cis–regulation and mis–regulation – Insights into genomic regulatory mechanisms underlying the control of *fgf8a* in zebrafish *Danio rerio*

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by

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ABSTRACT

Fibroblast growth factor 8 *(Fgf8)* is a potent vertebrate morphogen that plays decisive roles in multiple developmental processes, such as cellular proliferation, survival, differentiation, growth and migration. *Fgf8* regionalizes the forebrain and has organizing activity in the midbrain–hindbrain boundary (MHB). Fgf8 induces limb formation and maintains the apical ectodermal ridge (AER), it controls development of the branchial arches and craniofacial skeleton, and its expression in the presomitic mesoderm (PSM) regulates axis elongation, and defines somite boundaries. It follws that the spatiotemporal activity and expression levels of Fgf8 should be tightly controlled, and this control likely occurs at multiple levels – from transcription to regulation of signaling pathways triggered by Fgf8. This thesis constitutes an analysis of mechanisms controlling the activity of Fgf8, and shows that slight modifications in its expression can alter multiple phenotypic traits.

Comparative analysis of mammal:teleost conserved chromosomal regions combined with enhancer detection revealed functional chromosomal units called genomic regulatory blocks (GRBs). GRBs usually contain a developmental control gene, or target gene, whose expression is under the control of numerous highly conserved noncoding elements (HCNEs) distributed over a large area that can stretch into and beyond adjacent phylogenetically and functionally unrelated genes, called bystander genes, expressed in patterns unrelated to the target gene. Rearrangements in evolutionarily conserved GRB may have serious consequences during development and thereby contribute to human developmental disease.

In all vertebrates, as well as the ascidian *Ciona intestinalis*, the *Fgf8* gene is located in a block of conserved synteny, which also contains the bystander gene *Fbxw4*. The teleost genomes contain an additional gene upstream of *fgf8, slc2A5,* which was lost from the mammalian lineage, while the fish have lost *BTRC*, which is found in all tetrapod genomes. In addition, a whole genome duplication in the teleost lineage resulted in two *fgf8* paralogs, *fgf8a* and –*b*. Along the *fgf8a* synteny block a number of HCNEs was detected, located in an area of about 200kb, including inside and around adjacent genes. Functional analysis of these HCNEs in transgenic zebrafish lines suggested that all of them acting as enhancers directed expression of the reporter GFP protein into domains characteristic for *fgf8a* expression, but not for *fbxw4* or *slc2a5*. Even though in teleosts the *fgf8a* locus is inverted with respect to tetrapods, and HCNEs are located on the other site of the gene as a result,

they still exhibit specific *fgf8a* activity. Therefore, *fgf8a* and the bystander genes *fbxw4* and *slc2a5* genes and *cis*–regulatory elements of *fgf8a* constitute a genomic regulatory block (GRB), conserved in all teleost genomes.

Insertion of tumor viruses in the proximity of developmental control genes can cause misregulation of the gene and lead to cancer in the mouse. Long terminal repeats (LTRs) of murine tumor viruses contain steroid response elements, which can act as a long–range enhancers. Analysis of four enhancer detection lines in the zebrafish, where integrations of engineered murine proviral vectors had occurred in a 100kb region around the *fgf8a* gene, caused multiple developmental defects, which were linked to LTR activity. While the expression pattern of YFP reporter protein in all *fgf8a*[CLGY] lines mimicked, at least partially, the endogenous expression domains characteristic of *fgf8a*, fish from the transgenic lines showed extensive phenotypic disorders, including pigmentation anomalies, craniofacial, axial and dermal skeleton defects, and abnormal accumulation of subcutaneous and visceral fat. These phenotypic changes were connected to elevated expression of *fgf8a* and became manifest during metamorphosis. In addition, transgenic fish had an 80% higher risk of malignant lesions in the brain. The strength of the observed phenotype appeared to be dependent on the distance of the integration site from *fgf8a*. Progression of tumorigenesis tended to be more severe in males of one line, suggesting androgens as mediators of altered *fgf8a* activity. Morpholino knock–down experiments targeted to androgen receptor transcript in this transgenic line could reduce the observed elevated *fgf8a* level.

Fgf8 signaling is also regulated at the protein level by specific antagonists, the sprouty proteins. A new member of this family was identified in zebrafish by enhancer detection. A transgenic line was isolated with an expression pattern overlapping *fgf8a* in forebrain, dorsal diencephalon, MHB, branchial arches, pectoral fin and PSM. Mapping by inverse PCR identified this locus as *sprouty1*. Sprouty1 is a downstream modulator of Fgf signaling and its expression is attenuated by the small molecular inhibitor of FGF receptor 1, SU5402.

Together these findings highlight complex regulatory mechanisms underlying the control, in all vertebrates, of *Fgf8,* a potent morphogen, which controls multiple aspects of animal form and function.

GENERAL INTRODUCTION

Fibroblast growth factors (FGFs) and their signaling

Fibroblast growth factors (FGFs) constitute a family of polypeptide ligands, which by binding to specific tyrosine kinase receptors (RTKs) activate cytoplasmic signal transduction pathways, controlling in time and space a wide range of developmental and physiological processes, including growth, migration, differentiation and proliferation of cells from all three germ layers. In the context of a individual multicellular organism, changes in FGF signaling can lead to pathogenesis, malformations, and tumor development and –progression, while in evolution, altered regulation of Fgfs may have contributed to the astonishing variety of animal form.

Fibroblast growth factors were originally identified as potent mitogens in cultured fibroblasts (Gospodarowicz 1974). The vertebrate Fgf family contains twenty–five members, FGF1 – 25. All members of this family are characterized by high homology in a central core of 120 –140 amino acids, which fold into twelve antiparallel β–strands that form a three–dimensional cylindrical barrel, and have more variable amino– and carboxy terminal stretches (Ago et al. 1991; Coulier et al. 1997; Zhu et al. 1991).

FGFs stimulate cellular responses by binding to the extracellular domains of their specific fibroblast growth factor receptors (FGFRs 1-4), leading to receptor dimerization and phosphorylation of the cytoplasmic domain of the FGFR. Molecular association between FGF and its receptor requires direct involvement of heparin and heparan sulfate glycosaminoglycans (HSGAGs) in a specific complex on the cell surface, which is essential for biological activity (Ornitz 2000; Powers et al. 2000; Raman et al. 2003).

Identification of Fgf8

Fibroblast growth factor 8 was identified as androgen–induced growth factor (AIGF) in the conditioned medium of the androgen–dependent mouse mammary Shionogi carcinoma cell line SC–3. SC–3 cells, which require androgen for growth stimulation, and presence of androgen is also necessary to induce secretion of AIGF (Tanaka et al. 1992). Sequence analysis revealed high similarity to other fibroblast growth factors, and AIGF became the eighth member of the fibroblast growth factor family (Ohuchi et al. 1994). Restricted and

dynamic expression of FGF8 during embryogenesis and morphogenesis suggests a unique and important role in the development of multiple embryonic structures.

