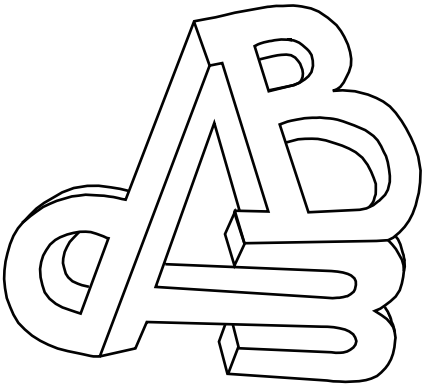
Bmp signaling is at the heart of vertebrate left-right asymmetry



Manon Verhoeven

The research described in this thesis was performed at the Hubrecht Institute of Developmental Biology and Stem Cell Research, part of the Royal Netherlands Academy of Arts and Sciences (KNAW), within the Graduate School of Developmental Biology, Utrecht, the Netherlands. This study was supported financially by the Dutch Organisation for Scientific Research (NWO), department General Life Sciences (ALW).

ISBN/EAN: 9789490122010
Print: Gildeprint BV, Enschede, the Netherlands

Financial support by the Netherlands Heart Foundation and the Juriaanse Foundation for the publication of this thesis is gratefully acknowledged.

Bmp signaling is at the heart of vertebrate left-right asymmetry

Bmp signalering ligt in het hart van links-rechts asymmetrie in vertebraten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 2 april 2009 des ochtends te 10.30 uur

door

Manon Chrétienne Verhoeven
geboren op 11 december 1979 te Oss

Promotor: Prof. dr. H.C. Clevers

Co-promotor: Dr. J. Bakkers

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Chapter 1

General Introduction

Manon Verhoeven
Jeroen Bakkers

Adapted from : Bakker J, Verhoeven MC, Abdelilah-Seyfried S. Shaping the zebrafish heart: from left-right axis specification to epithelial tissue morphogenesis. Submitted.

Although vertebrates appear bilaterally symmetric on the outside, various internal organs are asymmetric with respect to their position and/or their orientation based on the left-right (L/R) axis. This is a highly conserved feature and the normal asymmetric arrangement (situs solitus) occurs in well over 99% of humans. Interestingly, individuals who have all their organs arranged in a mirror-image fashion (situs inversus) generally face no harmful physiological consequences and often go through life undiagnosed. On the other hand, situs ambiguus produces a mixture of normal and abnormal L/R pattern and usually manifests multiple congenital anomalies including asplenia or polysplenia, intestinal malrotation, pulmonary isomerism and various complex heart defects (figure1).

The timing of alterations in L/R patterning information is also of great significance with respect to the phenotype. Disruptions occurring early in the signaling cascade, when overall L/R patterning is established are predicted to have global effects, whereas later modifications are expected to affect only one or some organ(s). A global reversal of L/R identity leads to a situs inversus, whereas a randomization of the L/R patterning information as well as ambiguous (bilateral or absent) information

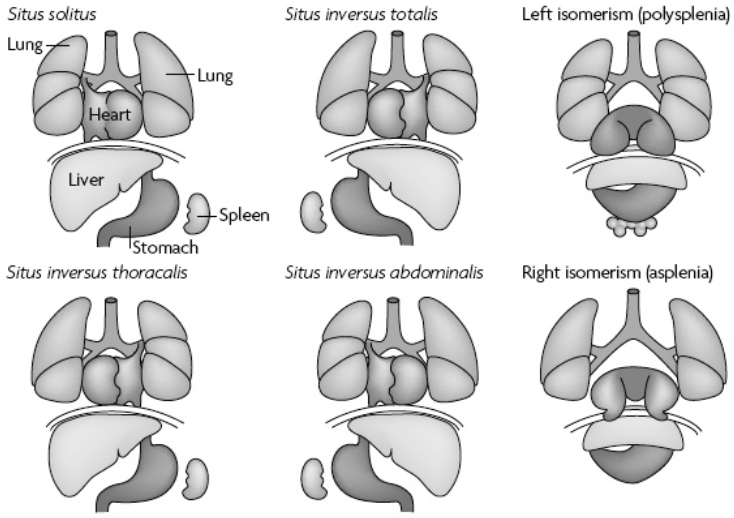

Figure 1 Human laterality disorders. Schematic illustration of normal left–right body asymmetry (situs solitus) and five laterality defects that affect the lungs, heart, liver, stomach and spleen. A left or right isomerism reflects a duplication of the left or the right side respectively. Adapted from (Fliegauf, 2007).

results in either a randomized positioning of organs (organ discordance or situs ambiguus) or a mirror-image duplication of organs (isomerism).

In humans, clinically significant laterality defects occur at least 1 in 10,000 births (Peeters and Devriendt, 2006). The significant morbidity and mortality associated with laterality disease almost always are attributed to complex congenital heart defects, reflecting the extreme susceptibility of the developing heart to disturbances in L/R patterning.The process of symmetry breaking is thought to improve organ

packaging in a limited space through handed positioning and looping, but is also necessary for proper functioning of many organs. One striking example of that is the heart, the main subject within this thesis. The subdividing organization into left and right chambers is essential for its function and if L/R asymmetry is abnormal, a wide spectrum of congenital heart defects can arise. They range from atrial septal defects (ASD) or ventricular septal defects (VSD) to life-threatening malformations such as double outlet right ventricle (DORV), double inlet left ventricle (DILV) or a transposition of the great arteries (TA) (Ramsdell, 2005) (figure2).

The lack of variability of the body situs under normal conditions within and across vertebrate species implies that the determination of L/R identity is controlled by a highly conserved pathway. The L/R patterning can be subdivided into three distinct phases. The first involves the initial break in L/R symmetry and focuses on actions in and surrounding the nodal area. Subsequently, the second phase converges on the propagation of the L/R information from the nodal area to the lateral plate mesoderm (LPM). And finally, this asymmetric information needs to be interpreted by individual organ primordia in the third phase, resulting in the asymmetric morphogenesis of several organs.

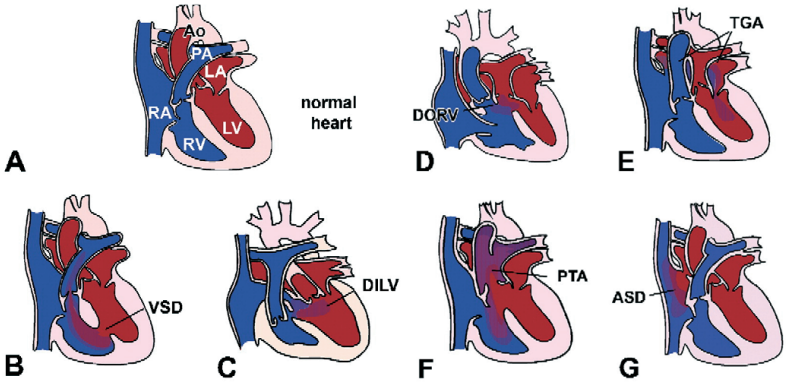

Figure 2 Congenital heart defects that are frequently associated with laterality disease. A schematic depiction of a normal heart is shown in panel A. Red and blue indicate oxygenated and deoxygenated blood, respectively, and purple shading in panels B–G indicates mixing of oxygenated and deoxygenated blood. A ventricular septal defect (VSD) is shown in panel B. An atrial septal defect is shown in panel C. Double-inlet left ventricle (DILV) is shown in panel D. Double-outlet right ventricle (DORV) is shown in panel E. Transposition of the great arteries (TGA) is shown in panel F. Persistent truncus arteriosus (PTA) is shown in panel G. All hearts are drawn in ventral view. Adapted from (Ramsdell, 2005).

INITIAL BREAK OF SYMMETRY

With the discovery of Kartagener syndrome, a human genetic disorder exhibiting laterality defects, the connection between cilia and L/R patterning was highly expected. This syndrome displays some striking features like immotile respiratory cilia and sperm flagella that lack dynein arms (Afzelius, 1976). The connection

was furthermore supported when the gene causing one of the most described mouse mutant with randomized L/R determination turned out to be an axonemal dynein (Supp, 1997). The first conclusive evidence for the link between cilia and asymmetry was reported 10 years ago, based on the study of a Kif3b mutant mouse, encoding a kinesin motor protein (Nonaka, 1998). In wild type mice they observed a clockwise rotation of primary cilia found in node cells, creating a leftward flow of the extra-embryonic fluid within the nodal cavity. The role of this fluid flow was further emphasized when an artificial rightward flow was imposed to the embryo and proved to be able to reverse L/R patterning (Nonaka, 2002). After that, all arrows were pointed towards gaining insight in how this unidirectional fluid flow could be generated by the rotational movement of cilia. The answer came after detailed motion and morphological analyses of the node cilia and by fluid dynamic model experiments. The cilia were found not to protrude perpendicular to the node surface, but are tilted posteriorly. This leads to a more efficient sweep of the cilia towards the left as opposed to the right. For the mechanism of how the nodal flow determines L/R patterning, two models now exist. The first is based on the formation of a chemical gradient, by the asymmetric accumulation of a diffusible morphogen (Nonaka, 1998; Okada, 2005)(figure3). Nodal, Fgf, Shh and retinoic acid are proposed to act as such a morphogen (Tanaka, 2005), although this could not be fully supported by genetic mouse data. Nodal, for instance, has been recently shown to signal from node to LPM via an internal route rather than an external one (secretion in nodal cavity) (Oki, 2007). Either way, ultimately an elevated intracellular concentration of Ca^{2+} on the left periphery of the ventral node is initiated, which is then propagated laterally to fix the identity of the left side (McGrath and Brueckner, 2003).

The second model is founded on the physical stimulation of the nodal flow that is mechanistically sensed by immotile cilia situated in the peripheral region of the ventral node (figure3). These immotile cilia contain the Ca^{2+} permeable cation channel Polycystin-2 (Pkd2). Flexing these cilia will also lead to an elevation of intracellular Ca^{2+} on the left side of the node, which in turn can activate asymmetric patterns of gene expression (McGrath and Brueckner, 2003; Mercola, 2003; Tabin and Vogan, 2003; Yost, 2003). This second model was proposed as an explanation for the differences observed in phenotypes of mutant mice lacking cilia motility or those lacking cilia altogether. The absence of nodal flow due to absent monocilia consequently leads to an unsuccessful break in bilateral symmetry, whereas mutations affecting nodal flow due to immotile cilia result in randomized asymmetry. Both models however converge together when the leftward nodal flow is eventually translated into an asymmetric intracellular calcium signal at the left side of the node. This provides an important link between nodal flow and left-specific cell signaling. The question now emerges whether this mechanism is the event that breaks the initial symmetry, or whether other events precede the rotating cilia. When seen in an evolutionary broader context, a review of early symmetry-breaking steps in eukaryotes suggests a considerable role for intracellular polarity

in L/R specification at a subcellular level. There, randomly distributed key molecules, like ion transporters, become asymmetrically distributed during the first sets of cleavages, by the activity of intracellular motor proteins. H^+/K^+-ATPase activity was reported necessary for normal L/R asymmetry development, providing early L/R

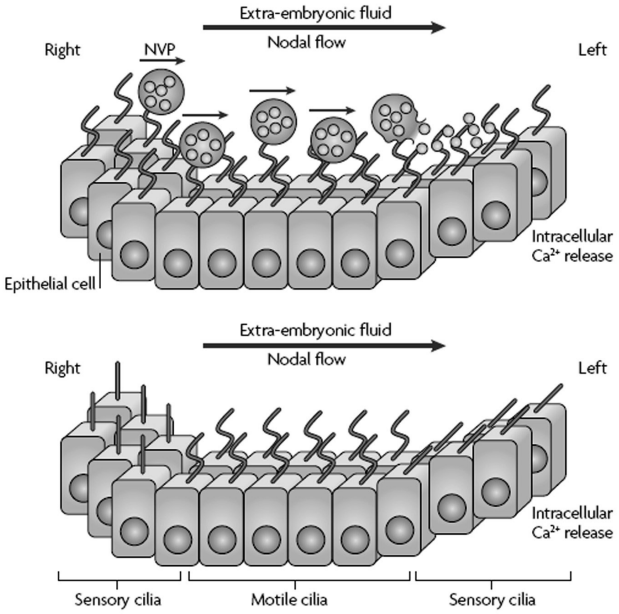

Figure 3 Current models for establishing left–right asymmetry.
By their vigorous circular movements, motile monocilia at the embryonic node generate a leftward flow of extra-embryonic fluid (nodal flow). The nodal vesicular parcel (NVP) model predicts that vesicles filled with morphogens (such as sonic hedgehog and retinoic acid) are secreted from the right side of the embryonic node and transported to the left side by nodal flow, where they are smashed open by force. The released contents probably bind to specific transmembrane receptors in the axonemal membrane of cilia on the left side. The consequent initiation of left-sided intracellular Ca^{2+} release induces downstream signalling events that break bilaterality. In this model, the flow of extra-embryonic fluid is not detected by cilia-based mechanosensation. In the two-cilia model, non-sensing motile cilia in the centre of the node create a leftward nodal flow that is mechanically sensed through passive bending of non-motile sensory cilia at the periphery of the node. Bending of the cilia on the left side leads to a left-sided release of Ca^{2+} that initiates the establishment of body asymmetry. Adapted from (Fliegauf, 2007).

cues based on differences in membrane potential in *Xenopus*, zebrafish and chick (Levin, 2005; Levin and Palmer, 2007; Shu, 2007). Different studies in zebrafish have described the role of Ca^{2+} signaling in setting up L/R asymmetry, including a role in Kupffer's vesicle formation and correct function, the regulation of cilia motility as well as the translation of the leftward fluid flow into asymmetric gene expression patterns (Bisgrove, 2005; Kreiling, 2008; Sarmah, 2005; Schottenfeld, 2007; Shu, 2007). Amphibians, fish and birds could represent transitional stages where both ciliary and intracellular mechanisms control the orientation of L/R asymmetry. In

species like mice, however, these intracellular mechanisms might no longer serve as early L/R cues and the leftward nodal fluid flow could be considered the initial break in body symmetry. For more details on this early phase in L/R specification we refer to two very detailed reviews on this matter by (Hirokawa, 2006) and (Levin and Palmer, 2007).

TRANSFER INFORMATION FROM NODE TO THE LPM

After establishing L/R identity within the node, it is essential to transfer this information to the LPM, because most organs that display L/R asymmetry are derivatives of the LPM. How this occurs has been studied extensively but is still not fully understood. The discovery of asymmetric gene expression patterns, in majority, in chick embryos opened the field to analyze molecular pathways. Some of the key players that have been reported with asymmetric expression patterns are Nodal, Lefty1 and 2, Pitx2, Bmp4, Shh and Fgf8.

During the early stages of L/R determination, chick embryos express *Shh* on the left side of the node prior to the left-sided *Nodal* expression and *Shh* has been shown to be both necessary and sufficient to induce nodal expression in the left LPM (Pagan-Westphal and Tabin, 1998). Bmp4 signaling in its turn is both necessary and sufficient to maintain *Shh* asymmetry within the node, and this regulation is mediated by a regulatory network, involving ActivinβB, Mtf2 and Mid1. Shh and Bmp4 proteins negatively regulate each other's transcription, resulting in a strict complementary gene expression pattern on either side of the node (Monsoro-Burq and Le Douarin, 2001).

Notch signaling has also been implicated in providing early polarity cues in various organisms, from nematodes to mammals. In mouse, chick and zebrafish Notch signaling has been shown to determine L/R asymmetry by inducing nodal expression surrounding the node. Notch-responsive elements were detected in the Nodal promoter, unveiling a direct relationship between the two (Krebs, 2003; Raya, 2003). The induction of asymmetric *Nodal* expression implies a requirement for an asymmetric domain of Notch activation in the left peri-nodal region, which in its turn is dependent on a transient accumulation of extracellular calcium (Raya, 2004).

Regulation of Nodal signaling

The initiation of *Nodal* expression in and around the node plays a central role in determining the L/R identity. As a member of the TGFβ super family Nodal signals through a complex of type I and type II receptors in the presence of an EGF-Cfc co-factor (Yan, 2002; Yeo and Whitman, 2001). Both genetic and biochemical studies have shown that Nodal and its orthologs are absolutely dependent for their biological activity on the presence of these co-factors in the plasma membrane (Saijoh, 2000; Shen and Schier, 2000). Nodal ligands have properties associated with a morphogen: they act over a distance to elicit dose-dependent responses

in an area of responsive cells (Ashe and Briscoe, 2006). The stability as well as the efficiency of Nodal processing are primary determinants of their signaling range (Beck, 2002; Le Good, 2005).

Active signaling results in the onset of downstream targets, like Lefty 1 and 2, Pitx2, Cyclops and Nodal itself. After *Nodal* expression is initiated in the node and in close vicinity around the node, a second wave of *Nodal* expression spreads through the left LPM in an anterior-posterior fashion. Nodal is currently the only signaling gene whose function in the node has been found essential for subsequent *Nodal* expression in the left LPM, thereby transferring L/R information to the rest of the embryo (Brennan, 2002; Saijoh, 2003). The question still remains whether the Nodal signal is relayed, or whether Nodal itself is transported. The expression pattern in the LPM is highly dynamic as well as transient, and consistent with this and its crucial role in various developmental processes, Nodal is very tightly regulated in numerous ways. Importantly, Nodal regulates *Nodal* expression through an auto-regulation mechanism that involves the transcription factor FoxHI (Long, 2003; Norris, 2002; Osada, 2000; Saijoh, 2000). Together with the activation of its own antagonists Lefty1 and 2, Nodal is at the midst of its own complex regulatory loop, which is proposed to be fundamental for the propagation of their asymmetric expression in the left LPM (Branford and Yost, 2002; Feldman, 2002; Meno, 1999; Meno, 1998; Saijoh, 2000). Specifically, Lefty induced by Nodal signaling antagonizes Nodal and dampens the activity or the response to Nodal signals in surrounding cells. This limits the effective range of Nodal, as well as the duration of Nodal signaling in a developing embryo (Chen and Schier, 2002). In Lefty2 deficient mice *Nodal* expression therefore persists longer and reaches farther than in wild types, and the asymmetric expression of the downstream target Pitx2 was also markedly upregulated (Meno, 2001). The antagonizing activity of Lefty represses right-sided *N*odal expression, based on the observation that mouse embryos deficient for Lefty1 show bilateral *Nodal* expression in the LPM. Lefty1 is expressed in various domains, but its expression in the midline is crucial for regulating Nodal signaling. The Lefty1 expression in the midline may function as a physical barrier to prevent the spread of asymmetric signals in the LPM to the inappropriate other side, thereby avoiding the right LPM to acquire a left-sided identity. It has also been suggested that the midline may function as a source of molecules that either direct or repress gene expression in adjacent tissues (Lohr, 1998; Lohr, 1997). To date, no single mechanism proposed to explain the regulation of L/R patterning is capable of accounting for the diversity seen in laterality defects in humans and model organisms (Bisgrove, 2000).The effect of the correct development of the midline structures on the concordance of heart, gut and brain L/R asymmetry have been studied and will be discussed later in this introduction.

