular interactions with other transcription factors, including paired- and POU homeodomain proteins that can interact with ETS domain proteins (Bradford et al., 2000; Fitzsimmons et al., 1996). Molecules of both classes are known to be essential for MHB development or function (Favor et al., 1996; Urbanek et al., 1997; Lun and Brand, 1998; Burgess et al., 2001), and Erm and Pea3 are attractive candidates for relaying Fgf signaling into the transcriptional regulation involved in MHB patterning.

Since single loss-of-function studies in mice deficient for either Pea3 or the Pea3 class ETS domain factor ER81 have not been reported to yield early developmental defects (Arber et al., 2000; Laing et al., 2000), it is likely that multiple loss-of-function studies are necessary to address the functional relevance of the Pea3 family of ETS domain factors in more depth.

5. Experimental procedures

5.1. Fish strains and maintenance

Zebrafish embryos were obtained from natural spawning and maintained under standard conditions (Westerfield, 1994). For all injection and transplantation experiments, fish were kept in Ringer's solution. Embryos were staged as described by Kimmel et al. (1995) or in hpf at 28°C. Heterozygous carriers of the ace^{ti282a} mutation (Reifers et al., 1998) or the noi^{tu29a} mutation (Lun and Brand, 1998) were identified by random intercrosses. Homozygous mutant embryos were obtained by heterozygote mating.

5.2. Live morphology

Embryos were anesthetized with 3-aminobenzoic acid ethyl ester (MESAB) and embedded in methylcellulose for photography.

5.3. Analysis of gene expression

Standard methods for whole-mount RNA ISH were used as described by Reifers et al. (1998). The following probes have been used: *pax2.1* (Krauss et al., 1991; Püschel et al., 1992), *fgf8* (Fürthauer et al., 1997; Reifers et al., 1998),

spry4 (Fürthauer et al., 2001), *fgf3* (Kiefer et al., 1996), *pea3*, and *erm* (Münchberg et al., 1999).

5.4. RNA injections

cDNAs of *fgf3*, *sprouty4*, and *lacZ* were subcloned into pCS2 + (Rupp et al., 1994), and linearized plasmid DNA was used for in vitro transcription (SP6 mMESSAGE mMACHINE kit (Ambion/AMS Biotechnology GmbH, Wiesbaden, Germany)). Injection solutions contained final concentrations of 0.2 M KCl and 0.2% phenol red. The amount of RNA injected was estimated based on the concentration of the solution and the calculated volume of a sphere of RNA solution injected into oil at the same pressure settings. RNA solution was backloaded into borosilicate capillaries prepared on a Sutter puller and injected into single blastomeres of two-cell stage embryos. The progeny of the injected blastomere was visualized by detection with a beta-Galactosidase specific antibody (Promega GmbH, Mannheim, Germany, 1:500) after ISH (Dornseifer et al., 1997).

5.5. Bead implantations

Bead implantations are described in Reifers et al. (2000b). Beads coated with Fgf8b (R&D Systems GmbH, Wiesbaden, Germany) were implanted at indicated regions of wild-type and *ace* mutant embryos between shield stage and 70% of epiboly. Embryos were fixed at tailbud stage and processed for ISH.

5.6. Inhibitor treatment

For inhibition of the Fgf pathway, wild-type embryos were treated with the chemical inhibitor SU5402, interfering with the ATPase domain of Fgf receptors (Calbiochem-Novabiochem GmbH, Schwalbach, Germany; Mohammadi et al., 1997). The inhibitor was dissolved to 8 mM in DMSO, aliquots were kept frozen at −20°C and applied at a final concentration of 16 μM in embryo medium at 28°C in the dark. After treatments, embryos were directly fixed for ISH.

5.7. Morpholino 'knock-down' experiments

To specifically block translation of mRNAs, morpholino-antisense oligomers (Nasevicius and Ekker, 2000) directed against the translational start sites of *fgf8* and *fgf3* were used (obtained from GeneTools, LLC, Corvallis, OR). The sequence of the oligos used was 5′-GAGTCTCATGTTTA-TAGCCTCAGTA-3′ (fgf8-MO) and 5′-CAGTAACAA-CAAGAGCAGAATTATA-3′ (fgf3-MO). Morpholinos were resuspended in water and stored as 5 mM stock solutions at −20°C. Working solutions were prepared in 0.2 M KCl and 0.2% phenol red. The injected amounts were estimated as described for RNA injections. Injected amounts of either fgf8-MO or fgf3-MO were 2.5 ng/embryo.

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