Fgf8 isoforms in diverse species

The exon–intron organization of *Fgf8* has been highly conserved during vertebrate evolution from teleosts to mammals. In many species multiple Fgf8 protein isoforms, which share a common carboxyterminal region, but possess different amino termini, are generated by alternate splicing of exon 1 (Crossley and Martin 1995; Gemel et al. 1996). Isoforms vary by their binding to FGFRs and hence in their biological function, and also in mitogenic and transforming capacity. In the mouse, multiple splice donor and splice acceptor sites suggest that at least seven Fgf8 isoforms can be generated that differ only at their amino terminus (Crossley and Martin 1995; MacArthur et al. 1995a). In humans four isoforms were identified that are identical to their murine counterparts in the common carboxyl region of the mature protein. They also reveal high homology to corresponding murine isoforms in the amino termini (Gemel et al. 1996; Ghosh et al. 1996; Valve et al. 2001). Significant diversity is also observed in the 3'–untranslated region of the mRNAs between human *FGF8* and mouse *Fgf8* genes (Ghosh et al. 1996). Chicken and *Xenopus Fgf8*, like the mouse and human counterparts, are also alternatively spliced, but there are only two isoforms (Haworth et al. 2005; Sato et al. 2001; Shim et al. 2005). Isoform b of the Fgf8 protein in human, mouse and chicken, due to its higher affinity for Fgf receptors, has a more potent biological activity than other isoforms, (Gnanapragasam et al. 2003; Guo and Li 2007). In the zebrafish genome, two copies of *fgf8* genes were identified (Jovelin et al. 2007; Kikuta et al. 2007), and *fgf8a* appears to have two splice variants (Inoue et al. 2006).

Fibroblast growth factor receptors and Fgf8

Four different fibroblast growth factor receptors (FGFR) were identified in vertebrates, including human, mouse, chicken and zebrafish. FGFR 1 – 3 have structural variants generated by alternatively spliced exons IIIb – b and IIIc – c, which results in two major isoforms containing different immunoglobin–like III domains in the extracellular region (Chellaiah et al. 1994; Itoh and Ornitz 2004; Johnson et al. 1991; Lee et al. 1989). FGFR4 is unique and has only one possible form of its IgIII domain (Vainikka et al. 1992). Alternative

splicing of FGFRs increases the functional diversity of these receptors. Ligand–binding specificity and tissue–specific expression properties of alternatively spliced forms of FGFRs vary considerably and are essential to the function of the Fgf signaling system (Powers et al. 2000).

FGFRs are widely expressed during vertebrate development, and their highly specific expression patterns suggest that each of them plays an important role. *Fgfr1* is expressed almost exclusively in mesoderm (Orr-Urtreger et al. 1991; Peters et al. 1992; Yamaguchi et al. 1992; Yamaguchi et al. 1994), while *Fgfr2* is predominantly localized in epithelia (Orr-Urtreger et al. 1993; Peters et al. 1992), *Fgfr3* is expressed in the developing central nervous system as well as in developing bones (Peters et al. 1993), whereas *Fgfr4* is expressed in the endoderm and the somitic myotome (Stark et al. 1991).

Diversity in Fgf8 signaling is achieved by differential binding affinity of different isoforms of the Fgf8 ligand to different splice variants of Fgfrs during embryogenesis, in adulthood, and also in developmental defects and pathological conditions. Fgf8 has high affinity to Fgfr2c (Ornitz et al. 1996), and to Fgfr1 (Scholpp et al. 2004). In zebrafish *fgfr1* belongs to the *fgf8* synexpression group, and the co–expression of both genes in presomitic mesoderm (PSM) is important during somitogenesis (Sawada et al. 2001). Human FGF8 isoforms mediate signaling by FGFR2c, FGFR3c and FGFR4 (Buratini et al. 2005a; Buratini et al. 2005b; MacArthur et al. 1995a; Valve et al. 2001). None of the FGF8 isoforms interact with the b splice form of FGFR1 – 3, nor with the c form of FGFR1 (MacArthur et al. 1995a; Valve et al. 2001).

fgf8 duplication and the fgf8/17/18–subfamily

A whole genome duplication, and concomitant further functional diversification of the duplicated genes are thought to underlie the astonishing phenotypic variation and evolution of the teleosts (Holland et al. 1994; Wolfe 2001). Duplication of the genes and, importantly, their regulatory elements, may have facilitated functional and spatial subfunctionalization (Force et al. 1999; Kikuta et al. 2007; Kleinjan et al. 2008; Li et al. 2005; MacCarthy and Bergman 2007; Vavouri et al. 2007).

A whole genome duplication occurred early in the evolution of the ray–finned fish (Actinopterygii) (Amores et al. 1998; Aparicio 2000; Jaillon et al. 2004), and the Fgf superfamily reveals traces of expansion and loss through large–scale duplications (Itoh and

Ornitz 2004; Popovici et al. 2005). *Danio rerio* has two paralogs for only about 20% of identified human genes (Postlethwait et al. 2000), suggesting an intense rediploidization process.

Three Fgf genes have been identified in *Drosophila melanogaster*, named *branchless* (Sutherland et al. 1996), *thisbe* (also known as *fgf8–like 1*) and *pyramus* (also known as *fgf8–like 2*) (Gryzik and Muller 2004; Stathopoulos et al. 2004), and two in *Caenorhabditis elegans* (*egl–17* and *let–756*) (Burdine et al. 1997; Coulier et al. 1997). A survey of Fgf genes in the basal urochordate, the ascidian *Ciona intestinalis*, reveals presence of only six ancestors of vertebrate FGFs. One of them, *Ci–Fgf8/17/18*, was assigned by phylogenetic analysis as an ortholog of vertebrate FGF8, FGF17 and FGF18 genes (Satou et al. 2002). During two subsequent whole genome duplications, the ancestral *Fgf8/17/18* became multiplied into the three members of the tetrapod *Fgf8/17/18* subfamily (Jovelin et al. 2007). *Fgf17* and *Fgf18* reveal a high level of homology to the main member of the subfamily, *Fgf8*, whose functions in many aspects of embryonic development seem to be highly conserved in relatively distant species, such as mouse and zebrafish. The teleost *Fgf8/17/18–*subfamily also includes *Fgf24* (Itoh and Ornitz 2004; Popovici et al. 2005), which has the highest sequence similarity to *Fgf18* in teleosts (Fischer et al. 2003).