The relation of Nodal and Lefty was suggested to resemble that of a 'reaction-diffusion system', a theoretical pattern-generating model. This model requires that the feedback inhibitor diffuses faster and spreads farther than the activator (Meno, 2001; Saijoh, 2000), which holds true for Lefty as opposed to Nodal (Sakuma, 2002).

The model harbors a 'self-enhancement and lateral-inhibition' (SELI) nature and produces 'self-organizing patterns' of expression. The SELI model is able to convert a small difference between two separated regions into a robust difference through local activation and long-range inhibition (Meinhardt and Gierer, 2000). Potentially, the nodal flow generates only a small difference in nodal expression around the node and the interplay of Nodal and Lefty converts this small asymmetry into an amplified and exclusively left-sided Nodal expression spanning the left LPM. With this model it is easier to comprehend how dramatic differences in gene expression between the left and right side of the embryo can be initiated by for instance leftward nodal flow. By construction of a mathematical model it is possible to both simulate and predict certain asymmetry determining events in mouse embryos (Nakamura, 2006).

The molecular basis for Lefty/Nodal antagonism appears to be based on the interactions of Lefty proteins with EGF-Cfc proteins as well as Nodal ligands, thereby blocking formation of receptor complexes (Chen and Shen, 2004; Cheng, 2004). In addition, the long-range action of Nodal was recently reported to be dependent on the growth and differentiation factor Gdf1, a member of the TGFβ super family. Gdf1 directly interacts with Nodal and thereby greatly increases its specific activity (Tanaka, 2007).

Role of Bmp signaling: positive, negative or both

Describing the relationship between nodal signaling and Bmp signaling, one comes across a variety of contradictions. One of these covers a very fundamental issue, namely, whether the effect of Bmps on nodal signaling is positive or negative. Studies in chick suggest that Bmp acts as a positive facilitator of the left-sided molecular cascade and is required for Nodal induction and its maintenance in the left LPM (Piedra and Ros, 2002; Schlange, 2002; Yu, 2008). Bmp is not sufficient for *Nodal* expression, since the positive effect of Bmp on Nodal was shown only effective when *Nodal* expression has first been initiated by Shh. The positive regulatory function of Bmps was proposed as indirect, acting by regulating Cfc asymmetric expression (Piedra and Ros, 2002; Schlange, 2002). Cfc functions as a co-factor rendering cells competent to respond to Nodal signaling (Gritsman, 1999). Notably, once *Nodal* expression has been activated in the LPM, it no longer requires Bmp signaling, or *Cfc* expression (Schlange, 2001). Consequently, this regulatory mechanism cannot account for the highly dynamic and transient pattern of *N*odal expression in the LPM. Experiments in mouse embryos have suggested a positive role for Bmp signaling in regulating Nodal as well (Fujiwara, 2002; Yu, 2008). In the absence of embryonic Bmp4, as well as culturing embryos in the presence of the Bmp antagonist Noggin, the expression of Nodal and Lefty2 is absent in the left LPM (Fujiwara, 2002).

On a contrary, a repressive function of Bmp signaling was described initially in chick and later supported by studies in other organisms. Caronte, a member of the Cerberus family of Bmp antagonists is expressed in the left LPM, and misexpression was shown sufficient to activate Nodal in the right LPM (Rodriguez Esteban, 1999;

Yokouchi, 1999; Zhu, 1999). Considering that Caronte acts upstream of Nodal and that several Bmps are symmetrically expressed in the LPM, this resulted in a model in which the expression of Caronte releases the initial Bmp-mediated repression of Nodal and thereby allowing *Nodal* expression on the left side of the LPM. The antagonistic function of Caronte was however questioned in the more recent studies describing a positive role for Bmp signaling, mainly because the expression domains of Caronte and Bmp2 in chick do not overlap. Also both Caronte and Bmp2 enhance *Pitx2* expression, and although Cfc factors are dependent on Bmp, overexpression of Caronte fails to suppress Cfcs (Piedra and Ros, 2002; Schlange, 2002). Whether Caronte establishes a Bmp gradient in the LPM, or whether the interaction between them is of a different nature, needs to be further investigated.

Studies in mouse embryos also attributed Bmp signaling a repressive role, when mice deficient for either Smad5 or Alk2, express Lefty1 at a very low level, while Nodal, Lefty2 and Pitx2 are bilaterally expressed (Chang, 2000; Kishigami, 2004). Recent work delivered additional evidence for a repressive effect of Bmps on *Nodal* expression in mouse. Two Bmp antagonists, Chordin and Noggin, were reported to limit Bmp signaling in the left LPM, and thereby relieving the repression of Nodal. This lead to a new model of asymmetric nodal signaling set up by Bmp antagonism instead of an active role for Bmp signaling (Mine, 2008).

In Xenopus, Bmp signaling was also proposed to repress *Nodal* expression, as inactive Bmp signaling on the right side led to bilateral *Nodal* expression. Constitutively active Bmp signaling, on the other hand, resulted in loss of *Nodal* expression (Ramsdell and Yost, 1999). Similar results were observed in the zebrafish (Chocron, 2007) and a model was proposed where Bmp represses Nodal by inducing Lefty1 within the midline which then prevents *Nodal* expression on the right side of the embryo. This does contradict earlier observations in chick embryos where Bmp signaling represses Lefty expression in the midline (Piedra and Ros, 2002; Schlange, 2002; Yokouchi, 1999). The foundation of this controversy may lie in the variations between species and/or the differences in experimental approach.

Arguments are presented and supported for both the nodal inducing as well as the Nodal repressive role of Bmp signaling and for now, this paradox mainly indicates the complexity of the regulatory mechanisms in establishing and maintaining L/R patterning. The experiments suggesting a repressive role for Bmps on *Nodal* expression in chick were carried out at early developmental stages when ectopic Bmp signaling can abolish *Shh* expression in the node, which in turn leads to loss of *Nodal* expression (Monsoro-Burq and Le Douarin, 2001; Rodriguez Esteban, 1999; Yokouchi, 1999). The studies describing a positive role for Bmp signaling focus on a later phase of L/R specification in chick. Bmp signaling therefore seems to regulate L/R asymmetry at different time points in development in chick and mouse and this hypothesis has recently been strengthened by studies in zebrafish. There, Bmp-dependent regulation of L/R asymmetry was distinguished in two distinct phases. First, shortly after Kupffer's vesicle has been formed during early segmentation, Bmp4 is required to represses the Nodal-like gene *southpaw* and as a consequence

regulate both cardiac and visceral laterality. Later during segmentation, a second wave of Bmp4 signaling is required for cardiac, but not visceral, laterality by positively regulating the expression of *cyclops, lefty1* and *lefty2* in the left cardiac field. Therefore, Bmp4 seems to display opposing activities on the left and the right side and acts both upstream and downstream of Nodal signaling in the zebrafish (Chocron, 2007).

Together, these data suggest Bmp signaling have multiple functions in L/R patterning in mouse, chick and zebrafish, probably due to their dynamic expression in various tissues at different developmental time points (Chocron, 2007; Fujiwara, 2002). The data therefore might not contradict each other, but all add different components to a very complex regulatory network surrounding Bmp and Nodal signaling.

SITUS-SPECIFIC ORGANOGENESIS

A crucial feature in establishing asymmetric organogenesis is the interpretation of L/R specific information that is initiated around the node and subsequently propagated through the LPM. This information needs to be interpreted by different organ primordia, and is often translated in asymmetric gene expression patterns in or surrounding these primordia. As a result cells will undergo asymmetric morphogenesis, which is tightly regulated and very specific for each organ. There are at least three different ways to generate an asymmetric organ. The first is looping or rotation out of the midline, mainly seen in organs that originally form as a tube (heart & gut). In the second, an originally symmetric organ develops side-specific differences in size or branching pattern (i.e. lung). And third, one side of a bilateral symmetric structure undergoes regression, and only one side remains (venous system)(Casey and Hackett, 2000).

Since the establishment of L/R identity occurs far earlier than the actual asymmetric morphogenesis, transiently expressed genes like nodal and lefty are not sufficient to transfer the L/R information to different organ primordia. The homeobox gene Pitx2, acting downstream of nodal signaling, has been extensively reported over the years to fulfill this task (Logan, 1998; Yoshioka, 1998).

Interpretation of LR information mediated by Pitx2

Pitx2 is expressed in anterior mesoderm and the left LPM in mouse, chick, *Xenopus* and zebrafish, and described as a direct target of Nodal. (Campione, 1999; Logan, 1998; Piedra, 1998; Ryan, 1998; Yoshioka, 1998). Unlike Nodal, left-sided *Pitx2* expression is maintained until much later in development and is still evident in primordia of both heart and gut (Campione, 2001; Essner, 2000). In the absence of Nodal *Pitx2* expression is maintained by Nkx2 (Shiratori, 2001). In zebrafish there are two isoforms, Pitx2a and Pitx2c, which display distinct expression patterns, differential regulation and have non-overlapping functions during asymmetric organ development (Essner, 2000). Mammalian Pitx2 has three variants but only the Pitx2c isoforms seems to be cardiac-specific. Mouse studies have shed significant light

over the last years on the role Pitx2c plays in heart asymmetry. Pitx2 null mouse embryos display severe defects in valve formation, sinuatrial morphogenesis and atrioventricular connections, including double outlet right ventricle and transposition of the great arteries (Kitamura, 1999; Liu, 2001). However, this knockout as well as the isoforms-specific Pitx2c knockout shows normal cardiac looping, suggesting no specific role for Pitx2c in this process (Kitamura, 1999; Liu, 2001; Liu, 2002). Studying myocardial-specific Pitx2 knockout revealed its function in mediating L/R atrial identity and asymmetrical ventricular and outflow tract remodeling in a dose-dependent manner (Ai, 2006; Franco and Campione, 2003; Tessari, 2008). Specifically, Pitx2 is required to suppress a SA node transcriptional program in the left side of the developing heart, combined with a repression of *Bmp10* expression in the left atrium. This suggests that Pitx2 represses a default right transcriptional pathway which differentiates molecularly and morphologically the left and right SA regions. Since Pitx2 is a transcriptional activator, these actions might be mediated by activation of one or more repressive pathways (Tessari, 2008). On the other hand, Pitx2 was found to promote cardiomyocyte maturation in the second heart field and thereby contributing to the expansion and subsequent remodeling of the right ventricle and the outflow tract (Ai, 2006; Tessari, 2008). Pitx2 is certainly not only involved in asymmetric heart morphogenesis, but is also critical in for instance gut looping, in asymmetric gonadal development in both sexes of mouse and chick embryos, and anterior pituitary development (Davis, 2008; Essner, 2000; Guioli and Lovell-Badge, 2007; Kelberman and Dattani, 2006; Kurpios, 2008; Shiratori, 2006). The mechanism how Pitx2 regulates asymmetric organogenesis continues to be full of open questions. The discovery of downstream targets of Pitx2 remains crucial in unraveling this matter. New light could then be shed on how Pitx2 can regulate the asymmetry of different organs both individually of in coordination.

Concordance among different organs

When considering the relationship between the directionality of heart looping and asymmetry of the intestinal organs, a high degree of concordance can be seen. In addition to the cardiac looping, the positioning of the visceral organs and the direction of gut looping can all be predicted by the initial direction of the heart tube (cardiac 'jogging'); cardiac and visceral organ laterality are linked under normal conditions. In homozygous *iv* -/- progeny, 50% displays *situs solitus* and 50% *situs inversus,* and the same pattern of inheritance is observed in human patients suffering from the Kartagener syndrome (Supp, 1997). This suggests that although laterality is perturbed, the linkage among asymmetric organs remains.

In both humans and model organisms, very diverse laterality defects can be seen, which exhibit different degrees of discordance in brain, heart and gut asymmetry. This suggests that the L/R orientation of each of these organs can be regulated separately (Bisgrove, 2000). Misexpression of Shh for instance, a key regulator early in the L/R signaling cascade, on the right side of the embryo reverses asymmetric gene expression patterns in precursors of both heart and viscera. The reversals,

however, in the heart and the gut are uncoordinated, suggesting that each organ interprets the signal in its own way (Schilling, 1999).
That initial heart tube position (direction of jogging) and the chirality of heart looping, as well as the gut looping are highly correlated was determined in various wild type zebrafish strains. Discordance was studied in several zebrafish mutants resulting in different categories. Normal development of midline structures seems to be a requirement for coordination between heart jogging, heart looping and intestinal looping to occur (Chin, 2000). Heterotaxia phenotypes are obviously not only found in mutants affected in their general L/R patterning, also mutants for genes involved in a organ-specific process can show an ambiguous situs. Misexpression of Bmp4 on the right side reverses the heart, but the visceral organs are unaffected, consistent with a function for Bmp signaling specifically in the heart field (Schilling, 1999).

Cardiac Jogging

In vertebrates, the heart is the first organ to develop asymmetrically. Many reviews nominate the onset of cardiac looping as the visible sign of L/R asymmetry in vertebrate embryos. Since the establishment of L/R asymmetry has been studied extensively over the past decade, we now know that the morphological symmetry is already broken prior to cardiac looping, when the heart tube begins to form. Zebrafish is so far the only model organism in which this phenomenon has been studied and the early process of heart tube formation has thereby been extensively described. The heart tube consists of an outer myocardial layer and an inner endocardial layer, which are both derived from bilateral populations of mesodermal cells that fuse in the midline to form a cardiac cone. This cone has its base on the yolk and its central cells will give rise to the future ventricle (atrial pole), whereas the peripheral cells will form the future atrium (venous pole) (Stainier, 2001). Until recently, the model for breaking the bilateral symmetry in heart morphogenesis was based on the simultaneous extension of the cone into a tube and the tilting of the cone. This results in a leftward displacement of the developing tube, a process that is referred to as cardiac jogging. The mechanism of this cardiac jogging however is not fully understood, what is known however, is that it is dependent on L/R signaling and it is predicting the direction of the cardiac looping. Therefore a leftward displacement of the heart tube will result in a rightward (D-) loop, which occurs under normal circumstances, and a reversed rightward displacement of the tube will result in a reversed leftward (L-) looping ((Chen, 1997) fig.4). The tilting of the tube as it was described might be oversimplified and recently it was reported that much more complex cellular behaviors are involved during tube morphogenesis. The zebrafish proved very useful for studying tissue dynamics required for the transition from a cardiac cone into a heart tube. Simultaneously with the extension and the leftward displacement, the cardiac cone undergoes a rotational movement in a clockwise fashion and an involution of myocardial cells at the right-posterior border of the cardiac cone (Rohr, 2008; Smith, 2008). These movements result in a redistribution of the cells, where the cells initially restricted to the left half of the cone end up

largely in the dorsal part of the heart tube. So one could speak of a conversion of the L/R polarity of the cone into a dorsal-ventral (D/V) polarity of the tube. Similar studies were done in mouse and chick, were Pitx2 was used as a marker for the left cardiac crescent, combined with labeling cells in that part of the heart. The left cardiac tube in both mouse and chick was demonstrated to contribute to the left atrium, the ventral portion of both ventricles, and to the ventral-left part of the outflow tract (OFT). Here, the L/R polarity in the linear tube is maintained in the sinoatrial region but is converted into D/V polarity in the ventricles and OFT (Campione, 2001). The fact that the left side of the heart tube ends up on the dorsal side in zebrafish embryos and on the ventral side in mouse and chick, reflects more similarity than would seem, it merely represents the differences in early orientation

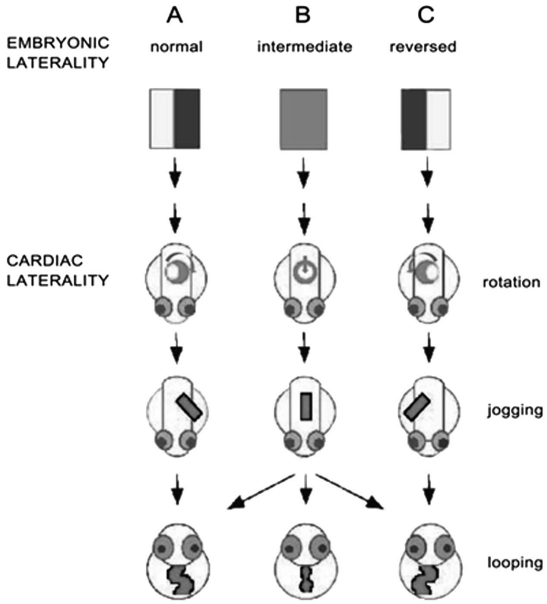

Figure 4 Model of the decisions in cardiac laterality. (A) Normally, the primitive heart tube reads the embryonic left-right signals and the heart cone rotates to the left. This leads to left-sided jogging and, later, right-sided looping. (B) If the embryonic left-right signals are lost or the heart fails to interpret embryonic left-right asymmetry, no rotation is observed. The heart then fails to jog. Looping will have no directionality and the heart may bend to the left, right or not at all. (C) If the embryonic left-right signals are reversed and the heart cone rotates to the right. This causes right-sided jogging and left-sided looping. Adapted from (Chen, 1997).

of the heart tube with respect to the D/V axis. Zebrafish hearts will continue to elongate and move over the yolk, so that the original dorsal part of the tube ends up on the ventral side of the embryo.

The involution of the right-posterior border of the cardiac cone results in an inversion of the right half of the cone, placing the basal surface of the involuting tissue toward the lumen of the nascent heart tube. When disrupting L/R signaling by means of a

Spaw morpholino, the orientation of the involution was randomized, whereas the process of tissue involution itself appeared unaffected (Rohr, 2008).
Hyaluronan synthase 2 (Has2), previously already described to be required for cell migration of mesodermal cells (Bakkers, 2004), proves to be critical in zebrafish for both the first rotational movement as well as the leftward displacement of the venous pole of the heart (Smith, 2008). Other genes are bound to be required for such complex movements, and prior to these events, multiple genes are asymmetrically expressed in and surrounding the cardiac field, including *bmp4, lefty1* and *2,* and *pitx2.* The asymmetric expression of *bmp4* within the left LPM as well as the left side of the cardiac field (Chen, 1997; Chocron, 2007) results in asymmetric Bmp signaling in these regions. Although the role of Bmp signaling in L/R identity has been determined already more than 10 years ago, the mechanism of its involvement is still not fully understood, probably due to involvement in various processes. With respect to the process of rotation Bmp signaling was shown to be required to direct the migration of cardiac progenitor cells. During their migration the posterior cardiac progenitor cells accelerate, suggesting these cells move toward a localized source of a chemoattractant. Manipulating Bmp signaling, by implanting Bmp beads on either side of the embryo, redirected the cardiac jogging toward the bead (Smith, 2008). The exact function of these early movements still needs to be determined. Are they necessary and/or sufficient for cardiac looping or are other processes involved? It will be very useful to investigate these movements in different mutants that have affected jogging, looping or both to learn more about the coordination of these processes.