The single ancestral copy of *Fgf8* found in tetrapods underwent duplication during the teleost genome duplication, and in zebrafish and stickleback two copies of the *fgf8* gene were identified, *fgf8a* (on chromosome 13 in zebrafish) and *fgf8b* (on chromosome 1 in zebrafish) (Jovelin et al. 2007; Kikuta et al. 2007). The duplication event was followed by lineage–specific subfunctionalization (Jovelin et al. 2007). Expression of *fgf8b* (previously known as a *fgf17a* (Reifers et al. 2000a)) begins later compared to *fgf8a* and its transcript is not detectable during gastrulation. Expression of *fgf8b* largely overlaps with *fgf8a*, and it is expressed in a subpopulation of *fgf8a*–positive cells, namely those with high level of *fgf8a* transcripts. Thus, *fgf8b* is present at the midbrain–hindbrain boundary (MHB), the optic stalk and anteromedial margin of the maturing somites. Later, *fgf8b* transcripts are detectable in the otic vesicle, hyoid cartilage and the dorsal diencephalon close to the forming epiphysis (Reifers et al. 2000a).

Fgf8 loss–of–function mutants

During embryogenesis, *fgf8a* is expressed in organizing centers. Its expression starts at early stages of gastrulation, and is highly dynamic and spatially restricted (Furthauer et al. 2004; Reifers et al. 1998). The zebrafish loss–of–function mutant of *fgf8a*, *acerebellar* (*ace*), dies at around 2 – 3 days because of multiple malformations and heart oedema. Homozygous *ace* mutants fail to develop the MHB and cerebellum (Reifers et al. 1998), they have ear defects (Leger and Brand 2002; Phillips et al. 2001), heart abnormalities (Reifers et al. 2000b), and forebrain and optic stalk defects (Shanmugalingam et al. 2000).

Zebrafish *acerebellar* constitutes a splice mutation, which deletes 107 base pairs corresponding to exon 2, from the mRNA. A G to A mutation inactivates the splice donor site, leading to skipping of exon 2 and a shift in the open reading frame, and as a result to a premature stop codon. Thus *ace* protein lacks the amino acids encoded by exon 2 and 3, which are required to activate the receptor and which are conserved between Fgf8 proteins (Lorenzi et al. 1995). The *acerebellar* phenotype can be mimicked by antisense morpholinos against the *fgf8a* mRNA, targeted to the start codon (Araki and Brand 2001).

In mouse *Fgf8* is initially expressed in the primitive streak, and is essential for cell migration during gastrulation. In the *Fgf8* knockout mouse mutant, epiblast cells undergo epithelial–to–mesenchymal transition, but fail to move away from the streak. As a consequence no endodermal or mesodermal structures are formed, and mutant embryos do not proceed through gastrulation (Sun et al. 1999).

Fgf8 is important for early vertebrate embryogenesis

An essential role of Fgf8 was revealed in gastrulation, somitogenesis, brain development, and also in limb and craniofacial morphogenesis. Regions where high levels of Fgf8 transcript are detected are known to direct patterning and outgrowth of embryonic structures, and Fgf8 has been recognized as a morphogen acting in organizing centers in the embryo.

In situ hybridization analysis reveals that *Fgf8* is predominantly expressed in the primitive streak region in mouse (Crossley and Martin 1995) and *fgf8a* in the embryonic shield, the zebrafish equivalent to the Spemann organizer (Reifers et al. 1998). Further prominent expression domains include the MHB (Irving and Mason 2000; Jaszai et al. 2003; Mason et al. 2000; Picker et al. 1999; Reifers et al. 1998), the rostral forebrain

(Shanmugalingam et al. 2000; Walshe and Mason 2003b), limb ectoderm and apical ectodermal ridge (AER) (Boulet et al. 2004; Lewandoski et al. 2000; Mahmood et al. 1995; Moon and Capecchi 2000; Ohuchi et al. 1997; Sun et al. 2002), and branchial arch ectoderm and the craniofacial skeleton (Abu-Issa et al. 2002; Albertson and Yelick 2007; Hall et al. 2006; MacArthur et al. 1995a; Tucker et al. 1999a; Tucker et al. 1999b; Walshe and Mason 2003a).

Fgf8 is an organizer of brain development

Vertebrate brain patterning depends on an organizing center located at the isthmus, a constriction in the embryonic midbrain–hindbrain region. Specialization of the mesencephalon (midbrain) and metencephalon (rhombomere 1 at the anterior end of the hindbrain) and anterior–posterior patterning of this region starts during gastrulation, with the beginning of zygotic gene expression of *engrailed*–related *En1* and *paired*–related *Pax2*, which distinguishes these two regions of the brain (Bouchard et al. 2000; Hanks et al. 1995; Schwarz et al. 1997; Wurst et al. 1994). *Otx2* and *Gbx2* are also required for positioning of the isthmus, by antagonistic actions (Li and Joyner 2001; Liu and Joyner 2001; Simeone 2000).

Grafting experiments in chick revealed isthmus activity in patterning of the midbrain and cerebellum. Tissue containing the midbrain–hindbrain boundary, when grafted to caudal forebrain, can induce a cell identity change and guide development of forebrain towards an ectopic midbrain, and when the isthmus is transplanted into posterior hindbrain it induces the development of ectopic cerebellar structures (Marin and Puelles 1994; Martinez et al. 1991).

Fgf8 protein can induce ectopic expression of genes in the forebrain that are normally expressed in the isthmus. Signals from the ectopic isthmus–like organizing center induce formation of ectopic midbrain, in a mirror image of normal midbrain (Crossley et al. 1996a; Martinez et al. 1999). Thus, Fgf8 produced in the isthmus is necessary for establishment and maintenance of a signaling center in the MHB (Chi et al. 2003; Mason et al. 2000; Rhinn and Brand 2001). In mammals, Fgf8 is also involved in cortical patterning (O'Leary et al. 2007) and in zebrafish for establishment of correct retinotopic projections (Picker and Brand 2005).

Fgf8 determines size and shape of somites and somite boundaries

In vertebrates, the primary segmented tissue of the body axis, the vertebrae, ribs, skeletal muscle and dorsal dermis are derived from blocks of paraxial mesoderm, the somites that become patterned during early embryogenesis. Somitogenesis requires strict temporal and spatial regulation by the segmentation clock through oscillating gene expression and a wavefront formed by gradient expression of *Fgf8* (Baker et al. 2006a; Baker et al. 2006b). Somite formation starts in the presomitic mesoderm (PSM) in the tail that forms two parallel bands alongside the posterior notochord. PSM does not exhibit any segmentation, but displays rhythmic expression of particular genes (Pourquie 2003), and this activity persists throughout somitogenesis (Jouve et al. 2002). Somite formation proceeds in a strict anteroposterior sequence such that a new pair of somites is regularly added in a rostro–caudal fashion until a fixed species–specific number of somites is reached (Collier et al. 2000; Schnell et al. 2002; Stickney et al. 2000).

Several genes reveal dynamic expression in the PSM, with the oscillatory frequency equal to the time necessary to form one somite (McGrew et al. 1998; McGrew and Pourquie 1998; Palmeirim et al. 1997). During the formation of somites, bands of periodic expression of the *hairy1* basic helix loop helix (b–HLH) transcription factor (Palmeirim et al. 1997) and *lunatic fringe* (*l–fng*) (McGrew et al. 1998) sweep along the PSM, triggered by the segmentation clock. Notch–Delta signaling also defines somite boundaries (Conlon et al. 1995; Jiang et al. 2000; Jiang et al. 1998; McGrew et al. 1998).

Fgf8 protein is expressed at high levels only in the posterior PSM, and through subsequent *Fgf8* mRNA decay generates a gradient along the somites with the highest level in the posteriormost region (Dubrulle and Pourquie 2002; Dubrulle and Pourquie 2004). Fgf8 generates a moving wavefront along the AP axis of the PSM, which controls both segment boundary position and axial identity (Dubrulle et al. 2001; Sawada et al. 2001). High levels of Fgf8 at the posterior region of PSM are required to maintain the newly formed PSM cells in an immature and undifferentiated state. During maturation of the somite, while the cells move away from their origin in the PSM, FGF8 levels progressively decrease, and cells reach a threshold of FGF signaling, called the determination front, at a certain level of the PSM, which activates the segmentation process (Delfini et al. 2005). By decreasing levels of Fgf signaling, for example by incubation of zebrafish embryos in the small molecule inhibitor of Fgfr, SU5402, abnormally large somites are formed, while the

opposite effect is observed after transplantation of Fgf8–soaked beads into the paraxial mesoderm in the tailbud region (Sawada et al. 2001). In zebrafish, and presumably other teleosts as well, posterior mesoderm is not regulated by *fgf8* alone, but requires in addition the teleost–specific *fgf24* (Draper et al. 2003).

A further important function in determining the wavefront in the PSM and in the formation of somites is played by retinoic acid (RA), which generates a gradient antagonistic to Fgf8. RA attenuates *Fgf8* expression in paraxial mesoderm, and through this controls somite boundary position (Diez del Corral et al. 2003). Collectively, these findings show that vertebrate axis formation depends crucially on the number of *Fgf8* transcripts per cell, which by way of mRNA decay results in the formation of a protein gradient in a posterior to anterior direction.

Fgf8 initiates and maintains vertebrate limb outgrowth and patterning

The outgrowth and patterning of the vertebrate limb bud is the result of a reciprocal interaction between the mesoderm and a specialized region of the ectoderm, the apical ectodermal ridge (AER), which rims the distal tip of the limb bud (Lewandoski et al. 2000; Mahmood et al. 1995; Martin 1998; Sun et al. 2002), and which promotes proximal–distal (PD) limb outgrowth (Capdevila and Izpisua Belmonte 2001). Signals emanating from the AER act to maintain the underlying mesoderm, called the progress zone (PZ), in a highly proliferative and undifferentiated state, and cells from the PZ are assigned progressively more distal positional values during limb growth. Several *Fgf* genes are expressed in the developing limb in AER–specific patterns: *Fgf4* (Moon et al. 2000; Sun et al. 2000)*, Fgf8* (Lewandoski et al. 2000; Moon and Capecchi 2000)*, Fgf9* (Colvin et al. 1999; Colvin et al. 2001) and *Fgf17* (Amsterdam et al. 1999; Martin 1998; Xu et al. 2000). In mouse individual loss of function of *Fgf4*, *Fgf9* and *Fgf17* has no effect on limb development, whereas inactivation of *Fgf8* causes limb truncation, identifying it as the gene with a primary role in limb development (Lewandoski et al. 2000). It is expressed in the ectoderm of the prospective limb territory prior to morphological outgrowth of the limb bud in both mouse and chick, and is maintained throughout the AER during the period of limb development (Mahmood et al. 1995). Fgf8 also maintains Sonic hedgehog expression in the zone of polarizing activity (ZPA) in the limb bud (Crossley et al. 1996b), another organizing center.

Removal of the AER results in the cessation of limb bud growth, thus causing limb truncation along the proximo–distal axis and lack of distal skeletal elements (Mahmood et al. 1995). Substitution of the surgically removed AER by Fgf8 protein maintains limb bud outgrowth in mouse embryos (Crossley et al. 1996b; Mahmood et al. 1995), suggesting that Fgf8 signaling mediates AER activity (Crossley et al. 1996b; Vogel et al. 1996). The duration of *Fgf8* expression in the AER of the developing limb has also a function in the development of digits, as prolonged signaling leads to formation of additional phalanges by elongation and segmentation of the penultimate phalanx, and to formation of an additional joint. Thus, high levels of Fgf8 inhibit tip formation and promote elongation of digit primordia, whereas artificial attenuation of Fgf signaling induces premature tip formation (Sanz-Ezquerro and Tickle 2003).

Teleost pectoral fins are homologous to tetrapod forelimbs (Sordino et al. 1995), and *fgf8a* is expressed in AER during fin development. However, its disruption does not cause any developmental defects in the fin, which are normal in *acerebellar* mutant larvae (Reifers et al. 1998). The crucial role in the fish AER is instead performed by *fgf24*, a teleost specific member of the *fgf8/17/18* family, and zebrafish with loss–of–function mutation in the *fgf24* gene accordingly lack pectoral fins (Fischer et al. 2003).

Fgf8 function in skeletal development

Fgf signaling also plays important roles during the development of the skeleton. Perturbations of Fgf signaling during craniofacial skeleton development leads to multiple alterations of the head skeleton, and form a genetic background of various dysmorphology syndromes (Ornitz and Marie 2002; Passos-Bueno et al. 1999).

Fgf8 is required for the patterning of mesenchymal cells of cephalic neural crest, and regulates morphogenesis of the craniofacial skeleton. In the absence of Fgf signaling, no pharyngeal and neurocranial cartilages are formed (Creuzet et al. 2005; Crump et al. 2004; Walshe and Mason 2003a). Neural crest cells participating in the formation of head bones and cartilages can be divided into a *Hox*–negative anterior domain, which predominantly yields bones and cartilages of the face (facial skeletogenic neural crest, FSNC), and a posterior domain, where *Hox* genes of the four first paraloguous groups are activated, and which generate a small part of the head skeleton, the hyoid cartilage (Couly et al. 1996; Couly et al. 1993). Rhombomere 3 (r3) partially participates in both domains, although its

contribution to branchial arches is minor, since most of the neural crest cells derived from r3 normally undergo apoptosis (Graham et al. 1994; Graham et al. 1993). Exogenous Fgf8 protein is able to rescue development after experimental excision of the FSNC through recruitment of cells from r3 (Creuzet et al. 2005). Craniofacial defects are connected with *Fgf8* function in proliferation, migration and survival of neural crest cells (Creuzet et al. 2004; Crump et al. 2004; Walshe and Mason 2003a).