Cardiac Looping

In zebrafish, after the initial displacement of the newly formed heart tube towards the left, the tube further extends anteriorly after which it shifts back towards the midline. Another rotational movement was described, restoring D/V polarity back to L/R polarity. Thereby, the original left side ends up on the left side of the looped heart again (Baker, 2008). Whether these movements are conserved in other species is currently unknown. Subsequently, the tube loops, and the heart assumes the basic configuration necessary for further development into a multi-chambered pump. This process depends on both genetic factors as well as biophysical mechanisms, and consists of two main phases: C-looping and S-looping. In chick, during C-looping, the heart transforms from a straight tube into a C-shaped tube via two deformations: ventral bending and dextral torsion. Due to these motions, the original ventral surface of the straight tube becomes the outer curvature, while the original dorsal surface becomes the inner curvature of the looped heart. During S-looping, the atrium moves superior to the ventricle, resulting in a basic final form, which can then be divided into multiple chambers by septation. All this bending and twisting clearly requires mechanical forces and many extracellular matrix (ECM) proteins therefore engage in this process (Manner, 2000; Manner, 2004). Many hypotheses for looping have come and gone, like the involvement of differential

cell proliferation and cell death, differences in cell numbers contributed from the left and right cardiac primordial, and the influence of flow dynamics. Also the role of pressure administered by the cardiac jelly was investigated, as well as changes in cell shape between the outer and the inner curvature, but all hypotheses have been both supported and contested. It is very likely that the process of cardiac looping involves a combination of several different mechanisms, some of which may be redundant. The current hypothesis accepts that within C-looping, the ventral bending is primarily a process intrinsic to the heart tube, while the dextral torsion is driven by external forces (Taber, 2006). Throughout the years, the mechanisms of S-looping have received relatively little attention.

As mentioned, the process of cardiac jogging exhibits a high degree of prediction for the directionality of subsequent cardiac looping. However, when the heart tube fails to 'jog' it does not automatically result in a failure to loop; it can also lead to a randomization of the looping (Chen, 1997). This indicates that these processes can be separated and they are regulated by both common regulatory mechanisms as well as separate ones.

Laterality of the visceral organs

The digestive system is also subjected to L/R positional cues, resulting in the asymmetrical positioning of for instance liver and pancreas with respect to the midline and a bending of the intestine for proper space management in the abdominal cavity. In zebrafish, the first leftward bend of the developing intestine is referred to as gut looping, and takes place between 26-30 hpf. During this process, cells from the neighboring right LPM migrate ventrally, whereas cells from the left LPM move dorsally toward the embryonic midline, and the LPM thereby pushes the gut leftwards with respect to the midline. These LPM movements have been proven to be autonomous and under the control of L/R signaling (Horne-Badovinac, 2003). In *Xenopus*, asymmetric gut morphogenesis has been attributed to the asymmetric, but proliferation-independent, elongation of the right side of the gut tube, which leads to a concave left side and a convex right side (Muller, 2003). However, neither of these cellular mechanisms appears to be responsible for the looping of the gut in chick or mouse (or higher vertebrates). The chirality of the midgut looping in the chick embryo is determined by the asymmetrical architecture of the dorsal mesentery, a structure connecting the gut tube to the dorsal side of the body wall. This architecture is asymmetric by changes in the ECM and cell-cell adhesion and these cell properties are instructed by the transcription factors Pitx2 and Isl1, specifically expressed on the left side, and Tbx18, specifically expressed on the right side (Davis, 2008; Kurpios, 2008).

Clearly, the asymmetric morphogenesis of different organs is a result of a complex cascade of processes that differ greatly between species. What is important, however, is to distinguish between mechanisms that affect all asymmetric organs in general or one in specific. Abnormal L/R development of any of the internal organs with respect of other organs, referred to as discordance or heterotaxia, is

as previously mentioned often accompanied by severe pathology. It is therefore interesting to identify and pursue genes that are involved specifically in the asymmetric morphogenesis of individual organs.

Bmp4 is specifically involved in cardiac laterality

Different lines of evidence suggest that Bmp links the L/R axis to the asymmetric heart morphogenesis. Bmp4 is asymmetrically expressed on the left side of the cardiac field and the developing heart tube in both Xenopus and zebrafish, and its asymmetry is dependent on Nodal signaling (Breckenridge, 2001; Chen, 1997; Chocron, 2007; Schilling, 1999). Specific transgenic manipulation of Bmp4 expression in the heart showed that symmetric *Bmp4* expression leads to cardiac randomization, whereas inhibition of Bmp signaling prevents cardiac looping in Xenopus (Breckenridge, 2001). Studying the relationship between Bmp signaling and cardiac laterality in mouse was relatively unsuccessful, since Bmp2 null embryos suffer from multiple defects and nonspecific cardiac malformation, and Bmp4 mutant embryos arrest before gastrulation (Winnier, 1995; Zhang and Bradley, 1996). In zebrafish, 21 different mutants were examined with a defect in the cardiac jogging process and subsequent looping. In these mutants symmetric *bmp4* expression was associated with a failure to jog and a higher expression on the right side was associated with a reversed jog. *bmp4* expression in zebrafish is only asymmetric in the cardiac field and surrounding LPM and only in a small time window, around the 20-22 somites stage. Manipulating Bmp signaling at various time points indicated that the jogging process requires intact Bmp signaling up until the 22 somite stage, whereas for instance visceral laterality can only be perturbed in earlier stages of development (Chocron, 2007).

Timing and context of Bmp signaling are critical for its function

Over the years, it has become evident that the mere presence of a Bmp signal is not sufficient to regulate a developmental process, but timing, duration, and local concentration are of the essence as well. This subtle regulation can be achieved by inhibition by extracellular proteins, membrane-bound co-receptors or by cross-talk with other signaling pathways (van Wijk, 2007)(figure5).

Extracellular modulators, like Noggin, Chordin and Follistatin, can bind Bmp ligands directly, preventing their interaction with the Bmp receptors. Co-receptors can either inhibit Bmp signaling by interfering with receptor complex formation or enhance signaling by presenting Bmp ligands to the receptor complex (Balemans and Van Hul, 2002; Samad, 2005). Besides signaling through Smad proteins, Bmp signaling can also be mediated through Tak1 activating p38 MAPK, RAS, ERK and JNK (Nohe, 2004). More than 20 Bmp-related proteins have been identified and are subdivided based on their structures and functions. Bmp ligands can be secreted extracellular as homo- and heterodimers, creating multiple possible combinations of signaling proteins. Interestingly, Bmp2 and Bmp4 belong to the same subdivision, and even their functions are exchanged between different species; mouse Bmp2

seems to represent Bmp4 in the zebrafish. Both are expressed specifically in the AVC myocardium and regulate valve formation, as mentioned previously. Also, zebrafish Bmp2 functions during dorsoventral patterning in a role analogous to that of Bmp4 in mouse embryogenesis. Bmp4 homozygous mutant mouse embryos die early in development, display disorganized mesoderm and strongly truncated posterior structures (Winnier, 1995), similar to the phenotype observed in the *bmp2b/swirl* mutant. This supports the idea that zebrafish Bmp2 resembles mouse Bmp4 in function, rather than mouse Bmp2 (Kishimoto, 1997). Bmp signaling is highly defined by affinity of the multiple components for each other. Three different type I and three different type II receptors have been reported that form a complex and specifically bind Bmps, but some with higher affinity than others. The fact that Bmps are secreted proteins with often broad expression domains makes it necessary for receptors to be able to distinguish between them. Different type I or type II receptors can be expressed by responding cells to create a specific signal. Also the extracellular modulators bind different Bmps with different affinity (Zimmerman, 1996). A deficiency of some Bmp ligands results in very severe defects, while others display viable phenotypes with minor defects (van Wijk, 2007). This indicates a high level of redundancy among some of the Bmp proteins, most often clustered in the same group. In zebrafish, many genes are also duplicated, resulting in some cases in more redundancy. Differences between duplicated genes were also observed, and clearly, duplicated genes are not by nature the same genes exerting the same functions (Monteiro, 2008). Bmpr2 is duplicated in the zebrafish, and Bmpr2a, but

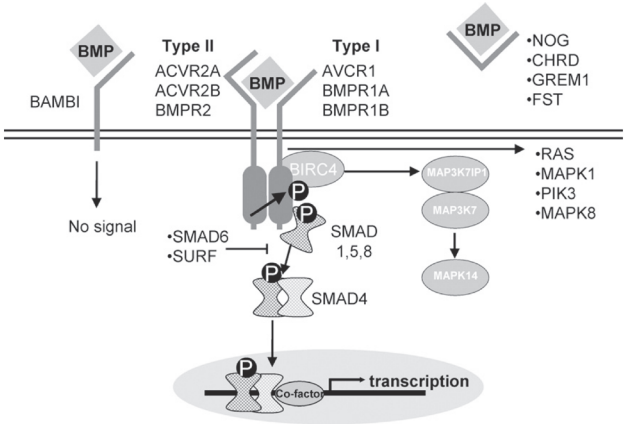

Figure 5 Bmp signaling pathways. Bmps form a heteromeric complex with type I and type II Bmp receptors. Subsequent to this complex formation the type II receptor phosphorylates the type I receptor, upon which the Smad1, Smad5 or Smad8 are phosphorylated. Phosphorylated Smad forms a complex with the common Smad4 and is transported into the nucleus. Besides signaling via Smads, the Bmp signal can also be transduced via MAP3K7(Tak1)/MAP3K7IP1 (Tab1), RAS, MAPK1 (ERK) or PIK3. Extracellularly, Bmps can be inhibited by secreted inhibitors, like Noggin (NOG), Chordin (CHRD), Gremlin (GREM1)and Follistatin (FST), or by the decoy receptor Bambi, which lacks the intracellular domain for signal propagation. Bmp signal transduction is intracellularly inhibited by Smad6 or Smurf (Adapted from (van Wijk, 2007).

not Bmpr2b, was shown to mediate induction of *lefty1* in the posterior notochord by Bmp4 emanating from the KV. Bmpr2a possessed a higher affinity for Bmp2 and Bmp4, but Bmpr2b was determined more efficient in transducing Smad dependent signaling. Bmpr2b was thereby more important for induction of *spaw* expression in the left LPM. This differential interpretation of Bmp signaling through Bmpr2a and Bmpr2b is essential for the correct establishment of LR asymmetry (Monteiro, 2008). Responsiveness to a particular Bmp ligand can vary between different developmental stages, for example by responding cells producing different ligand-binding type II receptors in time. Timing and context are critical when such a small number of signaling pathways can elicit such diverse or opposite outcomes. This also makes it difficult to rely solely on experiments using chronic overexpression or simple knockout strategies, but a more subtle approach is not always available or time-efficient.

Overall, the understanding of the role of Bmps signaling in L/R asymmetry is emerging more and more. We found a role for Bmp signaling during general L/R specification as well as a later role during heart morphogenesis possibly by guiding the cardiac progenitor cells in the correct direction. There is still a lot to learn about how Bmps regulate L/R patterning and heart morphogenesis. In addition it will be important to unravel new genes regulating organ morphogenesis as a response to L/R patterning.

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Chapter 2

The identification of genes required for left-right patterning and heart morphogenesis using a forward genetic approach

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ABSTRACT

Vertebrates display striking left-right (L/R) asymmetries in the placement of many internal organs, which is a result of the interpretation of asymmetric gene expression by organ primordia of various internal organs, like the heart and several intestinal organs. Complex, congenital heart defects often cause significant morbidity and mortality in individuals with laterality disease. A lot of questions surround the regulation of asymmetric morphogenesis of the heart and hardly anything is known regarding genes that specifically regulate cardiac laterality. Finding novel genes involved in this earliest break in cardiac symmetry would provide answers on organ-specific laterality. For this purpose, we used forward genetics in zebrafish and screened for mutants with specific defects in the asymmetric positioning of the linear heart tube. We developed a two-step screening protocol allowing us to identify mutants in which the laterality of the heart is affected while the laterality of the other organs is not. In the two screens we identified in total 20 putative mutants with perturbed jogging, and of those at least 4 mutants were heart-specific. Here, we report here the analysis and positional cloning of one mutant with a general L/R patterning defect and two heart-specific mutants. We describe the recovery of the first *spaw* mutant, the first *bmpr1a* mutant and a mutant that most likely encodes for a novel gene involved in cardiac L/R patterning.

INTRODUCTION

In vertebrates, many internal organs are positioned asymmetrically with respect to the left-right (L/R) axis, the heart being the first one to display this asymmetry. The break of the initial bilateral symmetry has been the subject of extensive studies (see chapter 1). A ciliated structure, called the node in mice or Kupffer's vesicle in zebrafish, generates a fluid flow from right to left and plays a crucial role in L/R patterning in most vertebrates (reviewed in (Hirokawa, 2006). Central in these early stages of asymmetry establishment is Nodal, a member of the TGFβ superfamily. The leftward nodal flow creates an asymmetric distribution of various genes, including *Nodal*, which is subsequently propagated to the left LPM, resulting in the asymmetric expression of downstream genes, like *Lefty1* and *2*, *Cyclops*, *Bmp4* and *Pitx2* (Levin, 2004). Asymmetric gene expression is downstream interpreted by organ primordia of various internal organs, like the heart and several intestinal organs. Under normal conditions organ asymmetry is correlated, resulting in most cases in a *situs solitus*, and occasionally in a *situs inversus.* The absence of specific L/R information often results in either symmetric organ morphogenesis or a 'randomization' of organ laterality. This randomization implicates that organs are able to interpret L/R signals individually, resulting often in very severe congenital defects. The significant morbidity and mortality of laterality disease almost always are attributed to complex, congenital heart defects. This prevalence indicates that the developing heart is extremely susceptible to disturbances in embryonic L/R patterning (Ramsdell, 2005).

Despite this clinical significance, a lot of questions surround the regulation of asymmetric morphogenesis of heart and hardly anything is known regarding genes that specifically regulate cardiac laterality. Bmp signaling has been reported to be involved at two separate time points: (I) early in establishing L/R patterning in the whole embryo and (II) later in development to direct heart morphogenesis (Chocron, 2007). The heart is the first organ to develop asymmetrically and this is evident by the leftward placement of the linear heart tube (Chen, 1997). Finding novel genes involved in this earliest break in cardiac symmetry would shed more light on organ-specific laterality. For this purpose, we used forward genetics in zebrafish and screened for mutants with specific defects in the asymmetric positioning of the linear heart tube. Zebrafish has been shown extremely useful for forward genetics screens, finding novel genes based on various phenotypical assays (Driever, 1996; Haffter and Nusslein-Volhard, 1996; Odenthal, 1996; Schier, 1996; Solnica-Krezel, 1996; Stemple, 1996; van Eeden, 1996). Previously, two screens were carried out searching for mutants with heart laterality defects. The first one was based on the re-evaluation of a collection of previously identified zebrafish mutants. When rescreened, 21 mutations were found to disrupt cardiac laterality, based on direction of the cardiac jog. All these mutants were characterized by additional phenotypes, predominantly gastrulation defects and affected midline structures (Chen, 1997). The laterality of other organs was not determined in these mutants, therefore it is

impossible to conclude anything about the heart specificity of the mutations. The strategy of a follow up screen, performed by the same lab, was aimed to identify novel mutants defective in organ laterality. For this screen, the position of the linear heart tube, the cardiac looping as well as positioning of intestinal organs were evaluated. Seven recessive mutations, affected in L/R patterning of one or all organs, were isolated and in contrast to the mutants from the previous screen, these mutations do not cause any other obvious defects. None of these mutants, however, displayed a defect specific in heart laterality (Chen, 2001).
Overall, the results from previous forward genetics screens on L/R patterning indicate that (1) the vast majority of the mutants with affected cardiac jogging display randomization (Chen, 1997) and (2) The majority of mutations that affect cardiac jogging, also affect other organs and these mutants loose the correlation between heart and gut chirality (Chen, 2001).
To identify genes that are downstream of Nodal signaling and required for asymmetric development of the heart, we performed two independent forward genetics screens. Our objective was to distinguish between mutations that perturb L/R asymmetry within the whole embryo and therefore affecting all organs, and mutations that specifically affect laterality of the heart. We focused on the second group, for much less was known about genes specifically determining asymmetry in a single organ. We therefore developed a two-step screening protocol allowing us to identify mutants in which the laterality of the heart is affected while the laterality of the other organs is not. In the two screens we identified in total 20 putative mutants with perturbed jogging, and of those we could at least verify 4 mutants to be heart-specific. We report here the analysis and positional cloning of two heart-specific mutants and one mutant with a general L/R patterning defect.

MATERIALS & METHODS

Mutagenesis
TL male fish were treated with N-ethyl-N-nitrosourea (ENU) as described previously (van Eeden, 1999) and outcrossed to wild type TL female fish, to generate F1 families. F2 families were generated by random incrossing of F1 fish, and mutant phenotypes were analysed in F3 embryos. In the second screen described, TL males were treated with ENU and then outcrossed to transgenic Fli-GFP females from an AB background. Of each F2 family between 1 and 4 lays were screened harboring approximately 40 embryos.

Determination of cardiac situs
Between 25 and 29 hpf the direction of the linear heart tube (jogging) was analysed live and designated as left (normal jog), midline (no jog) or right (reversed jog), using the neural tube as a midline reference. Embryos were divided into three groups based on their jog and in the presence of PTU (1-phenyl-2-thiourea) they were allowed to develop until 48 hpf. Groups were then fixed separately in 4% paraformaldehyde + 4% sucrose in PBS-tween to be processed for subsequent *in situ* hybridization.

In situ hybridization

Whole-mount *in situ* hybridization (ISH) was performed as described (Thisse, 1993). *foxa3* was used to analyze the asymmetry of the intestinal organs (gut looping and position of the liver and the pancreas) and *cmlc2* was used to confirm the position of the jogging and determine the orientation of the heart looping at 48 hpf. The assess correct chamber differentiation *anf (nppa), tbx2b, bmp4, has2, amhc, vmhc* markers were used. Embryos were subsequently cleared in Murray's (benzylalcohol:benzylbenzoate 1:2) and stored at 4°C.

Mapping

The mutant derived from the first screen was outcrossed to a WIK background. Mutants derived from the second screen however were mapped directly on the TLxAB F2 family. On all mutants described, bulked segregate analysis on genomic DNA of 48 pooled mutant and sibling embryos was carried out as previously described (Michelmore, 1991). Two polymorphic microsatellite markers (SSLPs) per chromosome were tested on both pools and linkage was determined at a single embryo level. Fine mapping of the several mutants described were all performed with SSLPs, details can be found in the results section.