Zebrafish *acerebellar* heterozygous adults show various craniofacial defects, such as ectopic bone growth, aberrant cranial suturing and asymmetrically missing cartilages, likely because of *fgf8a* haploinsufficiency (Albertson and Yelick 2005; Albertson and Yelick 2007). However, increasing *Fgf8* activity is also associated with ectopic bone formation, and vertebral–fusion defects were recently described and linked with high level of *Fgf8* expression in vertebral bodies in the mouse. *Fgf8* knock–in to the *Tbx1* domain resulted in viable and fertile mice with hypoplasia in the two anterior cervical vertebrae and fusion of vertebral bodies (Vitelli et al. 2006). *Fgf8* is widely expressed during early stages of skeletal development, and is involved in cartilage and bone formation (Huang et al. 2003; Moon and Capecchi 2000; Walshe and Mason 2003a; Xu et al. 1999). Addition of exogenous Fgf8 protein to mouse bone marrow cultures significantly promotes cell proliferation and subsequent osteoblastic differentiation, suggesting a function in bone formation in vivo (Valta et al. 2006). Interestingly, increased expression of *Fgf8* in androgen–dependent breast and prostate tumors is correlated to occasional ectopic cartilage and bone–like structures in the tumors (Kaufman et al. 1984; Valta et al. 2006).

Fgf signaling is controlled by negative regulators, the Sprouty proteins

Fgf and Egf signaling is mediated by specific receptor tyrosine kinases (RTKs), whose activity is orchestrated in space and time by negative and positive regulators (Fernig and Gallagher 1994; Kramer et al. 1999; Reich et al. 1999). Sprouty (Spry) proteins inhibit RTK signaling through negative feedback loops, and their expression is stimulated by the ligand whose pathway they inhibit (Mason et al. 2004).

Sprouty was identified in *Drosophila* as a regulator of tracheal branching and eye development (Casci et al. 1999; Hacohen et al. 1998). Vertebrate genomes including the teleosts contain four *sprouty* homologs (de Maximy et al. 1999; Huebert et al. 2004; Mailleux et al. 2001; Minowada et al. 1999; Tefft et al. 1999; Wang et al. 2006). Through

their negative regulation of Fgf signaling, Sprouty proteins have important regulatory functions during embryogenesis and in adulthood, and regulatory defects can lead to various developmental disorders and cancers (Basson et al. 2005; Goodnough et al. 2007; Kwabi-Addo et al. 2004; Sutterluty et al. 2007; Wang et al. 2006).

The Sprouty proteins are regulated at the transcriptional level, but also through posttranslational modification, and through cellular localization. Endogenous Sprouty proteins are localized in the perinuclear region, in vesicles, and in the plasma membrane (Impagnatiello et al. 2001; Yigzaw et al. 2001). Upon growth factor stimulation of RTK Sprouty protein is translocated to the plasma membrane, where several Sprouty binding partners are located (Gross et al. 2001; Lim et al. 2000; Lim et al. 2002; Sasaki et al. 2003). Sprouty specifically inhibits the Ras/MAP/ERK signaling pathway (Shaw et al. 2007), but does not affect the phosphoinositide 3–kinase (PI3K) and other MAPK pathways (Yusoff et al. 2002). The exact mechanism and place of action of Sprouty proteins in the pathway remains controversial: Sprouty may act downstream of RTK and upstream of Ras or at the level of Raf (Gross et al. 2001; Hacohen et al. 1998; Leeksma et al. 2002; Reich et al. 1999; Yusoff et al. 2002). Nevertheless, the Sprouty proteins add an additional level of regulation to the control of Fgf signaling.

CLGY enhancer trap lines

Insertional mutagenesis and enhancer detection using retroviral vectors constitute an efficient method to characterize known as well as unknown regulatory genes involved in developmental processes and oncogenic transformation in mouse and zebrafish (Amsterdam 2003; Amsterdam et al. 1999; Ellingsen et al. 2005; Nakamura et al. 2005; Sivasubbu et al. 2007; Theodorou et al. 2007; Wang et al. 2007).

In a large–scale screen in our labolatory, a zebrafish proximal *gata2* promoter (Meng et al. 1997) was ligated into an engineered Moloney murine leukemia virus (MLV) derived CL vector (Naviaux et al. 1996), upstream of a gene encoding yellow fluorescent protein (YFP), thus creating the **CL–GATA2–YFP** (CLGY) enhancer detection vector (Ellingsen et al. 2005). The virus was pseudotyped with the Vesicular Stomatitis Virus G protein (VSV–G), enabling infection of zebrafish cells (Burns et al. 1993; Gaiano et al. 1996a). When the provirus inserts in the proximity of long–range *cis*–regulatory elements, the *gata2* promoter becomes activated and expresses YFP in a tissue specific manner. YFP expression patterns

mimic domains characteristic for the gene normally regulated by these non–coding elements (Ellingsen et al. 2005). Integration sites of the CLGY provirus were identified by obtaining genomic sequences flanking the provirus through linker mediated PCR (LM–PCR) (Wu et al. 2003). Subsequently, sequences were mapped to the zebrafish genome assembly in the Ensembl database (www.ensembl.org/Danio_rerio), using BLASTN (Ellingsen et al. 2005).

During the large–scale enhancer detection screen around 1240 transgenic lines with tissue–specific YFP expression pattern were generated. While many of the mapped insertions occurred at a small distance from genes with developmental functions, others occurred far away from such genes (Ellingsen et al. 2005).

Among CLGY enhancer detection lines generated in our laboratory, a few showed phenotypic effects in adulthood. Insertions in four such lines were mapped near the *fgf8a locus* and cause pigment pattern anomalies and body shape defects. The proviral integrations near *fgf8a* CLGY508, 657, 667 and 1030 (Paper I and III) and *spry1* CLGY786 (Paper IV) are listed in Table 1.

Table 1. CLGY enhancer detection transgenic zebrafish lines used during the research in this thesis. Chromosomal positions are based on zebrafish genomic assembly Ensembl Zv7.

CLGY line	Chromosome position	Flanking seq. length (bp)	ID (bp)	Target gene	Distance from the promoter	reference
508	chr:13 20563141–20563300	160	159	*fgf8*	9.952bp downstream	(Kikuta et al. 2007)
657	chr:13 20493685–20493754	70	70	*fgf8*	79.498bp downstream	(Kikuta et al. 2007)
667	chr:13 20525014–20525158	145	145	*fgf8*	48.094bp downstream	(Kikuta et al. 2007)
1030	chr:13 20596055–20596268	214	212	*fgf8*	23.016bp upstream	(Kikuta et al. 2007)
786	chr:14 868323–868595	273	271	*spry1*	3.333bp upstream	Paper IV

AIM OF STUDY

In light of available data, Fgf8 appears to be an important morphogen, and crucial developmental regulator. Its function in initiation and patterning of brain, craniofacial skeleton, limb formation, somitogenesis and many other embryonic structures has been extensively explored for the last decade, but very little is understood of how the gene is regulated. Given that the concentration of its mRNA often decides the fate of a cell within a given regulatory network, transcriptional control of *Fgf8* must be precise.

The aim of the present study was to investigate aspects of the regulation of *fgf8a* expression using zebrafish as a model system. In particular:

- To define regulatory elements controlling the transcription of *Fgf8*, their location, and the transcriptional patterns they direct in transgenic fish.
- To characterize a number of retroviral insertions that appeared to interfere with normal *Fgf8* signaling, and an investigation of the phenotypic consequences.
- To map the proviral insertion in an enhancer trap line with expression pattern similar to *fgf8a* and characterize the identified gene.

The combination of zebrafish as an animal model and emerging knowledge about the organization of genes and regulatory elements into genomic functional and structural chromosomal units involved in regulation of developmental genes allowed investigation of *cis*–regulation of the *fgf8a*, and this thesis provides observations which might help to explain the genetic mechanism of human split hand/foot malformation 3 (SHFM3).

In more general terms, this investigation intended to gain insight into the *cis*–regulatory activity mediated by highly conserved noncoding elements (HCNEs), which control regulation of *fgf8a* in zebrafish. This thesis provides details of organization of HCNEs in the *fgf8a* locus and shows that these elements are distributed within and beyond bystander genes in the region.

Finally this study highlights that exogenous sequences derived from retroviruses inserted into a genomic region dedicated to regulation of a morphogen can lead to

significant changes in gene activity, phenotypic traits, and might serve as a model for exaptation of newly inserted regulatory sequence in evolution.

LIST OF PAPERS

Paper I

Genomic regulatory blocks encompass multiple neighboring genes and maintain conserved synteny in vertebrates.

Hiroshi Kikuta, Mary Laplante, Pavla Navratilova, **Anna Z Komisarczuk**, Pär G. Engström, David Fredman, Altuna Akalin, Mario Caccamo, Ian Sealy, Kerstin Howe, Julien Ghislain, Guillaume Pezeron, Philippe Mourrain, Staale Ellingsen, Andrew C. Oates, Christine Thisse, Bernard Thisse, Isabelle Foucher, Birgit Adolf, Andrea Geling, Boris Lenhard and Thomas S. Becker.

Genome Res. 2007 May;17(5):545-55

Paper II

Roles for teleost-specific and pan-vertebrate noncoding elements in *cis*- regulation of zebrafish *fgf8a*

Anna Z Komisarczuk, Koichi Kawakami and Thomas S Becker

Paper III

Modulation of multiple phenotypic traits in zebrafish by insertion of steroid responsive retroviral sequences in the *fgf8a* genomic regulatory block.

Anna Z Komisarczuk, Adam Amsterdam, Nancy Hopkins, Stefanie Topp, Laure Bally Cuif, Rüdiger W Schulz, and Thomas S Becker.

Paper IV

Enhancer detection and developmental expression of zebrafish *sprouty1*, a member of the *fgf8* synexpression group.

Anna Z. Komisarczuk, Stefanie Topp, Christian Stigloher, Marika Kapsimali, Laure Bally–Cuif and Thomas S Becker.

SUMMARY OF RESULTS

Paper I – Genomic regulatory blocks encompass multiple neighboring genes and maintain conserved synteny in vertebrates.

Chromosomal areas containing long–range regulatory elements (>1Mb), are "protected" from evolutionary breaks, which would otherwise disturb regulation of developmental control genes (Becker and Lenhard 2007). While this observation challenges the random breakage theory of chromosomal evolution (Nadeau and Taylor 1984), comparative analysis of multiple genomes revealed numerous very short, "hidden" synteny blocks containing tandem repeats, suggesting that these regions had broken repeatedly during vertebrate evolution. To describe this process the term "breakpoint reuse" was coined, which indicates that multiple breaks have happened repeatedly in these fragile regions, between long "protected" regions of conserved synteny.

But why are long blocks of conserved synteny kept intact? The conserved genomic segments are enriched for highly conserved noncoding elements (HCNEs) which control the expression of target developmental gene(s). Thus, the target gene plus its multiple regulatory inputs in the form of HCNEs create a regulatory, functional domain, called a genomic regulatory block (GRB). GRBs also contain so–called "bystander" genes, phylogenetically and functionally unrelated transcriptional units that are not under the specific control of the regulatory elements, and are often expressed in patterns that are distinct from that of the target gene. Thus, developmental control genes are often regulated by elements inside or beyond other genes, from distance of hundreds of kilobases away, and this type of arrangement has been conserved for several hundred million years.

Fgf8 is kept in a syntenic block with its downstream neighbor *Fbxw4*, in all vertebrates, and also in *Ciona* genomes. The ancestral vertebrate synteny block underwent duplication in teleosts, and in zebrafish there are two copies of *fgf8*, *fgf8a* on chromosome 13 and *fgf8b* on chromosome 1. Orthologs of *POLL* and *NP_056263.1* genes present in human *FGF8* locus were lost in the zebrafish *fgf8a* block, and the *FBXW4* ortholog was lost from the *fgf8b* locus. *NPM3* is found elsewhere on zebrafish chromosome 13, separated from both *fgf8* loci.

Enhancer detection in zebrafish allows visualization of *cis*–regulatory content of the region into which the insertion has occurred. Four insertions were mapped to the *fgf8a* GRB

on chromosome 13, and all of them exhibit *fgf8a*–like patterns. The organization of regulatory elements in the proximity of the *Fgf8/fgf8a* has recently been analyzed in mouse and in zebrafish, but the results in this thesis suggest that the *fgf8a* regulatory block is much larger, including also the adjacent genes, *slc2a* and *fbxw4*.

Paper II – Roles for teleost-specific and pan-vertebrate noncoding elements in cis-regulation of zebrafish fgf8a

The introns of zebrafish *fgf8a* and and the intergenic regions towards the neighboring genes *slc2a5* and *fbxw4* contain numerous regulatory elements tested before (Inoue et al. 2006; Inoue et al. 2008), although these do not explain the expression of *fgf8a* in for example the somitic mesoderm. Comparative analysis of zebrafish *fgf8a* locus with other vertebrate species reveals the presence of highly conserved noncoding elements (HCNEs) in an area of approximately 200kb around *fgf8a*, including in the adjacent genes *fbxw4* and *slc2a5*.

These putative regulatory sequences around *fgf8a*, and in the adjacent genes and sequence beyond them were tested in multiple transgenic lines in zebrafish. Sequences were amplified and cloned upstream to zebrafish *gata2*:GFP in the destination vector based on the Tol2 transposone. 22 regulatory sequences were tested form zebrafish and one conserved only in tetrapods, which in a mouse assay directed reporter expression to the AER (Beermann et al. 2006).

Tested elements, based on the obtained results, were divided into three groups: 1. Negative elements, which did not exhibit regulatory activity. 2. Positive elements, which show specific *fgf8a* pattern in highly reproducible manner, and 3. Positive elements, which show *fgf8a* specific expression, but variations between lines are observed. The majority of positive elements directed expression of green fluorescent protein in pattern consistent with *fgf8a* expression domains, including the elements located in the adjacent *slc2a5* or *fbxw4* genes, which are expressed in skin cells and ubiquitously, respectively, and beyond these genes.