RNA injection, mutagenesis

Mutant RNA was created by using Quickchange protocol. Wild type and mutant RNA were injected at 1-2 cell stage in the yolk, in various concentrations. Dorsalization phenotypes were scored between 24 and 48 hpf as described previously (Kishimoto1997).

Time-lapse and cell tracking

Progeny from homozygous mutants with cmlc2:GFP in the background were mounted in glass-bottom 6-well plates in 0.25% agarose in embryo medium at 22 somites in the presence of the anesthetic MS-222 (16mg/ml). Confocal imaging was performed using a Leica SP2 confocal laser scanning microscope with 40x magnification. Duration of the time-lapse was on average 10 hrs, acquiring stacks every 5 minutes. Embryos were subsequently collected from the agarose and fixed at 48 hrs for ISH and antibody staining. Data from the time lapse was analysed with Volocity software and automated 3D cell tracking was performed with Image J software. Rotation was calculated by measuring the angle with the LR axis of four imaginary lines connecting four individual cells at the start and the end of the time-lapse.

RESULTS

Forward genetics screen results in 3 new laterality mutants

To find genes that regulate heart morphogenesis in response to L/R patterning we performed two independent forward genetic screens in zebrafish. We screened the F3 generation for mutants in which the heart fails to jog to the left, since this is the first symmetry breaking event during zebrafish heart development. Since the heart is easily visible in the transparent zebrafish embryo we decided to screen the embryos live, analyzing the direction of the heart tube from a frontal view of the embryo. In most cases, the live observation was subsequently confirmed by in situ hybridization (ISH) with *cmlc2*, which is expressed in al cardiomyocytes. A first screen was performed by us at the Max-Planck Institute in Tübingen as part of the ZF-Models program. During this screen approximately 790 genomes were screened and in total 16 putative mutants were discovered and verified by ISH (table1). Surprisingly, only 8 of those were recovered in the next generation (7/8 in very low frequencies). These 8 mutants were crossed to a polymorphic background. Meanwhile, to determine which mutants specifically affected cardiac laterality, the positioning of other organs was evaluated. We found two mutants, *nk107-08* and *ou115-06*, to be heart-specific. Unfortunately, of the 8 mutants only one mutant could be recovered in the polymorphic background, the *ns061-05* mutant, which was determined to affect L/R patterning of multiple organs. The follow-up screen was performed at the Hubrecht Institute and was based on a two-step protocol; screening the embryos live as previously, directly followed by assessing the laterality of other organs to determine heart-specificity. For efficiency, putative mutants were already present in a polymorphic different background (TLxAB), creating the opportunity to proceed with positional cloning without the need for a new generation. We found 4 mutants after screening 108 genomes, 2 of which were found heart-specific (*hu119* and *hu305*: table2). We decided to proceed with the characterization and positional cloning of these two heart-specific mutants, *hu119* and *hu305*, as well as the *ns061-05* mutant, which displayed general L/R defects.

ns061-05 affects embryonic L/R patterning

As described above the heart tube in the *ns061-05* mutant embryos at 27 hpf remains positioned at the midline instead of jogging towards the left (fig.1B). Surprisingly, a majority of the *ns061-05* mutants do not show any obvious morphological differences with their wt siblings at 48 hpf (fig1C). Heart looping of mutant embryos, selected by the cardiac jogging defect, was analyzed at 48 hpf by *in situ hybridization* (ISH). We observed that heart looping was strongly affected in the mutant embryos and varied from either normal looping, reversed looping to no/reduced looping (fig.1D-H). To address whether the defects in heart morphogenesis can affect the specification of chamber versus atrioventricular canal myocardium we performed ISH using appropriate marker genes. Myocardial specification is normal in the *ns061-05* mutant, since both *nppa* and *bmp4*, markers for chamber

Table 1 Mutants discovered in ZF-Models screen 2005, with phenotype and recovery

ID	% no jog	Additional phenotype	Verified ISH	Carriers retrieved
Class I				
NZ051-03	29% (10/35)	No	yes	no
NK107-08	25% (7/28)	No	yes	yes*
NS053-06	23% (6/26)	No	yes	yes*
NS064-04	29% (7/24)	No	negative	x
NY098-02	25% (5/20)	No	no	no
NS061-05	29% (7/24)	No	yes	yes (4/14)
OU047-02	29% (14/48)	No	yes	yes*
OU055-04	21% (11/52)	No	yes	yes*
OU110-01	26% (12/47)	No	yes	yes*
OU115-06	30% (26/87)	No	yes	yes*
Class II				
NQ042-05	21% (10/48)	Cardiac edema and blood accumulation	negative	x
NL143-02	50% (10/20)	Cardiac edema	yes	yes*
NK125-05	32% (8/25)	C1 dorsalization	yes	no
NI105-05	29% (8/28)	C1 dorsalization, cardiac edema, stringy heart	no	no
NI112-08	23% (10/43)	Small head and reduced heart beat	yes	no
NL099-03	19% (14/72)	Small head and reduced heart beat	yes	no
NX010-01	20% (5/25)	Cardiac edema, small head and reduced heart beat	negative	x
NI117-02	29% (18/62)	Curly tail, swirl upon touch	yes	no
Class III				
NZ097-03	22% (5/23)	Unknown	negative	x
NY082-01	26% (7/27)	Unknown	yes	no

Table 1 Mutants were all identified based on live observation of the initial position of the linear heart tube. Mutants in bold font were recovered in the next generation, 7 of 8 only in very low frequencies (*). The mutant in bold font as well as underlined was recovered after outcrossing. Two mutants were found heart-specific (*italic*).

Table 2 Mutants discovered in Hubrecht screen 2007, with phenotype

ID	% no jog	Additional phenotype	Verified ISH	Gut laterality in mutants
Class I				
HU304-01	25% (32/130)	Unknown	yes	No gut loop and duplication liver/pancreas
HU202-04	24% (29/121)	Unknown	yes	No gut loop and duplication liver/pancreas
Class II				
HU305-09	10% (11/107)	C1 dorsalization 27% (29/107)	yes	Unaffected
HU119-02	23% (29/126)	Cardiac edema	yes	Unaffected

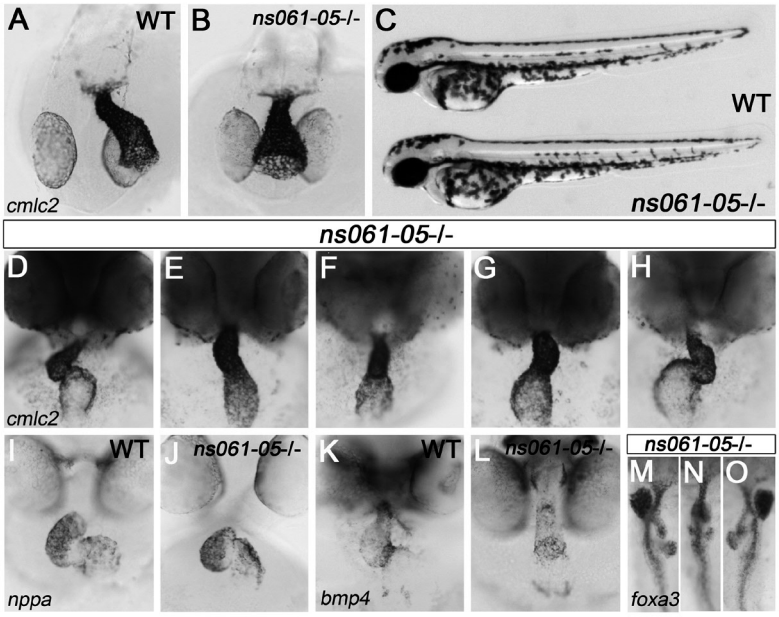


Figure 1 Dorsal view of wild-type sibling (A) and *ns061-05* mutant stained at28 hpf for *cmlc2* expression to reveal the positioning of the linear heart tube. (C) *ns061-05* mutants (bottom) display no obvious additional phenotypes at 48 hpf compared to wild-types (top). *ns061-05* mutants selected on their no jogging phenotype were stained for *cmlc2* expression at 48 hpf to determine the heart looping. Phenotypes observed ranged from normal D-looping (D), reverse L-looping (H) and reduced or absent looping (E,F,G). *nppa* expression in wild-type (I) and *ns061-05-/-* (J) and *bmp4* expression in wild-type (K) versus *ns061-05-/-* (L). (D-L) All are frontal views. Gut looping and positioning of the liver and pancreas, visualized with *foxa3* at 48 hpf from a dorsal view, in *ns061-05* mutants selected on their no jog phenotype. Observed phenotypes were normal visceral laterality (M), as well as reversed visceral laterality (O) and no visceral laterality (N).

differentiation and atrioventricular canal respectively, are expressed normally (fig.1I-L). As mentioned above, the laterality defects observed in *ns061-05* mutant embryos are not confined to the heart. Mutant embryos selected for the absence of cardiac jogging in addition displayed a randomization in the direction of gut looping (fig.1O,N).

ns061-05 mutants displays a high degree of heterotaxia

Under normal conditions, there is correlation between the laterality of different organs. This means that the positioning and looping of the heart tube is highly predictable for the direction of gut looping and the position of other organs. Normally in wild-type embryos the heart tube jogs to the left and loops towards the right side. In addition, the gut loops towards the left side with the liver on the left and pancreas on the right site with respect to the midline (*situs solitus*). When L/R information in the embryo is present, but incorrectly orientated, a complete reversion of all organs can be observed (*situs inversus*). When the L/R information is absent, homogenized or cannot be interpreted by the tissues involved, the correlation in laterality of the different organs is often lost and this is referred to as situs ambiguous or heterotaxia (Chen, 1997). To determine whether the correlation

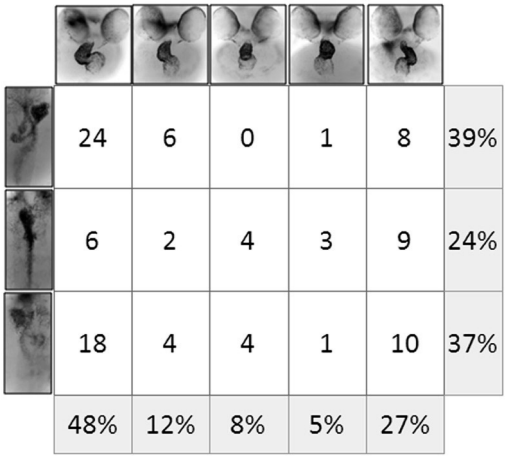


Figure 2 *ns061-05* mutants selected on a no jog phenotype were assayed at 48 hpf for both cardiac and visceral laterality by means of ISH with *cmlc2* and *foxa3* (n=100).

in organ laterality is affected in the *ns061-05* mutants, we subjected them to an ISH using a cocktail of two probes (*cmlc2* and *foxa3*). *Cmlc2* is expressed in the myocardium while *foxa3* is expressed in the developing gut, the liver as well as the pancreas allowing the simultaneous visualization of the heart and the intestinal organs. *ns061-05* mutant embryos with the linear heart tube positioned at the midline were subsequently cultured until 48 hpf and fixed for ISH. The directionality of the heart looping as well as the directionality of the gut looping was assessed for each individual embryo. We observed that in the majority of the *ns061-05* mutants

(59%) the correlation between the direction of heart looping and the direction of gut looping was lost (heterotaxia)(fig.2). These results suggest that in the *ns061-05* mutants the heart and the gut respond differently to the early L/R signals or that the L/R signal is absent.

The ns061-05 allele encodes for the nodal-related southpaw

In order to identify the genetic lesion in *ns061-05* mutants, genetic mapping was performed with simple sequence length polymorphism markers (SSLPs). We observed linkage of the mutation with the SSLP marker *z4299* located on chromosome 5. Scanning the genomic region we observed that the *southpaw* (*spaw*) gene is located 1.27 Mb away from z4299. *spaw* is the ortholog of nodal in the zebrafish and hence a very appropriate candidate for this mutation affecting L/R asymmetry. Previously reported data on a *spaw* morpholino revealed that *spaw* is necessary for correct cardiac jogging as well correct positioning of other visceral organs. By genomic sequencing of all coding exons of the *spaw* gene, we identified a missense mutation in exon 3, which was linked to the mutant phenotype (48/48) (fig.3). This mutation resulted in a cysteine to phenylalanine substitution at position 401, which concerns one of the seven highly conserved cysteines, characteristic for members of the TGFβ superfamily. This particular cysteine is predicted to be

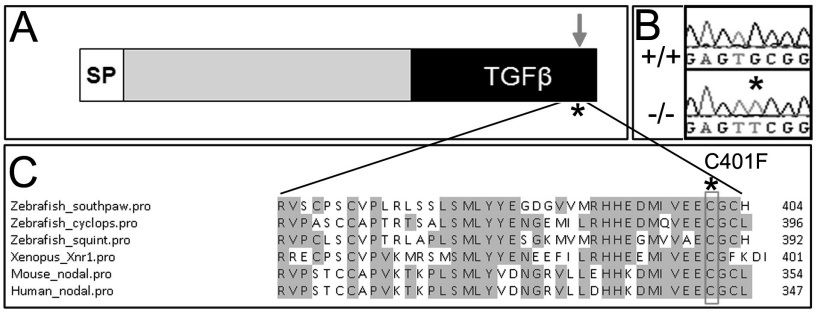


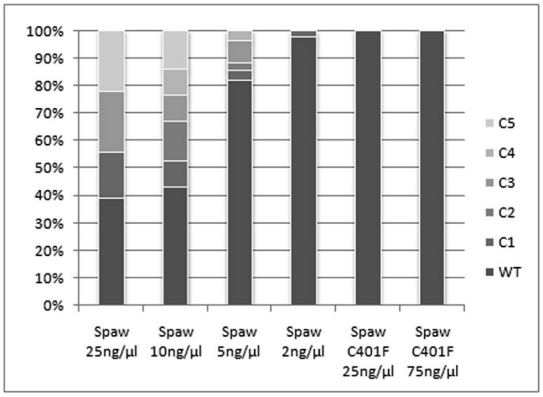
Figure 3 (A) Representation of the *spaw* gene, SP= signaling protein. The mutation is located at the end of the gene in the TGFβ-domain (* and arrow). (B) The mutation results in a G->T substitution at position 401. (C) The conservation of the protein sequence compared to different nodal-related genes in different species (gray). The conserved cysteine that is mutated in the *ns061-05* mutant is indicated by a box.

essential for intramolecular di-sulfide binding of the protein (SMART accession number: SM00204). We therefore predicted that the C401F mutation in Spaw protein would render it non-functional.

To verify this we injected wild-type *spaw* RNA as well as mutated *spaw C401F* RNA into wild-type embryos. Overexpression of wild-type *spaw* RNA results in a severe dorsalization phenotype, comparable to the effect of overexpression of the closely related *squint* and *cyclops* (Gritsman1999). When we injected 25ng/µl *spaw* RNA, 61% of the embryos displayed dorsalization ranging from less severe C1 to most severe C5 (Kishimoto, 1997). However, injection of 25-75 ng/µl *spaw C401F* did not

have any effect on embryo development, demonstrating that the *C401F* missense mutation identified in *ns061-05* inactivates the Spaw protein (fig.4A). In Addition, we observed a partial rescue of the jogging phenotype when we injected wild-type *spaw* RNA in *ns061-05* mutants (fig.4B). Since the severe dorsalization caused by injecting wild-type *spaw* RNA obscures the cardiac jogging, we did not inject higher concentrations to obtain a better rescue.

From our observations that (1) the genetic lesion responsible for the laterality defects observed in the *ns061-05* mutants maps to *z4299* located on chromosome 5 (2) a missense mutation (*C401F*) of a conserved cysteine residue was identified



	% No jog
ns061-05 uninjected	29% (n=109)
ns061-05 + *spaw* RNA 2ng/µl	22% (n=135)

Figure 4 Wild-type embryos were injected with either wild-type *spaw* RNA or mutated *spaw* RNA (n>30). Potential of wild-type *spaw* RNA to rescue mutant phenotype in *ns061-05* mutants.

in the *spaw* gene, located on chromosome 5 in close proximity to *z4299* (3) *spaw C401F* is inactive in embryos when compared with wt *spaw* (4) morpholino knock down of *spaw* in zebrafish results in comparable laterality defects as observed in *ns061-05* (5) injection of wt *spaw* into *ns061-05* embryos partly rescues the laterality defects we conclude that *ns061-05* encodes for *spaw.* As a reference to its no jogging phenotype we renamed the *ns061-05* allele into *straightforward*^{t30973} *(sfw*^{t30973}*)*.

Analysis of MZ sfw mutants

To address whether spaw has any additional essential function we tried to raise *sfw*^{t30973}mutant embryos up to adulthood. We observed that about 95% of *sfw* mutants survived to adulthood without any obvious defects and were fertile. Embryos from an incross of two homozygous *sfw* mutant fish displayed very similar phenotypes as observed in zygotic *sfw* mutant embryos, demonstrating that there is no major contribution of maternal *spaw* RNA and protein.

Nodal signaling is required for cardiac rotation, jogging, but not for cardiac looping

The jogging process has been recently described in more detail and subdivided into a simultaneous migration of the cardiac progenitor cells into the anterior-left direction and a clockwise rotation of the entire heart cone (Smith, 2008). Both BMP signaling and the expression of *has2* were found crucial for the leftward displacement as well as the rotational movement of the cone. Manipulating BMP signaling up to the 18-somite stage, as well as knocking down *has2*, results in an affected cardiac jogging, followed by a perturbed cardiac looping. To determine whether the rotation is correlated to the direction of the heart tube (jogging) as well as the direction of the cardiac looping, we investigated the rotational movements in the MZ*sfw* mutants. These mutants all fail to jog, but display a subsequent randomized cardiac looping. We analyzed the wild-type embryos and mutants, both in *tg(cmlc2:egfp*) background by means of time-lapse imaging between 20 and 30 hpf. In wild-types, we observed the directional migration of posterior cells that results in a clockwise rotation (reported previously by Smith2008) (fig.5C-C''). In the MZ*sfw* mutants, all cells move in the anterior direction and no rotation was observed (fig.5D-D''). The rotation was quantified in degrees and wild-type embryos displayed on average 39.7°±6,5° (n=5) rotation, while MZ*sfw* mutants displayed on average 0.7°±3.8°(n=10) (fig.5A,B). In wild-types, the posterior cells move at a much higher speed, travelling a longer distance than anterior cells. Although no rotation was observed in MZ*sfw* mutants, posterior cells in MZ*sfw* mutants cardiac cones still travel a longer distance than anterior cells and thereby move at a higher speed (fig.5D-D''). The overall speed of cardiomyocytes in MZ*sfw* mutants was reduced and more meandering was observed compared to wild-types. The subsequent cardiac looping was determined for each individual MZ*sfw* mutant at 48 hpf. We found, as expected, randomization of the cardiac looping, with normal D-loops (3/10), reversed loops (2/10) and absent cardiac looping (5/10). This indicates that the rotation is indeed correlated to the direction of the cardiac jogging, but not to the direction of the cardiac looping. Nodal signaling is necessary for both the rotation and the jogging, but not for cardiac looping.

Mutant hu305 encodes for bmpr1a (alk3)

In addition to the cardiac jogging defect, *hu305* mutants displayed a characteristic C1 dorsalization and thereby lack their ventral tail fin (Table 2 and fig.6E,F). Although the C1 dorsalization and the cardiac jogging defect were correlated, cardiac jogging was only compromised in 10% of *hu305* mutants indicative of a partial penetrance (fig.6C). The direction of heart looping as well as the orientation of the intestinal organs were evaluated at 48 hpf. Gut looping and the position of the liver and pancreas with respect to the midline seem unaffected in the *hu305* mutant (fig.6G,H). The heart looping, however, showed some variation. Most of the mutants with a normal left jog at 28 hpf, displayed a normal heart loop to the right (fig.6J), while 26% displayed a reduced loop to the right (n=9/34) (fig.6K). Mutants

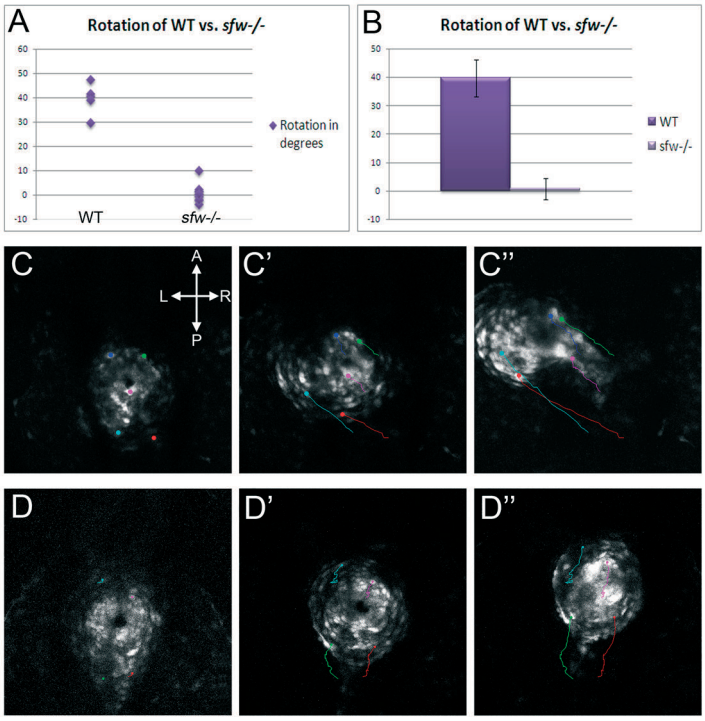


Figure 5 Scatter plot of quantification of clockwise rotation in wild-type (n=5) versus *sfw* mutants (n=10) (A). Quantification of clockwise rotation of wild-type versus *sfw* mutants (mean ± STD). The difference in rotation is significant ($p<0.0001$) (B). (C-C'') Selected images of a confocal time lapse recording of a representative wild type embryo, with individual cells marked (green and blue for anterior located atrial cells, red and cyan represent posterior located atrial cells and pink for ventricular cells located medial). (D-D'') Selected images of a confocal time lapse recording of a representative *sfw* mutant, with individual cells marked (cyan and pink for anterior located atrial cells, red and green represent posterior located atrial cells). Recordings are in a dorsal view with anterior to the top.

with affected jogging all showed a reduced heart loop at 48 hpf (fig.6L). Because C1 dorsalization is very characteristic for mutants in the BMP signaling pathway, we anticipated that *hu305* could encode for a gene in this particular pathway. To rule out certain known genes we performed complementation assays with the *acvr1* mutant, the *cpt* mutant (Smad5) and the *bmp4*^{stop} mutant. Complementation was present in all crosses, indicating that hu305 does not encode for *acvr1*, *smad5* or *bmp4* (table3). In order to identify the responsible gene we proceeded with genetic mapping with SSLP markers, using mutants selected for their C1 dorsalization. Linkage was determined to chromosome 13 between *z6259* (19 recombinants in 1140 meioses) and *z9868* (156 recombinants in 1140 meioses). This genomic region contains the *bmpr1a* gene, which would be a good candidate gene. Sequencing the coding regions of *bmpr1a* revealed a L337R substitution in exon 10 (0 recombinants in 1140 meioses), located in the very conserved kinase domain of this type I BMP receptor (fig.7).

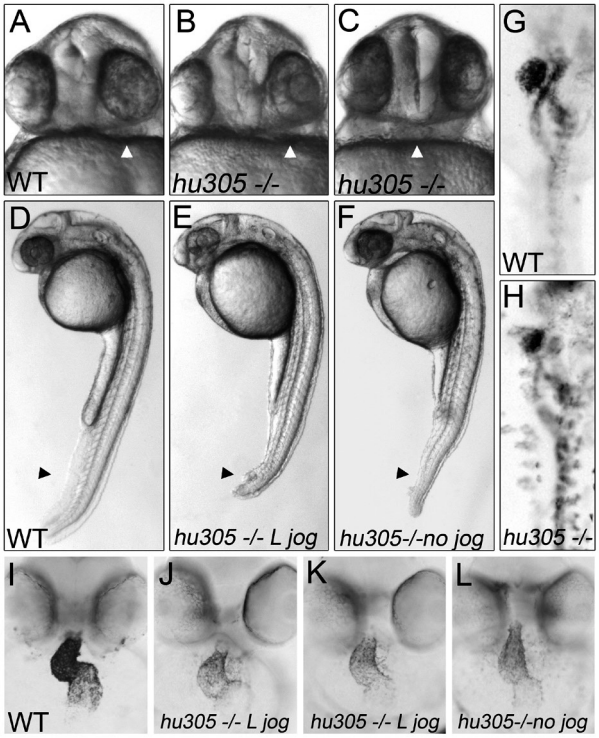


Figure 6 Pictures of live embryos at 28 hpf from a frontal view, (A) wild-type, (B) *hu305* mutant with a normal leftward jog and (C) an *hu305* mutant with aberrant jogging. Pictures of live embryos at 28 hpf from a lateral view, (D) wild-type, (E) *hu305* mutant with C1 dorsalization and a normal leftward jog and (F) an *hu305* mutant with C1 dorsalization combined with an aberrant jogging. Wild-type siblings (G) and *hu305* mutants (H) were stained for *foxa3* expression to reveal the positioning of the visceral organs. Wild-type siblings (I) and *hu305* mutants (J,K,L) were stained for *cmlc2* expression to reveal the direction of cardiac looping. Both staining were performed on embryos fixed at 48 hpf. Position of the visceral organs was assessed from a dorsal view, the heart looping from a frontal view.

Table 3 Complementation assay *hu305*

Cross	Complementation
hu305+/- x *acvr1*+/-	+
hu305+/- x *cpt*+/-	+
hu305+/- x *bmp4*+/-	+

In order to confirm the loss of function of the protein, we subsequently cloned *bmpr1a* and mutated L337R *bmpr1a*. Interestingly, injection of 10 ng/µl of *bmpr1a* in wild-type embryos revealed a dorsalizing phenotype. To circumvent this, we injected a low dose (2ng/µl) of wild-type bmpr1a RNA, which did not result in a phenotype, and tried to rescue the C1 dorsalization observed in the *hu305* mutant embryos (table4). Injection of hu305 mutants with wild-type *bmpr1a* RNA resulted

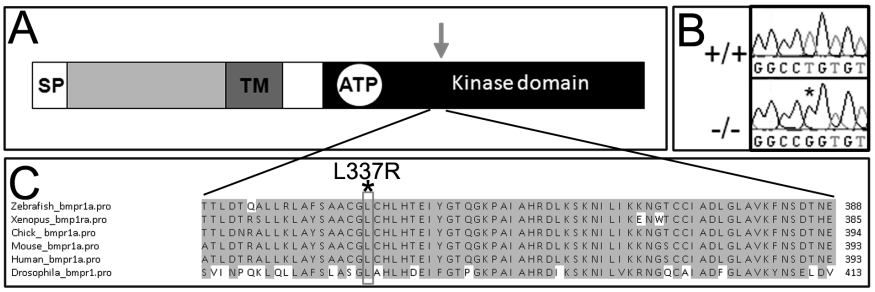


Figure 7 (A) Representation of *bmpr1a*, SP= signaling protein, TM= transmembrane domain, ATP= ATP binding domain. The mutation is located in the kinase domain (arrow). (B) The mutation results in a T->G substitution at position 337. (C) The conservation of the protein sequence compared to *bmpr1a* in different species (gray). The conserved leucine that is mutated in *hu305* mutants is indicated by a box.

in a partially rescue of the *hu305* mutant phenotype. When injecting the mutated L337R RNA, we did not observe any rescue of the C1 dorsalization, demonstrating that the L337R mutation renders Bmpr1a inactive. Taken together we conclude that the phenotypes observed in the *hu305* mutant are caused by reduced or a loss of Bmpr1a activity.

Cardiac laterality is specifically affected in hu119 mutants

hu119 mutant embryos were characterized by hearts remaining at the midline instead of jogging towards the left side (Table 2). In embryos with perturbed cardiac jogging, we could clearly observe a very distinctive space between the embryos head and the yolk (fig.8B). The midline position of the linear heart tube was confirmed by ISH using *cmlc2* to mark the myocardium (fig.8C,D). To determine the laterality of other organs, *hu119* mutant embryos were selected based on their no-jog phenotype at 28 hpf and subjected to ISH to visualize both heart looping and positioning of the intestinal organs at 48 hpf. Although cardiac looping was severely affected in *hu119* mutants (fig.8F), the positioning of the liver and the pancreas with respect ot the

Table 4 Rescue of the *hu305* mutant phenotype

	n	Phenotype	Genotype		*hu305-/-*
			sibling	mutant	with wt phenotype
Uninjected *hu305*	141	Wild-type	136	5	13%
	33	C1	0	33	
hu305 + wt *bmpr1a* 2ng/µl	136	Wild-type	121	15	35%
	29	C1+C2	1	28	
hu305 + L337R *bmpr1a* 2ng/µl	124	Wild-type	124	0	0%
	55	C1+C2	14	41	

midline and the looping of the gut were unaffected (fig.8H). When investigating the mutant unlooped hearts in more detail, we found no differences in the specification of both ventricle and atrium (fig.8I,J). The smaller ventricle in the *hu119* mutant most likely reflects a defect in chamber ballooning, rather than a defect in specification of ventricular cells. *bmp4* and *tbx2b* are both expressed in the myocardium of the differentiated atrioventricular canal (AVC) and *nppa* is expressed exclusively in the differentiated chamber myocardium (fig.8K,M,O). Although the heart looping was affected in the *hu119* mutants, the differentiation of the myocardium in chamber

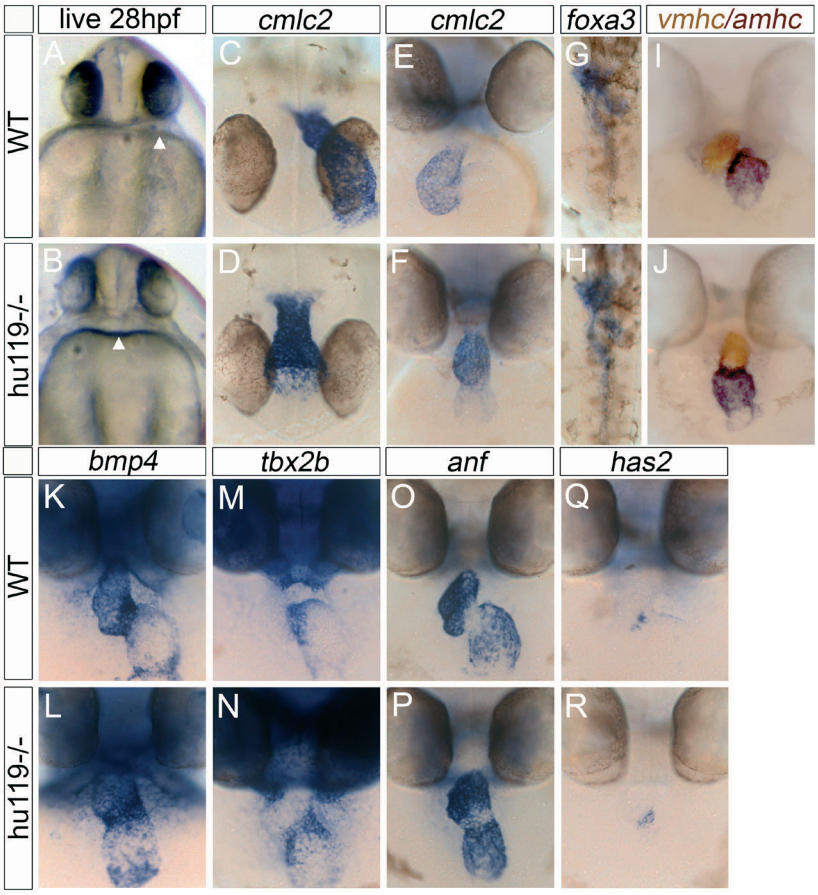


Figure 8 Live observation of wild-type siblings (A) and *hu119* mutants (B) at 28 hpf from a frontal view to assess the positioning of the linear heart tube. The orientation of the heart tube can be visualized from a dorsal view by staining for *cmlc2* expression at 28 hpf in wild-type siblings (C) and *hu119* mutants (D). Wild-type siblings and *hu119* mutants were subsequently fixed at 48 hpf for the following stainings; *cmlc2* to assess the direction of the cardiac looping (E,F) and *foxa3* to determine the positioning of the visceral organs (G,H). To analyze the specification of ventricle versus atrium myocardium embryos were double stained for *amhc/vmhc* (I,J) and to analyze the specification of chamber versus atrioventricular canal (AVC) myocardium embryos were stained for *bmp4* (K,L), *tbx2b* (M,N) and *nppa* (O,P). The specification of the AVC endocardium was assessed by staining the embryos for *has2* (Q,R). *foxa3* was assessed from a dorsal view, the rest from a frontal view.

vs. AVC was normal (fig.8L,N,P). In addition we observed in *hu119* mutants a normal *has2* expression in the developing endocardial cushions, structures that give rise to the atrioventricular valves (fig.8R). Together these results suggest that the gene, which is affected in *hu119* mutants, specifically is involved in heart morphogenesis and does have little or no additional roles in other aspects of cardiac development. To identify the responsible gene in the *hu119* mutant, genetic mapping was performed with simple sequence length polymorphism markers (SSLPs). Linkage was determined to a region on chromosome 7 between marker *z11085* and *z1206*. A snp in the *mespb* gene (snp1) was found to be in close proximity of the hu119 mutation (31 recombinants in 1700 meioses) (fig.9).

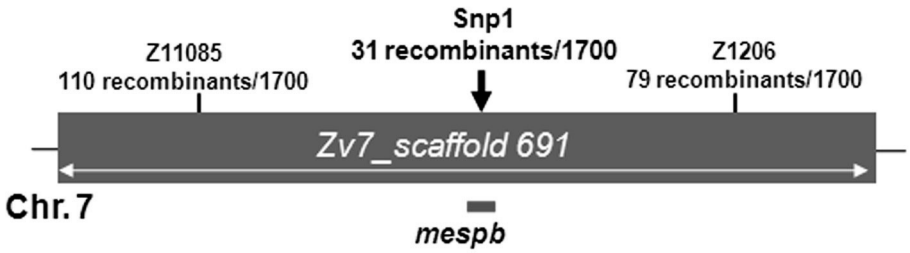


Figure 9 Representation of scaffold 691 on chromosome 7. Two flanking SSLP markers are depicted (z11085 and z1206) accompanied by the number of recombinants found divided by the number of meioses tested. Snp1 was found closest to the mutation, by approximation (31/1700)x100x0.7=1.28 Mb.

DISCUSSION

Here, we describe the recovery and identification of mutants that affect either general L/R asymmetry within the whole embryo or specifically at the level of heart morphogenesis. We performed two independent forward genetics screens, based on the position of the linear heart tube between 26 and 29 hpf. In total, we screened well over 900 genomes and found in total 20 candidate mutants. However, of the 16 candidate mutants initially identified with perturbed cardiac jogging in the first screen, only one of them appeared to be a heritable mutation in subsequent generations. A possible explanation for this could be that we initially found a very high degree of false positives. Cardiac jogging is a very sensitive process, and different wild-type strains show different degrees of background jogging defects (Chin, 2000). When the angle of the heart tube with the midline was measured in Tübingen wild-type embryos, the same strain used for our initial screen, it was found to be closer to the midline when compared to other wild-type lines (Oregon and brass). In addition we observed in approximately 10% of the Tübingen wild-type embryos a heart position at the midline or to the right (R.Postel, personal communication). Similar problems in recovering putative cardiac L/R mutants have been reported by others. Chen et al reported that during the original 1996 Tübingen and Boston screens, 24 candidate mutants were initially identified with randomized cardiac L/R looping, however none of these were recovered in subsequent generations (Chen, 1997). The first successful screen using cardiac jogging as an assay, reported in 1997, was founded on the re-evaluation of a collection of 279 mutants with various other embryonic defects (Chen, 1997). In a follow up screen Chen et al screened 750 genomes and isolated 7 L/R mutants (Chen, 2001). Out of both screens, only one mutant displayed a linear heart tube positioned at the midline in combination with normal gut looping. This mutant turned out to be another allele of the *alk8/laf* mutant also described in Chapter 2 (Chen, 2001).

The second screen we performed proved much more successful, with 4 candidate mutants identified, 2 of which were found to affect specifically heart laterality. It is striking, however, that these mutants were found after screening only 108 genomes. The differences between both screens concern the ENU mutagenesis strategy and the wild-type fish strain that was used (TL versus Tübingen). Combining the results obtained from both screens suggests that there will be only few genes that are specifically required for heart morphogenesis as a response to L/R patterning.