Although the *fgf8a* locus is inverted in teleost genomes with respect to tetrapods, *cis*–regulatory elements located upstream to *fgf8a*, in *slc2a5* and beyond, are located far downstream in the human *FGF8* locus in the neighbor gene *BTRC*. However, these elements, located across an evolutionary breakpoint, exhibited *fgf8a* specific activity.

Paper III – Modulation of multiple phenotypic traits in zebrafish by insertion of steroid responsive retroviral sequences in the fgf8a genomic regulatory block.

Integration of the pseudotyped MLV–derived CLGY vector in the *fgf8a/fbxw4* locus in zebrafish was observed to lead to phenotypic defects. One line, *fgf8a*^{CLGY1030}, was mapped approximately 22.8kb upstream of *fgf8a*, and three lines, *fgf8a*^{CLGY508}, *fgf8a*^{CLGY657} and *fgf8a*^{CLGY667} downstream from the gene at distances between 10 to 79.5kb from the *fgf8a* initiation codon, and one of these into the adjacent *fbxw4* gene (Table 1). Individuals from *fgf8a*^{CLGY} lines exhibit pigment stripe anomalies in adulthood, a phenotype that was previously described in the *hagoromo* (*hag*) zebrafish mutant, generated by proviral integration in the fifth intron of the *fbxw4* gene (Kawakami et al. 2000).

All *fgf8a*^{CLGY} lines represent alleles of *hagoromo*, with variable severity of the phenotype, from almost normal stripes in allele *fgf8a*^{CLGY657}, increasing in allele *fgf8a*^{CLGY667} and *fgf8a*^{CLGY508}, to the most extreme in *fgf8a*^{CLGY1030}. Investigation of pigment formation revealed that the larval pattern is not affected in mutants. Defects appear during metamorphosis, and adult fish have stripes that are branched, fused and wavy. The scales, a typical adult feature in zebrafish, are polymorphic and vary in size, and form on the body flank, and fail to develop in the dorsal and ventral regions of body.

In addition, changes in body shape in *fgf8a*^{CLGY1030}. is caused by accumulation of subcutaneous fat and severe malformations of the vertebral column and subtle craniofacial changes. The vertebral centra undergo fusion, and hemal and neural spines have multiple outgrowths and changed oriented towards the vertebral column. A decreased dose of *fgf8a* in *fgf8a+/-* fish also was found to exhibit mild vertebral column malformations. Skeletal defects in the *fgf8a*^{CLGY} mutants appear during metamorphosis, and are associated with higher level of *fgf8a* expression in the tissue surrounding the notochord.

Increased levels of the *fgf8a* expression are detected in larval and adult cerebellum. Knock–down experiment with morpholino targeted to androgen receptor transcript rescued elevated *fgf8a* expression in mutant larvae, suggesting that increased level of *fgf8a* in *fgf8a*^{CLGY} mutants can be at least partially mediated by androgens.

Paper IV – Enhancer detection and developmental expression of zebrafish sprouty1, a member of the fgf8 synexpression group.

During a large–scale enhancer detection screen, the CLGY786 line was recovered, where YFP expression pattern overlapped with that of fibroblast growth factor 8 a (*fgf8a*). The proviral vector in this line was mapped 3.333kb upstream to a zebrafish gene, which was identified as a member of the zebrafish *sprouty* (*spry*) family, and phylogenetic analysis determined this gene to be a homolog of vertebrate *Spry1* genes.

The full–length cDNA of *spry1* comprises 1270 nucleotides, containing a single open reading frame of 879bp, and encodes a predicted protein of 292 amino acid residues with a molecular weight of 27.7kDA (bankit1053103 EU379656). Amino acid sequence analysis showed conserved domains and motifs characteristic for Sprouty proteins.

Comparison of the *spry1* endogenous expression pattern by in situ hybridization and YFP reporter protein reveals significant similarities, and confirmed that enhancer trap line CLGY786 is a faithful reporter of endogenous *spry1*. Expression of *spry1* reveals a striking correlation with the *fgf8a, spry2* and *spry4* domains. In the brain it is expressed in telencephalon, dorsal diencephalon, midbrain–hindbrain boundary and rhombomeres. In the head region *spry1* positive cells are located in branchial arches and later in craniofacial skeleton and gill epithelium, and also in the optic stalk and ventral retina. In the trunk *spry1* is expressed in the lateral line, pronephros, and newly formed somites, neural plate, and later in notochord and tail fin fold.

spry1 expression is attenuated in the *fgf8/acerebellar* mutant and also by the small molecule inhibitor of receptor tyrosin kinase, SU5402, attesting an immediate regulation by Fgf activity and showing that sprouty1 is a negative regulator of Fgf signaling.

GENERAL DISCUSSION

This thesis investigates aspects of the regulation of a prominent vertebrate gene, fibroblast growth factor 8 (*Fgf8*) and one of its downstream regulators, sprouty1 (*Spry1*). During the early phases of my studies, I participated in the genomic mapping of a number of enhancer detection insertions during a large–scale screen that culminated in the realization that groups of genes have been held together in evolution in all vertebrate genomes by regulatory sequences (Paper I). In particular, I mapped and characterized the retroviral insertions *fgf8a*^{CLGY1030} and *spry1*^{CLGY786}, which provided the basis for experiments resulting in papers III and IV. To further characterize the organization of regulatory elements of the zebrafish *fgf8a* gene, and by extension other vertebrate *Fgf8* genes on the basis of the highly conserved nature of these regions, I characterized the function and location of individual regulatory elements controlling the expression of *fgf8a*. For reasons of a better flow of argument, this study has been inserted as paper II.

Organization of the genome into functional regulatory units

Comparison of the genomes of evolutionary distant vertebrate species like human and zebrafish reveals the presence of long chromosomal blocks in which the order of genes, as well as interspersed short stretches of conserved non coding sequence are conserved to a much higher degree than would be assumed under a random breakage model. Enhancer detection insertions in several such conserved blocks suggested that the regulatory elements spanning this sequence often are devoted to the regulation of a single developmental control gene within this chromosomal segment, the target gene, but not other, unrelated, non–developmental genes, the bystander genes. These blocks were recognized as functional units and were named genomic regulatory blocks (GRBs). The target genes are generally known from the literature and are those with essential roles during development, such as transcription factors, growth factors, their downstream regulators, and others. Many of these genes have multiple roles during development and therefore their expression must be strictly controlled on multiple levels, beginning with transcription regulation. Meanwhile, the bystander genes are usually functionally unrelated to target genes, and their expression pattern, often ubiquitous and/or low level, is different from the often highly restricted patterns of the target gene. Thus, the regulatory activity of clusters of highly conserved noncoding elements (HCNEs) spanning the GRB is directed toward the target gene, and

usually not the bystander genes, even if the latter contain these HCNEs. The result is colinear organization of genes and *cis*–regulatory sequences in the genomes of different species, maintained by evolutionary pressure, which had previously been hypothesized (Ahituv et al. 2005; Kleinjan and van Heyningen 2005; Mackenzie et al. 2004).