Many questions still surround issues of organ-specific laterality and the interpretation of L/R information. We have shown here that our method of screening in zebrafish proves successful for finding genes regulating organ laterality. We can thereby distinguish between genes affecting embryonic L/R patterning and genes that specifically regulate heart morphogenesis.

ns061-05 encodes spaw and affects L/R patterning of the embryo

We report here the identification of the *ns061-05 (sfw^{t30973})* mutant, which encodes the *spaw* gene. *Spaw*, the ortholog of nodal, has been studied previously and was shown to be a key player in early L/R patterning (Long, 2003). To our knowledge, this is the first report describing the isolation and characterization of a *spaw* mutant. Injection of the previously published *spaw* morpholino results in laterality defects similar to the laterality defects that we have described here for the *sfw* mutant (Long, 2003). The *spaw* morpholino injected embryos, however, display a randomized cardiac jogging (left, no and right jogging, while the *sfw* mutants are characterized by their complete absence of cardiac jogging. This might be due to a dose-dependent response, combined with the self-regulatory capacities of *spaw*. In *sfw* mutants, we anticipate that *spaw* is completely inactive and incapable of positively regulating itself, abolishing L/R asymmetric information in the LPM. The symmetry in the embryo is thereby translated into midline cardiac jogging. Partial knockdown as well as non-homologous distribution of the *spaw* morpholino could be responsible for the randomized cardiac jog reported by others. This phenotype could however also result from non-specific toxicity of the morpholino injection. Considering a loss-of-function of a nodal-related gene, the phenotype of the *sfw* mutant is relatively mild, and very specific for L/R patterning. During normal development, *spaw* transcripts are first detectable during early somitogenesis in paraxial mesoderm. Closely related genes *cyclops* and *squint* on the other hand are expressed already during gastrulation, fulfilling a major role in the induction of mesoderm. Injection of *spaw* RNA results in defected dorso-ventral patterning. A similar effect was reported for overexpression of *squint* (Gritsman, 1999). Since the TGFβ domains of all three genes are very much alike, overexpression of *spaw* could result in ectopic Nodal signaling during gastrulation and induce mesoderm formation.

In addition, we describe that Nodal signaling is required for the clockwise rotation during cardiac jogging, an observation supported by two independent reports analyzing rotation in *spaw* morphants and LZ*oep* mutants, respectively (Baker, 2008; de Campos-Baptista, 2008). The dynamic expression pattern of *has2*, which shifts from bilateral to the left side during heart tube development, is required for the left-anterior displacement of the cardiac progenitor cells and perturbed nodal signaling has been shown to affect *has2* expression in the cardiac field (Smith, 2008). By analyzing the rotation in embryos that fail to jog, but are able to loop subsequently, we demonstrated that the direction rotation (clockwise or anti-clockwise) is correlated to the direction of the cardiac jogging, but not to the direction of cardiac looping. Therefore it would be very interesting to assess the rotation in a mutant with normal cardiac jogging, which subsequently displays perturbed cardiac looping.

hu305 encodes for bmpr1a and affects cardiac asymmetry specifically

Our finding that *hu305* encodes a novel zebrafish BMP receptor provides additional evidence for the importance of BMP signaling on establishing cardiac L/R asymmetry. BMP4 has been reported crucial for the heart to interpret L/R information (Chocron, 2007; Smith, 2008). *bmp4* expression was described predominantly on the left side of the heart, from before the onset of cardiac jogging all through the jogging process. This asymmetric expression was found perturbed in laterality mutants (Chen, 1997). Opposed to the late and rather mild defects observed in the zebrafish *bmpr1a*^{hu305} mutants, mice lacking *Bmpr1a* fail to complete gastrulation, and none survive past the E9.5 stage (Mishina, 1995). One explanation for this difference could be the observation that the *bmpr1a* gene is duplicated in the zebrafish genome (K. Smith, personal communication). Targeted deletion of Bmpr1a in either the atrioventricular canal myocardium (Gaussin, 2005) or the endocardium (Song, 2007) in the mouse demonstrated that Bmpr1a is required for the normal development, growth and survival of mesenchymal cells in endocardial cushions. To our knowledge, a role for *bmpr1a* in regulating cardiac morphogenesis and laterality has not been reported thus far. Future experiments should reveal possible functions for *bmpr1a* in this process. It would be particular useful to know which cells are responding to the Bmp4 signal present in the LPM and cardiomyocytes and the *bmpr1a*^{hu305} mutant could be very helpful in this.

hu119 affects cardiac asymmetry specifically

So far, the only mutants described affecting specifically asymmetric morphogenesis of the heart are members of the BMP signaling pathway (*acvr1*-/- and *bmpr1a*-/-). Although we have not pinpointed the mutation responsible for the *hu119* phenotype yet, we anticipate that this mutant will reveal a novel player in cardiac L/R asymmetry. Since *hu119* mutants are not characterized by any other phenotypes, like dorso-ventral patterning defects, it is unlikely that *hu119* encodes a member of the BMP signaling pathway. So far, all zebrafish mutants described affecting the BMP signaling pathway, also affect dorso-ventral patterning either mildly (C1 dorsalization; *acvr1*-/-, *cpt*-/-, *bmp4*^{stop}-/-, *bmpr1a*-/-) or severe (C5 dorsalization; *swl*-/-). More importantly, we have narrowed down the region in which the mutation is located, and although not all genes are annotated, no known BMP family members are located there. Re-evaluating the scaffold of interest will be necessary to identify candidate genes and to proceed with this project more efficiently.

In conclusion, we have recovered the first *spaw* mutant, the first *bmpr1a* mutant and a mutant that most likely encodes for a novel gene involved in cardiac L/R patterning. The Nodal pathway and the BMP pathway are both previously reported crucial for general L/R patterning and heart-specific L/R patterning, respectively. The fact that we retrieved mutants in both these pathways validates our method of screening. Although the *hu119* mutant is not yet cloned, it is still a unique mutant as far as we know. It is the only mutant described affecting heart-specific L/R asymmetry without displaying additional phenotypes, like dorso-ventral patterning defects.

We conclude that the current set-up of our forward screen works very efficiently and effectively and follow up screens are ongoing. Based on previous experience, it would be best to ignore those candidate mutants that affect general L/R patterning or affect dorso-ventral patterning and focus on those mutants that compromise cardiac jogging specifically.

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Chapter 3

Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points

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Dev Biol. 2007 May 15;305(2):577-88

ABSTRACT

Left-right (L/R) asymmetry is regulated by early asymmetric signals within the embryo. Even though the role of the bone morphogenetic protein (Bmp) pathway in this process has been reported extensively in various model organisms, opposing models for the mechanism by which Bmp signaling operates still prevail. Here we show that in zebrafish embryos there are two distinct phases during L/R patterning in which Bmp signaling is required. Using transgenic lines that ectopically express either *noggin3* or *bmp2b*, we show a requirement for Bmp signaling during early segmentation to repress *southpaw* expression in the right lateral plate mesoderm and regulate both visceral and heart laterality. A second phase was identified during late segmentation, when Bmp signaling is required in the left lateral plate mesoderm to regulate left-sided gene expression and heart laterality. Using morpholino knock down experiments we identified Bmp4 as the ligand responsible for both phases of Bmp signaling. In addition, we detected *bmp4* expression in Kupffer's vesicle and show that restricted knock-down of *bmp4* in this structure results in L/R patterning defects. The identification of these two distinct and opposing activities of Bmp signaling provides new insight into how Bmp signaling can regulate L/R patterning.

INTRODUCTION

The looping heart is the first organ in vertebrates to develop morphological L/R asymmetry (Wolpert, 2002). Cardiac looping is a critical event in the normal sequence of heart development. The significant morbidity of laterality diseases are almost always attributed to complex, congenital heart defects (CHDs) (reviewed in (Ramsdell, 2005). This repercussion indicates that the developing heart is extremely susceptible to disturbances in embryonic left–right (L/R) patterning. The lateral plate mesoderm (LPM) plays a crucial role in the establishment of L/R asymmetry in the heart, gut and brain (Liang, 2000; Long, 2003). Asymmetric expression in the LPM of many different factors, such as Pitx2, Nodals, Bmps, and Shh has been recognized and their role in L/R patterning has been extensively studied (reviewed in (Levin, 2005). Left-sided Nodal expression is required for the induction of Pitx2 and the Nodal antagonists Lefty1 and Lefty2 in the LPM.

Bone morphogenetic proteins (Bmps) were originally identified about 40 years ago by their ability to cause bone differentiation. They belong to a large subgroup within the transforming growth factor-β (TGF-β) gene super family (Urist, 1965). Bmps are secreted proteins that signal via a complex of type I and II receptors. In vertebrates, three type I receptors (Bmp type I A receptor, Bmp type I B receptor and ACVRI, also referred to as ALK3, ALK6 and ALK2/8, respectively) and three type II receptors (BmpRII, ActRIIA and ActRIIB) have been identified as Bmp receptors (reviewed in (Kishigami and Mishina, 2005). The extracellullar cysteine-rich proteins Chordin and Noggin can bind to Bmps and prevent their interaction with the receptors. After ligand binding to the receptor complex, the type I receptor phosphorylates the receptor Smads (Smad1,5 or 8 for Bmp signaling) which heterodimerize with Smad4 and translocate to the nucleus to activate target gene expression (Shi and Massague, 2003).

Bmp signaling has been shown to regulate different aspects of L/R patterning (see for review (Kishigami and Mishina, 2005). The roles for Bmp signaling during L/R patterning have been studied in various model organisms. In Xenopus, expression of a truncated ACVRI (ALK2) receptor on the right side of the blastula leads to heart reversal accompanied with bilateral Nodal expression. Left-sided expression of a constitutively active ACVRI receptor leads to heart reversal and loss of Nodal expression (Ramsdell and Yost, 1999). In the chick, Bmp4 is asymmetric expressed in Hensen's node. The strongest expression of *Bmp4* being on the right side, which in turn positively regulates Fgf8 expression and prevents initiation of Nodal expression in the right LPM (Roderiguez Esteban, 1999; Yokouchi, 1999). In mouse embryos deficient for Smad5, Lefty1 is expressed at a very low level, while Nodal, Lefty2 and Pitx2 are expressed bilaterally (Chang, 2000). Chimeric mouse embryos using Acvr1 (Alk2) null ES cells also show bilateral Nodal, Lefty2 and Pitx2 expression in the LPM (Kishigami, 2004). Together these data demonstrate that in Xenopus, chick and mouse Bmp signaling is required for right-sided laterality, via repression of Nodal expression in the right LPM. In a converse role, Bmp2 has been shown in the chick

to be a positive regulator of Nodal signaling in the left LPM (Piedra and Ros, 2002; Schlange, 2002). Analysis of mouse tetraploid chimeras using Bmp4 null ES cells as well as Bmp4 mutant embryos revealed that Bmp4 is required for left-sided Nodal expression in the LPM (Branford, 2000; Fujiwara, 2002). These seemingly conflicting results have led to the suggestion that there exist two distinct types of Bmp signaling of opposing nature (Kishigami and Mishina, 2005).

Previously, a screen for zebrafish mutants with laterality defects has been performed based on the position of the linear heart tube (cardiac "jogging"). The mutants *snailhouse*, *somitabun* and *lost-a-fin* were identified (Chen, 1997), and interestingly were later shown to correspond to *bmp7*, *smad5* and *alk8*, respectively (Bauer, 2001; Dick, 2000; Hild, 1999; Mintzer, 2001; Schmid, 2000). The mechanism of how Bmp signaling in the zebrafish regulates L/R patterning is not well understood. *Bmp4* expression is stronger in the left LPM and cardiac field in 20 somite stage embryos, and overexpression of *bmp4* on the right, but not on the left side, induces laterality defects (Chen, 1997; Schilling, 1999). However, no loss-of-function studies to address whether zebrafish Bmp4 is required for L/R patterning have been performed to date.

Here, using transgenic lines for temporally controlled overexpression of *bmp2b* or the Bmp inhibitor *noggin3*, we show that in the zebrafish there are two distinct phases of Bmp action to regulate L/R asymmetry. Furthermore, using antisense morpholino technology, we identify Bmp4 as the Bmp signal required during both phases. At first, shortly after Kupffer's vesicle has been formed during early segmentation, Bmp4 is required to repress expression in the right lateral plate mesoderm of the nodal-like gene *southpaw* and regulate both visceral and heart laterality. Later, during segmentation, a second wave of Bmp4 signaling is required for cardiac, but not visceral, laterality by regulating expression of *cyclops*, *lefty1* and *lefty2* in the left cardiac field. These results support a model in which Bmp4 has opposing activities on the left and right side and acts both upstream and downstream of Nodal signaling.

RESULTS

Alk8 is required for L/R patterning

Bmp signaling has been demonstrated to play different roles during the establishment of L/R asymmetry in various organisms, by using ectopic expression of Bmps and/or Noggin or by generating (chimeric) mutant mouse embryos (Branford, 2000; Chang, 2000; Fujiwara, 2002; Kishigami, 2004; Piedra and Ros, 2002; Ramsdell and Yost, 1999; Roderiguez Esteban, 1999; Schlange, 2002; Yokouchi, 1999). To investigate the requirement for Bmp signaling in zebrafish during the establishment of L/R asymmetry, we used the Bmp receptor mutant *lost-a-fin/alk8*. In the *laf* ^{m110} allele used here, a conserved extracellular cysteine residue is replaced by an arginine residue resulting in a strong hypomorph or null mutation (Bauer, 2001; Mintzer, 2001). Besides a defect in dorsoventral patterning, which is only mild due to the contribution of maternal mRNA, mutant embryos display a severe cardiac oedema and blood circulation ceases at 3 days post fertilization (dpf) (Bauer, 2001; Mintzer, 2001). Induction of *nkx2.5* expression in the bilateral cardiac fields is unaffected (suppl. fig. 1) and the linear heart tube is formed normally in *laf/alk8* mutant embryos. Differentiation of atrial and ventricle cardiomyocytes is normal, as reflected by appropriate expression of *ventricle myosin heavy chain* (*vmhc*) and *atrium myosin heavy chain* (*amhc*) (suppl. fig. 1). However the atrium remains at the midline and is never displaced towards the left (in 45 mutant embryos analyzed), a process referred to as cardiac "jogging" (fig. 1A-C) (Chen, 1997). Also, the morphology of the atrium appears affected since it is much broader compared with wt siblings (fig. 1B). Besides cardiac jogging, subsequent looping of the heart is also affected in *laf/alk8* mutant embryos, resulting in a linear "string-like" heart at 2 dpf (suppl. fig. 1). Besides laterality of the heart, we also investigated laterality of other organs in the *laf/alk8* mutants. *In situ* hybridizations using antisense *foxa3* show that laterality of the visceral organs is affected in *laf/alk8* mutant embryos, albeit at a much lower frequency than laterality of the heart (fig 1D-G). In addition, using *lefty1* expression as a marker, laterality of the dorsal diencephalon is affected in *laf/alk8* mutants but again at a much lower frequency than laterality of the heart (fig. 1H-K). The low penetrance of the laterality defect in the visceral organs and dorsal diencephalon is not likely due to the hypomorphic nature of the mutation since another recently described *laf/alk8* allele (*laf* ^{gl2}) with a premature stop codon gave similar results (data not shown)(Hogan, 2006).

Bmp signaling is required for L/R patterning at two different stages of development

Since the early Bmp signaling processes are normal in zygotic *alk8/laf* mutants (due to the presence of maternally supplied *alk8* mRNA), it was considered that the difference in tissue responsiveness to altered Bmp signaling may be due to a requirement for Bmp signaling at different developmental time points during L/R patterning. To address this possibility we generated transgenic zebrafish lines with

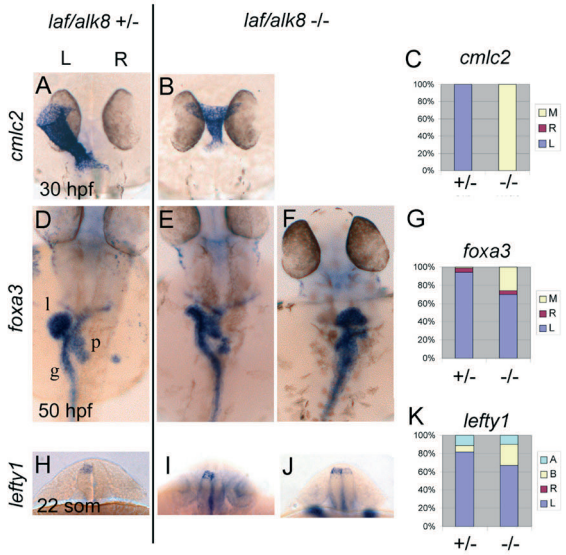

Figure 1 Laterality defects in *laf/alk8* mutant embryos. (A-C) Wild type sibling (A) and *laf/alk8* mutant (B) embryo at 30 hpf stained for *cmlc2* expression to reveal positioning of the heart and quantification of the results (C). (D-G) Wild type sibling (D) and *laf/alk8* mutant (E,F) embryos at 50 hpf stained for *foxa3* expression to reveal the positioning of the liver (l), pancreas (p) and gut (g) and quantification of the results. (H-K) Wild type sibling (H) and *laf/alk8* mutant (I,J) embryos at 22 somite stage stained for *lefty1* expression in the dorsal diencephalon and quantification of the results. All pictures are of dorsal views with anterior to the top, (L; left, R; right, B, bilateral, A; absent).

inducible *noggin3* or *bmp2b* expression. Levels of Smad phosphorylation in these transgenic lines, *tg(hsp70:noggin3)* and *tg(hsp70:bmp2b),* can be respectively reduced or increased within 2 hours, after shifting the embryos from 28.5°C to 37°C for 30 min (J.B. unpublished). Since Bmp signaling during gastrulation is required for cardiomyocyte induction, we chose the first time point at tailbud (tailbud) stage to apply the heat-shock. The second time point was chosen at the 15-18 somite stage, after laterality in the LPM has been established but preceding cardiac jogging. Laterality was analyzed using the nodal-like gene *southpaw* (*spaw*) which is expressed in the left LPM (Long, 2003), *lefty1* (notochord, left cardiac field and left dorsal diencephalon) and *lefty2* (left cardiac field) (Bisgrove, 1999; Long, 2003). When *tg(hsp70:noggin3)* embryos were heat-shocked at tailbud stage and analyzed for *spaw* expression in the LPM, most embryos exhibited bilateral *spaw* expression (92%, n=25) instead of expression restricted to the left LPM, as in wild-type embryos (fig. 2A and B and Table I). Applying the heat-shock at the 15-18 somite stage did not have any affect on *spaw* expression (fig. 2C). When ectopic *bmp2b* expression was induced in embryos at tailbud stage, the opposite phenotype was obtained, with absent *spaw* expression on both sides of the LPM (100%, n=39) (fig. 2D and Table I). Similarly, this effect was not observed when the ectopic *bmp2b* expression was induced at the 15-18 somite stage (fig 2E). These data support a model in which Bmp signaling is required at early segmentation stages to repress *spaw*

expression in the right LPM. Correct *spaw* expression in the left LPM is required for appropriate expression of other left-sided genes such as *pitx2* (Long, 2003; Shiratori, 2001). *Tg(hsp70:noggin3)* embryos heat-shocked at the tailbud stage lacked *pitx2* expression in the LPM (fig. 2F,G) whereas *tg(hsp70:bmp2b)* embryos heat-shocked at the tailbud stage had bilateral *pitx2* expression in the LPM (fig. 2I). These data suggest a Bmp dependent but Nodal independent regulation of *pitx2* expression in the LPM. A similar observation in the chick has been made, where Bmp and Caronte can synergistically induce *Pitx2* expression independent of Nodal (Schlange, 2002). Noggin or Bmp2b expression at the 15-18 somite stage does not affect *pitx2* expression in the LPM (fig. 2H,J). Again, these data support a model in which Bmp signaling during the early segmentation stage regulates L/R patterning in the LPM. *Tg(hsp70:noggin3)* embryos which were heat-shocked at tailbud stage or at the 15-18 somite stage lacked *lefty1* and *lefty2* expression in the cardiac field and showed strongly reduced levels of *lefty1* in the anterior notochord (fig. 2K-M and P-R). These data suggest that Bmp signaling is required at late segmentation stages for *lefty1* and *lefty2* expression.

Our next aim was to determine whether Bmp signaling would be sufficient to induce *lefty* expression. To investigate this we analysed *lefty* expression in *tg(hsp70:bmp2b)* embryos heat-shocked at tailbud or 15-18 somite stages. Although *lefty1* expression is induced in the notochord in the *tg(hsp70:bmp2b)* embryos, its expression is absent from the cardiac field (fig. 2N and O). In contrast to the *lefty1* expression, ectopic *lefty2* expression is induced in *tg(hsp70:bmp2b)* embryos when the heat-shock is applied at the 15-18 somite stage in both the right cardiac field (fig. 2T) and the notochord (fig. 2T') where *lefty2* expression is normally absent (fig. 2S). Together these data show a role for Bmp signaling during early segmentation stages in repressing *spaw* expression in the right LPM and a role during late segmentation stages in inducing *lefty1* and *lefty2* expression in the left cardiac field and the anterior notochord. These results suggest two independent requirements for Bmp signaling during early and late segmentation stages to regulate L/R patterning.

Bmp signaling regulates visceral organs and cardiac laterality independently

The previous results are suggestive that Bmp signaling regulates laterality of different organs independently by acting at different developmental stages. When the heat-shock is applied to *tg(hsp70:noggin3)* embryos at tailbud stage, gut looping is severely compromised and internal organ duplications are observed (fig. 2U and V). No effect on visceral organ laterality is observed when a heat-shock is applied at the 15-18 somite stage in these embryos (fig. 2W). This demonstrates that the early Bmp signal (early segmentation) is required for regulating visceral organ laterality. To define which developmental time point a Bmp signal is required to regulate cardiac jogging, *tg(hsp70:noggin3)* embryos were heat-shocked at different time points between the tailbud and the 28 somite stage, at which time the jogging process has started. Cardiac jogging is affected when *tg(hsp70:noggin3)* embryos were heat-shocked up to the 18-somite stage whereas a heat-shock administered

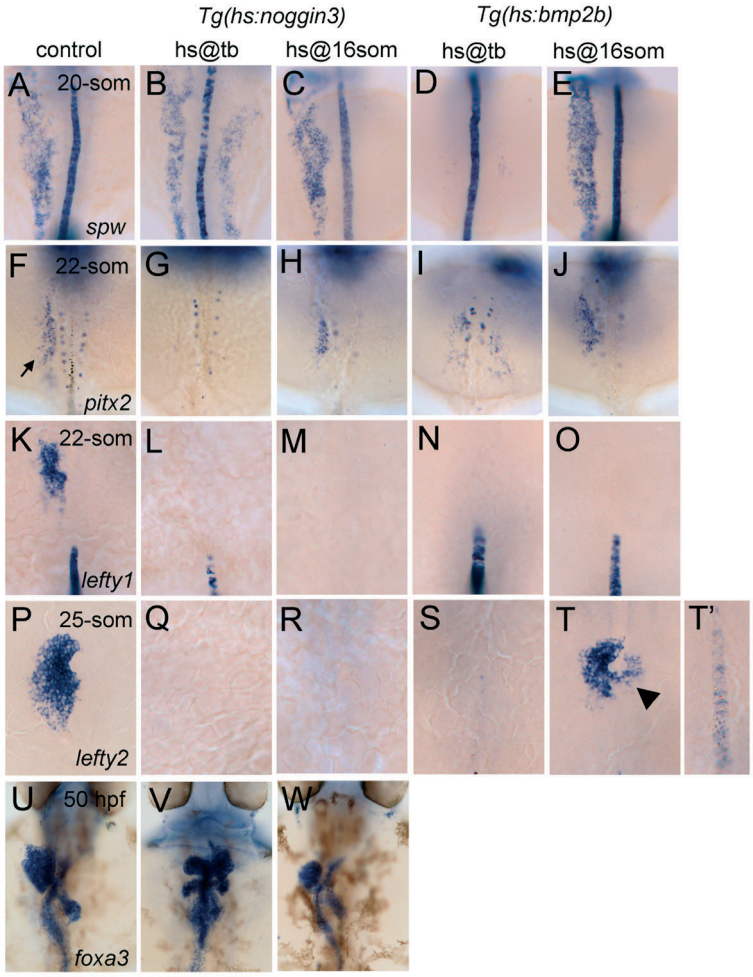

Figure 2 Bmp regulates L/R patterning at two distinct developmental time points. (A-E) *Spaw* expression in the LPM and *ntl* expression in the midline in wild type (A), *Tg(hs:noggin3)* (B,C) and *Tg(hs:bmp2b)* (D,E). Heat-shock induced *noggin3* expression at tailbud stage results in bilateral *spaw* expression (B) while *noggin3* induction at the 16-somite stage has no effect on *spaw* expression (C). Heat-shock induced *bmp2b* expression at the tailbud stage results in the absence of *spaw* expression (D) whilst *bmp2b* induction at the 16-somite stage has no effect (E). (F-J) At the 22 somite stage *pitx2* is asymmetrically expressed in the LPM (F, arrow). Left-sided *pitx2* expression is absent in *Tg(hs:noggin3)* embryos heat-shocked at the tailbud stage (G). Normal left-sided *pitx2* expression in *Tg(hs:noggin3)* embryos heat-shocked at the 16 somite stage (H). Bilateral *pitx2* expression in the LPM of *Tg(hs:bmp2b)* embryos heat-shocked at the tailbud stage (I). Normal left-sided *pitx2* expression in *Tg(hs:bmp2b)* embryos heat-shocked at the 16 somite stage (J). (K-O) *Lefty1* expression in the midline and left cardiac field at the 22-somite stage of a wild type embryo (K). Induction of *noggin3* (L.M) or *bmp2b* (N,O) at tailbud or 16-somite stage, results in the down regulation of *lefty1* in the cardiac field. In the notochord, *lefty1* expression is repressed by ectopic *noggin3* (L,M) and induced by *bmp2b* (N,O). (P-T') *Lefty2* expression in the left cardiac field of a 25-somite stage wild type embryo (P). Induction of *noggin3* at the tailbud (Q) or 16-somite stage (R) or the induction of *bmp2b* at the tailbud stage (S) results in the down regulation of *lefty2* expression. Induction of *bmp2b* expression at the 16-somite

stage results in ectopic *lefty2* expression in the right cardiac field (T, arrowhead) and in the notochord (T'). (U-W) *Foxa3* expression in the visceral organs of a wild type embryo at 50 hpf (U). Induction of *noggin3* at the tailbud stage results in visceral organ laterality defects, such as organ duplications and absence of gut looping (V) while ectopic *noggin3* expression induced at the 16-somite stage has no effect on the laterality of the visceral organs (W). All embryos are shown as dorsal views with anterior to the top of the panel.

at the 22 somite stage or later is ineffective (fig. 3A-J and supplementary table 1). This suggests that for proper cardiac jogging, a Bmp signal is required up to the 22-somite stage. In conclusion, these data demonstrate that cardiac laterality can be regulated independently in time from visceral organ laterality although both require Bmp signaling for appropriate L/R regulation.

Bmp4 is expressed in Kupffer's vesicle and the LPM

A number of Bmp ligands are expressed during zebrafish development (Martinez-Barbera, 1997; Nikaido, 1997). Bmp2b and Bmp7 are required for dorsoventral patterning during gastrulation (Dick, 2000; Kishimoto, 1997; Schmid, 2000). *Bmp4* is expressed in a ventral to dorsal gradient but is also present in the shield. During early segmentation stages, *bmp4* expression is restricted to an anterior and posterior expression domain (fig. 4A). In the posterior expression domain, *bmp4* expression is strong in the tailbud and LPM and weaker at the periphery of Kupffer's vesicle (the zebrafish structure equivalent to the mouse node and required for L/R patterning (Essner, 2002)). No difference in levels of *bmp4* expression between the left and right side was observed at this stage. At late segmentation stages, *bmp4* expression is confined to the cardiac field and LPM, as reported previously (Chen, 1997; Chin, 2000). In agreement with previous reports, we observed a transient up-regulation of *bmp4* expression in the left LPM compared with the right LPM (fig. 4C). In addition, we observed that the asymmetric *bmp4* expression in the LPM depends on the presence of Spaw since embryos injected with MOs directed against *spaw* display reduced levels of *bmp4* expression in the left LPM (fig. 4D). Although previous reports have suggested a role for Bmp4 in L/R patterning in the zebrafish, the mechanism by which Bmp4 is involved has not yet been elucidated (Branford, 2000; Chen, 1997; Schilling, 1999).

Morpholinos efficiently target bmp4

Since the effect of loss of Bmp4 function on L/R patterning in the zebrafish embryo had not been reported, we designed antisense MOs targeting the splice donor site between exon1 and exon2 of *bmp4* to disrupt production of functional Bmp4 protein (fig. 5A). To address whether injection of this MO could efficiently affect splicing of *bmp4*, we isolated mRNA from embryos injected with two different concentrations of the *bmp4* splice MO and analysed the splicing events by performing RT-PCR using different primers located either within the intron or located on exon 1 and exon 2 (fig. 5A). The results show a strong induction of unspliced *bmp4* mRNA in the MO-injected embryos. We also observed the presence of longer *bmp4* mRNA products,

which most likely are the product of incorrect splicing. The most prominent fragment produced by the MO injection was isolated (fig. 5D fragment b) and sequenced. This revealed an insertion of 54 bp originating from the intronic region, resulting in an insertion of 18 amino acids in the translated Bmp4 product. Since the open reading frame was not disrupted by the insertion, we tested the activity of the alternate form of Bmp4 protein by injecting synthetic *bmp4*^{splice} mRNA containing the 54 bp insertion. In contrast to wt *bmp4* mRNA, injection of the longer *bmp4*^{splice} mRNA had no effect on dorsoventral patterning even at concentrations more than 20 times higher than the wt RNA (fig. 5E and F and supplementary table 2). This

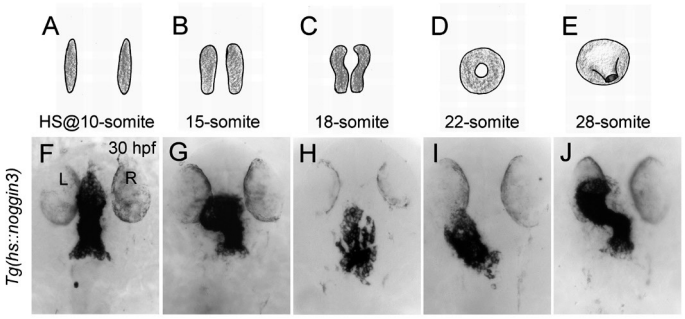

Figure 3 Ectopic *noggin3* expression prior to the 22-somite stage affects normal cardiac jogging. (A-E) Schematic depiction of the bilateral cardiac fields during heart tube formation at the indicated stages corresponding to the stage when embryos were heat-shocked. (F-J) Induction of *noggin3* in *Tg(hs:noggin3)* embryos by a heat-shock at the 10-somite stage (F), 15-somite stage (G), 18-somite stage (H), 22-somite stage (I) or 28-somite stage (J). All are dorsal views with anterior to the top of the panel showing *cmlc2* expression at 26-30 hpf. L; left, R;right

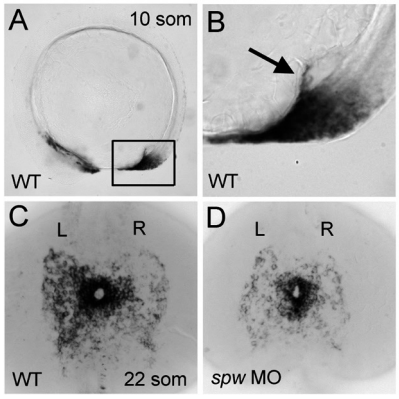

Figure 4 *Bmp4* expression at early and late somite stages. Lateral view of a 10-somite stage embryo showing *bmp4* expression in an anterior and posterior expression domain (A). Boxed area shown in panel (A) is enlarged in panel (B) and shows *bmp4* expression in and around Kupffer's vesicle. At the 20-22 somite stage (C), *bmp4* expression is confined to the cardiac field and LPM where it is stronger on the left compared with the right side. Injection of a *spaw* MO results in a down regulation of *bmp4* expression in the left LPM (D). Lateral views with anterior to the right (A,B) and dorsal views with anterior to the top (C,D). L; left, R; right

demonstrates that the 18 amino acid insertion results in an inactive Bmp4 protein. Injection of the *bmp4* splice MO resulted in mild C1 and C2 dorsalization, similar to that observed in *laf/alk8* mutant embryos (fig. 5G and H) (Bauer, 2001; Mintzer, 2001). The mild dorsalization was completely rescued by co-injection of *bmp4* splice MO with synthetic wt *bmp4* mRNA (fig. 5I) demonstrating that the *bmp4* splice MOs specifically prevent Bmp4 signaling.

Bmp4 is required for L/R asymmetry

To address whether Bmp4 is required for L/R patterning we studied *spaw* expression (fig. 6A,B and Table I). In *bmp4* morphant embryos, *spaw* expression in the LPM is bilateral, suggesting that Bmp4 is the ligand required during early segmentation stages to repress *spaw* expression in the right LPM. As a consequence, other genes which expression is confined to the left LPM are affected as well. *Pitx2* expression in the posterior LPM is absent after *bmp4* MO injection (fig. 6 C,D) and the asymmetry of the *cyclops* expression in the anterior LPM is lost (fig. 6 E,F). *Bmp4* expression in the LPM at the late segmentation stages overlaps with *lefty1* and *lefty2* expression. We observed that *lefty1* and *lefty2* expression is absent from *bmp4* morphant embryos (fig. 6G and H and data not shown). As a consequence, cardiac jogging was compromised in *bmp4* morphant embryos (fig. 6I and J). In agreement with the bilateral *spaw* expression and loss of *pitx2* expression in the LPM, gut looping was strongly affected and organ duplications were observed (fig. 6K and L). These phenotypes were very similar to those observed in *laf/alk8* mutants (fig. 1F) and *tg(hsp70:noggin3)* embryos induced at tailbud stage (fig. 2V). Consistent results were obtained with a second, previously described MO targeting the ATG of *bmp4* (Supplementary table 3) (Leung, 2005). All together, our results show that loss of Bmp4 in the zebrafish embryo results in L/R defects that can be attributed to a loss of Bmp signaling at the early segmentation stage as well as the late segmentation stage as described above.

Bmp4 is required in Kupffer's vesicle

Since *bmp4* expression at the early segmentation stages is found both in the LPM as well as Kupffers's vesicle (fig. 4B), we wanted to address the question of which tissue requires Bmp4 for repression of *spaw* in the right LPM. Injection of MOs in the yolk of the 512-cell stage embryos (3-4 hpf) results in the uptake of the MOs by the dorsal forerunner cells that will form Kupffer's vesicle (Amack and Yost, 2004). Utilising this process, fluorescein-labelled *bmp4* MOs where injected into the yolk at the 512 cell-stage. Injected embryos at 80% epiboly were selected for the specific uptake of the *bmp4* MO. Kupffer's vesicle morphogenesis was analysed At the 10-somite stage using *ntl* expression. No defect in Kupffer's vesicle morphogenesis was observed when the *bmp4* MO was targeted to the dorsal forerunner cells (fig. 7A,B). Moreover, expression of *left right dynein* (*dnah9* - Zebrafish Information Network), required for cilia function in Kupffer's vesicle (Essner, 2005), is not affected by loss of Bmp4 (fig. 7C,D). However, spaw expression was bilateral in this

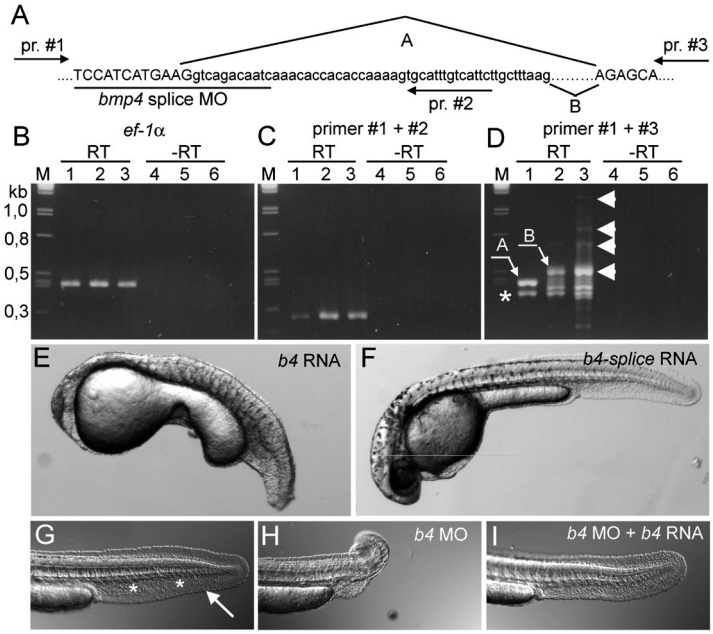

Figure 5 *Bmp4* morpholino knock down. (A) Genomic sequence of *bmp4* showing exon1 (capitalized), intron1 (non-capitalized) and exon2 (capitalized). Location of the *bmp4* splice MO is indicated and the primers used for RT-PCR are indicated by black arrows. (B-D) RT-PCR on mRNA from uninjected control embryos (lane 1) or embryos injected with 1 ng *bmp4* splice MO (lane 2) and 0.5 ng *bmp4* splice MO (lane 3) and control PCR (-RT) on control embryos (lane 4) or embryos injected with 1 ng *bmp4* splice MO (lane 5) and 0.5 ng *bmp4* splice MO (lane 6). PCR on the *ef1α* gene was used as a control for RNA isolation procedure and the RT reaction (B). PCR using the exon-intron primers 1 and 2 will amplify unspliced *bmp4* RNA (C) which is induced by *bmp4* splice MO injections. PCR using exon-exon primers 1 and 3 will results in the amplification of normal spliced *bmp4* mRNA (lane 1) or alternatively spliced *bmp4* mRNA after *bmp4* splice MO injection (lane 2 and 3). Band "A" in lane 1 indicates normal spliced *bmp4* mRNA and band "B" indicates alternatively spliced *bmp4* mRNA with 54 bp insertion (both determined by sequencing the fragments). The asterisk in lane 1 indicates non-specific amplification (determined by sequencing). Arrow heads indicate various alternatively spliced products induced by injection of 1 ng of *bmp4* splice MO. (E,F) Injection of 1,6 pg of wt *bmp4* mRNA results in a strong ventralization of the embryos (91%, n=116) (E), while injection up to 40 pg of the *bmp4* mRNA with the 54 bp insertion (*bmp4*-splice) has no effect (0% affected, n=54) (F). (G-I) Injection of 0.8 ng of *bmp4* splice MO results in the loss of ventral structures such as ventral tail fin (arrow) and blood islands (asterisks), both characteristics of mild C1 and C2 dorsalization (H) (in 49% of injected embryos, n=138) which can be rescued by coinjection of *bmp4* splice MO and 0.8 pg *bmp4* RNA (I) (15% C1 and C2 dorsalization, n=156).