Enhancer detection in the zebrafish uncovered a number of these GRBs, showing that they are the primary reason for conserved synteny among distantly related species, and also suggested that these functional units might help in the understanding of regulatory mutations underlying human disease.

Amongst 1200 CLGY enhancer trap lines generated in the laboratory (Ellingsen et al. 2005), a handful caused visible phenotypic defects in transgenic fish, and all of them were mapped to genes involved in Fgf signaling. In four of these lines, $fgf8a^{CLGY1030}$, $fgf8a^{CLGY508}$, $fgf8a^{CLGY667}$ and $fgf8a^{CLGY657}$ (hereafter collectively named $fgf8a^{CLGY}$ lines), the retroviral vector had integrated in the proximity of the *fgf8a*, and turned out to act similarly to tumor virus integrations in the equivalent genomic region in mouse mammary carcinoma (MacArthur et al. 1995b). One additional line, CLGY786, (hereafter $spry1^{CLGY786}$) was mapped upstream of *sprouty1*, a gene encoding a negative regulator of Fgf signaling. Homozygous individuals from all of these transgenic lines exhibit similar stripe anomalies in adulthood, resembling previously reported zebrafish *hagoromo* mutant (Kawakami et al. 2000), where proviral integrations were mapped into the fifth intron of the *fbxw4/hagoromo* gene. These observations prompted me to further investigate the *fgf8a* regulatory domain in zebrafish.

Highly conserved noncoding elements distributed in the fgf8a locus show specific regulatory function.

Multiple HCNEs were found in the 200kb region around *fgf8a*, including adjacent genes *slc2a5* and *fbxw4*. Regulatory function of these conserved elements was tested in the enhancer assay. Interestingly, all tested, positive elements direct reporter gene expression into domains that are characteristic for *fgf8a*, not for *slc2a5* or *fbxw4*. Interestingly, the most remote elements showed specific and reproducible patterns in all generated lines, including elements located across a teleost–tetrapod breakpoint.

Even elements that directed more variable patterns drove GFP in domains where *fgf8a* expression is known or where *fgf8a* has developmental function. In some of these domains

fgf8a is not easily detectable by in situ hybridization, suggesting a very low endogenous dose of this morphogen.

The most important and characteristic *fgf8a* domains, like MHB, AER or anterior myotomes are represented by multiple elements across the entire *fgf8a* regulatory domain, indicating a highly controlled, and perhaps robust, mechanism responsible for maintenance of embryo organizing centers.

Analysis of multiple developmental defects in the fgf8a^{CLGY} transgenic zebrafish activates fgf8a during metamorphosis

Four independent enhancer detection transgenic CLGY lines, which exhibit *fgf8a*–like YFP expression pattern, were mapped into a 100kb region around the gene, which represents a common insertion site in the mouse (Paper I). Detailed analysis of the phenotype of *fgf8a*^{CLGY} lines revealed specific developmental defects, presumably caused by insertion of the proviral vector near *fgf8a*. Similar pigment stripe anomalies to these observed in our *fgf8a*^{CLGY} were already reported and correlated with proviral integrations in the *fbxw4* gene (Kawakami et al. 2000). Genomic rearrangements downstream to the *Fgf8/FGF8* were described in mouse and human, and in both cases *Fbxw4/FBXW4* was linked with specific defects in digit formation. The function of *Fbxw4/fbxw4* in the limb or in pigment stripe formation is unknown and can be only speculated based on the observed phenotype, but is difficult to explain: *Fbxw4* is ubiquitously and weakly expressed in the embryo and in adult tissues, and its coding sequence was not affected in the mouse mutants. In contrast, function of the neighboring *Fgf8* as an inducer and organizer of limb development in mouse and chicken made this gene an attractive candidate for these defects, and the fact that its expression in the AER is attenuated in the *Dac* mutant, served only to reinforce this view. Although there are currently few indications for *fgf8a* function in pigment development in fish except that an insertion near *sprouty1* also can cause strip defects (see below), all other observed phenotypes may well be caused in the distinct vertebrate groups by regulatory mutations affecting *Fgf8*.

For example, Fgf signaling in bone and skin development and also in adipogenesis was demonstrated (Albertson and Yelick 2007; Hutley et al. 2004; Mandler and Neubuser 2004). The role of *Fgf8/fgf8a* in the development of the skeleton has been studied in mouse and zebrafish. A decreased dose of *fgf8a* causes craniofacial skeleton defects in *fgf8+/- ace* fish (Albertson and Yelick 2007), whereas increased levels lead to vertebral centra fusion

and formation of multiple outgrowths in zebrafish $fgf8a^{CLGY}$ mutants (Paper III) resembling the mouse phenotype when *Fgf8* was overexpressed in vertebral centra (Vitelli et al. 2006). In human hormone dependent tumors, overexpression of *FGF8* is connected to the formation of ectopic bones and cartilages (Kaufman et al. 1984; Valta et al. 2006). Axial skeleton defects have been previously described in a number of fish species, however the genetic background of these mutations is unknown. For example, certain breeds of the goldfish *Carassius auratus* display significant body shape changes and some of these are also characterized by fused vertebral centra, similar to those in the $fgf8a^{CLGY}$ mutants.

The observation that a simple regulatory sequence of a retrovirus can affect multiple aspects in the shape of a vertebrate animal may suggest that exaptation of exogenous sequences might have contributed to the divergence of vertebrate species. In fact, regulatory elements related to ancient transposable elements have been recruited by regulatory network in mammals and the human *Fgf8* locus was recently shown to harbor SINE retrotransposon-related elements that act as mammal-specific enhancers in brain development (Sasaki et al. 2008).

Sprouty proteins – negative regulators of Fgf signaling

A new member of the zebrafish *sprouty* family was found by enhancer detection, and the line $spry1^{CLGY786}$, where YFP reflects endogenous *spry1*, can be seen as a member of the *fgf8* synexpression group. Homozygous individuals from this line revealed a congenital pigment stripe phenotype and mild susceptibility to cancer not unlike that observed in the $fgf8a^{CLGY}$ mutants (data not shown). This observation, combined with what we know about Sprouty – Fgf interactions during development, this finding would support a role of *fgf8a* in the observed defects in $fgf8a^{CLGY}$ lines, and in the *hagoromo* mutant. Although this would imply two different mechanisms by which proviral insertion close to the gene can change its expression, in fact downregulation of gene transcription is what is most commonly observed: Large–scale insertional screens in zebrafish are potent tool for generating number of mutants which affect genes involved in the various developmental processes. Majority of identified mutations caused by proviral integrations, lead to deactivation or downregulation of the affected gene, even when insertions occur in noncoding sequence around the gene (Amsterdam et al. 2004; Gaiano et al. 1996b; Gross et al. 2005). Therefore, insertions close to the promoter of the *spry1* gene might cause reduction of gene expression. To confirm or

reject this hypothesis, more detailed studies will be necessary to determine the mechanism that leads to the observed phenotype in the *spry1*^{CLGY786} line.

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