experiment, suggesting that Bmp4 is required in Kupffer's vesicle for signaling to the LPM (fig 7E,F). To test if other Bmps besides Bmp4, are required in Kupffer's vesicle as well, we injected MOs that have been reported to target Bmp2b or Bmp7 (Lele, 2001). Although injection of the *bmp2b* or *bmp7* MOs results in severe dorsoventral patterning defects when injected at the 1-cell stage, no effect on *spaw* expression was observed when injected at the 512-cell stage to target Kupffer's vesicle (fig

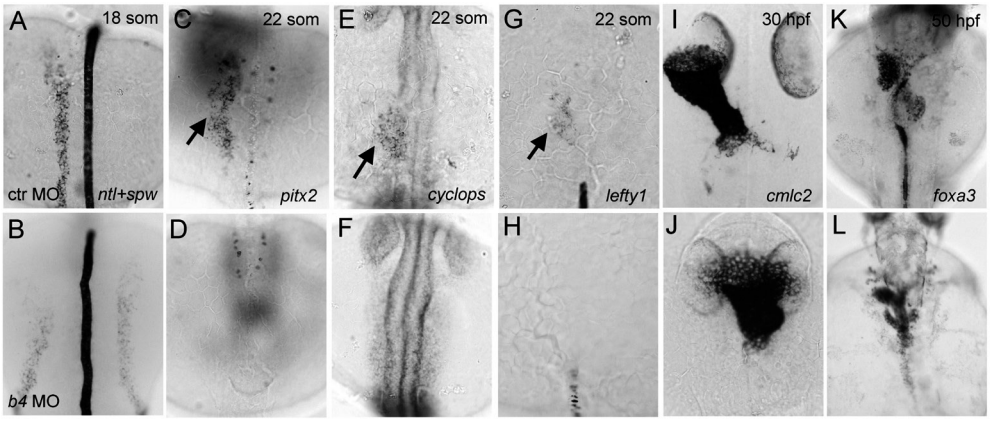

Figure 6 Bmp4 is required for L/R patterning. (A,B) *Spaw* expression in the left LPM and *ntl* expression in the notochord in control MO injected embryos (A) and bilateral *spaw* expression in *bmp4* splice MO injected embryos (B) at the 18 somite stage. (C,D) *pitx2* expression in the posterior LPM (arrow) of control MO injected embryos at the 22 somite stage (C) is predominantly absent or reduced when embryos are injected with *bmp4* splice MO (D). (E,F) Cyclops is broadly expressed but stronger in the left anterior LPM of wt or control MO injected embryos at the 22 somite stage (arrow in E). Although still being expressed, the asymmetry in *cyclops* expression is lost when embryos have been injected with *bmp4* splice MO (F). (G,H) Normal *lefty1* expression in the notochord and left cardiac field of 22 somite stage embryos (G) is absent from embryos injected with *bmp4* splice MO (H). (I,J) Normal cardiac jogging in control MO injected embryos (I) and loss of cardiac jogging in embryos injected with *bmp4* splice MO (J) revealed by *cmlc2* expression. Dorsal view, anterior to the top of 30 hpf embryos. (L,M) Visceral organ laterality revealed by *foxa3* expression in control MO injected embryos (L) and embryos injected with *bmp4* splice MO (M) resulting in organ duplications and failed gut looping.

7G,H). Since Bmp signaling during late segmentation stages is required for both *lefty1* and *lefty2* expression in the cardiac field, we investigated whether Bmp4 in Kupffer's vesicle is required for early *lefty1* expression preceding *spaw* expression in the LPM. At the 10 somite stage *lefty1* is expressed strongly in the notochord of wt embryos (fig. 7I) but is absent from the notochord in embryos injected with *bmp4* MO at the 512 –cell stage (fig. 7J). This effect on early *lefty1* expression in the notochord was also observed at the 22 somite stage. Embryos injected at the 512-cell stage with *bmp4* MO lacked *lefty1* expression in the notochord and the heart field during late segmentation (fig. 7K,L). Contrary to *lefty1* expression, *lefty2* expression in the heart field was not affected in those embryos (fig. 7M,N).

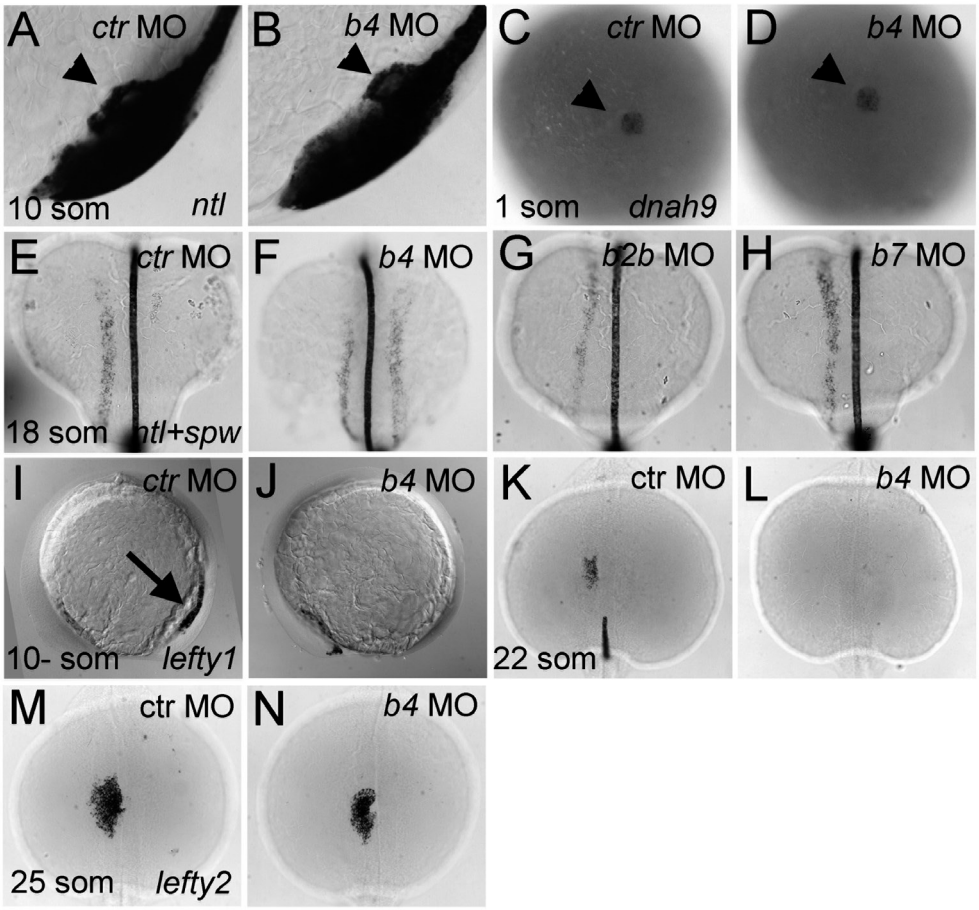

Figure 7 Bmp4 in Kupffer's vesicle is required for L/R patterning. All embryos shown here were injected at the 512 cell stage to target the *bmp4* MOs to the dorsal forerunner cells (D,F,C) that will form Kupffer's vesicle. (A,B) *Ntl* expression in 10 somite stage embryos marks Kupffer's vesicle. No difference in vesicle morphology was observed in DFC^{ctr} MO injected embryos (A) compared to DFC^{bmp4} MO injected embryos (B). Lateral views of tail bud, arrow head points to KV. (C,D) L/R dynein (*dnah9*) is expressed in Kupffer's vesicle. No difference was observed in DFC^{ctr} MO injected embryos (C) compared to DFC^{bmp4} MO injected embryos (D). ventral views of tail bud at 1 somite stage, arrow head points to Kupffer's vesicle. (E-H) Ectopic *spaw* expression in the right LPM upon DFC^{bmp4} MO injection (F) compared with DFC^{ctr} MO injected embryos (E). No effect on *spaw* expression was observed after DFC^{bmp2b} MO (G) or DFC^{bmp7} MO (H) injection. Dorsal views of 18 somite stage embryos.
(I-L) Lefty1 is expressed in the head region and in the notochord (arrow) at the 10 somite stage (I,J) and in the left heart field and notochord at the 22 somite stage (K,L). Lefty1 expression is absent from the notochord but normally present in the head region in DFC^{bmp4} MO injected embryos (J) when compared to of DFC^{ctr} MO injected embryos (I). At the 22 somite stage *lefty1* expression is absent from the DFC^{bmp4} MO injected embryos (L) compared to DFC^{ctr} MO injected embryos (K). (I,J) Lateral view of 10 somite stage embryos. Arrow points to the *lefty1* expression in the posterior notochord. (K,L) Dorsal views of 22 somite stage embryos. (M,N) Expression of *lefty2* in the LPM is not affected in the DFC^{bmp4} MO injected embryos (N) compared to DFC^{ctr} MO injected embryos (M). Dorsal views of 25 somite stage embryos.

DISCUSSION

Current models of the role of Bmp signaling during L/R patterning are dependent on the organism and experimental system used to study the process. In the chick embryo, right-sided *Bmp4* expression in Hensen's node is proposed to prevent *Nodal* expression in the right LPM (Roderiguez Esteban, 1999; Yokouchi, 1999). However, opposite roles for Bmp2 in maintaining *Nodal* expression in the left LPM have been demonstrated as well (Piedra and Ros, 2002; Schlange, 2002). In mouse embryos deficient for Bmp4, loss of *Nodal* expression has been observed, suggesting that Bmp4 is required for Nodal expression in the left LPM (Fujiwara, 2002). This loss of *Nodal* expression would be independent of the Bmp type I receptor Alk2, since chimeric mouse embryos composed of Alk2-deficient cells show bilateral *Nodal* and *Lefty* expression in the LPM (Kishigami, 2004). To explain these seemingly contradictory reports, it was suggested that Bmps might have multiple functions in L/R patterning due to their dynamic expression in various tissues at different developmental time points (Fujiwara, 2002).
In this study, we have addressed the regulation of L/R patterning by Bmp signaling in the zebrafish. Using this vertebrate model, we were able to distinguish two separate waves of Bmp signaling during L/R patterning at distinct developmental time points. Using heat-shock inducible lines, we were able to identify a requirement for Bmp signaling at the early segmentation stage. Bmp signaling at this stage is required for the inhibiting *spaw* expression in the right LPM and thereby regulates both visceral and cardiac L/R asymmetry. Furthermore, our morpholino experiments identified Bmp4 as the likely Bmp ligand responsible for this effect. Bmp4 is required in Kupffer's vesicle to repress *spaw* expression in the right LPM possibly via regulating *lefty1* expression. In addition, we were able to identify a second wave of Bmp signaling during late segmentation stages, required for regulating cardiac L/R asymmetry but not visceral asymmetry. Our results suggest a mechanism in which Bmp4 acts upstream of Nodal signaling by repressing *spaw* expression in the right LPM, and downstream of Nodal signaling at a later stage in the posterior LPM to regulate asymmetry in the heart field required for cardiac jogging.

Regulation of L/R patterning during early segmentation stages by Bmp signaling
Our results show that in *laf/alk8*-deficient embryos cardiac laterality is much more severely affected than visceral laterality (see fig. 1). Our experiments using the heat-shock inducible *noggin3* and *bmp2b* transgenic lines suggest that this might be due to different temporal requirements for Bmp signaling to control cardiac versus visceral laterality. In addition to zygotic expression by the embryo itself, the early embryo contains *alk8* mRNA of maternal origin. It has been demonstrated by knock down experiments using MOs targeting *laf/alk8* and by maternal zygotic *laf/alk8* mutants that the presence of such maternal mRNA compensates for the loss of zygotic *alk8* gene products during gastrula stages. Therefore accounting the for relatively normal dorsoventral patterning in zygotic *laf/alk8* mutant embryos (Bauer, 2001; Mintzer,

2001). Most likely, the presence of maternal *laf/alk8* mRNA is similarly responsible for the relatively weak defects of zygotic *alk8* mutants in visceral laterality, which is set up during early segmentation stages. As we have demonstrated here, cardiac laterality requires Bmp signaling during late segmentation stages and is therefore much more severely affected in zygotic *laf/alk8* mutant embryos.

Our data obtained with heat-shock inducible *noggin3* and *bmp2b* transgenes demonstrate a requirement for Bmp signaling at early segmentation for the repression of *spaw* expression in the right LPM. A second wave of Bmp signaling is also apparent at late segmentation stages to activate *lefty1* and *lefty2* expression in the left cardiac field (see below). Knock down of Bmp4 results in a bilateral *spaw* expression and reduced *pitx2* expression, suggesting that Bmp4 is the responsible Bmp ligand at the early segmentation stage (see fig. 6). In addition, the heat-shock experiments together with the *bmp4* morpholino injections show that Bmp4 is required for *lefty1* expression in the midline (fig. 2, 6 and 7). The recent proposed self-enhancement and lateral-inhibition (SELI) model, which demonstrates how left sided Nodal expression might be regulated, predicts that Lefty1 is required for repression of right sided *Nodal* expression (Nakamura, 2006). Indeed, mouse embryos deficient for *Lefty1* have bilateral *Nodal* expression in the LPM (Meno, 1998). Our data lead to a model in which Bmp4 is required to prevent nodal expression in the right LPM by inducing *lefty1* expression. A study using Bmp4-deficient mouse embryos suggests the opposite (Fujiwara, 2002). Mouse Bmp4 is required for node morphology and for transducing signals from the node to the left LPM. However, some similar phenotypes are reported for the Bmp4-deficient mouse embryos compared to our zebrafish results, such as the requirement for Bmp4 in inducing *Lefty* expression (Fujiwara, 2002). There are some major differences between the mouse and zebrafish system that could explain this controversy over Bmp4. First, while mouse Bmp4 is the major Bmp required during early mouse development, this is not what we find for zebrafish Bmp4. Our zebrafish *bmp4* morpholino experiments show only a mild defect in dorsoventral patterning and embryos survive without gross morphological defects. Zebrafish *bmp2b* mutants have a very severe dorsoventral patterning defect and do not survive past the 10 somite stage suggesting a more pronounced role for Bmp2b compared with Bmp4 (Kishimoto, 1997). Second, to avoid severe dorsoventral patterning defects our heat-shock experiments where performed after gastrulation when a Kupffer's vesicle has aL/Ready formed. Although our experiments suggest that Bmp4 is required in Kupffer's vesicle, no apparent defects in Kupffer's vesicle morphology were observed in *laf/alk8* mutants or *bmp4* MO injected embryos. We cannot exclude the possibility that Bmp4 is required for cilia function to generate a leftward flow in Kupffer's vesicle.

Regulation of L/R patterning during late segmentation stages by Bmp signaling

By using a heat-shock inducible *noggin3* transgenic line we were also able to pinpoint a requirement for Bmp signaling between the 18 and 25 somite stages for proper leftward cardiac jogging (fig 3). At the 22 somite stage, *bmp4* is asymmetrically

expressed in the left cardiac field and LPM (fig. 4) (Chen, 1997). The asymmetric *bmp4* expression is affected by defective midline formation (Chen, 1997) and, as we have shown here, by loss of the zebrafish nodal-related gene *spaw* (fig. 4). Our results with the knock down of Bmp4 (fig. 6) demonstrate that the left-sided Bmp4 expression in the LPM is required for *lefty1* and *lefty2* expression and cardiac jogging. This result is in agreement with an earlier study reporting that right-sided overexpression of Bmp4 in the zebrafish specifically affects laterality of the heart (Schilling, 1999). Our data from the experiments using the *tg(hsp70:noggin3)* embryos also demonstrate a requirement for Bmp activity for *lefty1* and *lefty2* expression in the left cardiac field. Our data from the experiments using the *tg(hsp70:bmp2b)* embryos show a different response for *lefty1* and *lefty2* to ectopic Bmp signaling. This difference was also observed when Bmp4 was specifically knocked down in Kupffer's vesicle (fig. 7). We currently do not understand how Bmp signaling at the late segmentation stage regulates *lefty1* and *lefty2* expression and controls cardiac morphogenesis. Detailed analysis of cardiac morphogenesis in combination with gain and loss of Bmp signaling will be required to address this question.

Asymmetric *bmp4* expression has also been observed in the developing heart tube of Xenopus, and heart specific over-expression of *XBmp4* or *XNoggin* results in randomized heart looping, indicating that the function for Bmp4 in establishing cardiac L/R patterning is conserved between zebrafish and Xenopus (Breckenridge, 2001). In addition, overexpression studies in Xenopus embryos revealed a strong interaction between Bmp4 and Lefty1 during L/R patterning (Branford, 2000). So far, this function for Bmp signaling during late segmentation that regulates specifically cardiac laterality, but not visceral laterality, has so far not been described in any mammalian organism. Mouse Bmp4 has a much earlier function in establishing L/R asymmetry in the node and the LPM (Fujiwara, 2002). Whether it also has a later function in establishing L/R patterning within the heart is not known, however, as asymmetric *mBmp4* or *mBmp2* expression within the cardiac field has never been reported. Tissue-specific and / or temporally controlled conditional knock out techniques will be necessary to give definitive answers.

ACKNOWLEDGEMENTS

We are very grateful M. Rebagliati and G. Krens for providing *southpaw* and control morpholinos, to B. Hogan for providing *laf* ^{gl2} embryos, to E. Weinberg, J. Yost, J-N Chen and M. Rebagliati for sending plasmids to K. Smith and M. Camacho for critical reading of the manuscript and members of the Bakkers laboratory for stimulating discussions. Work in J.B.'s laboratory was supported by the Royal Dutch Academy of Arts and Sciences, by the Netherlands Science Organization (ALW Grant #814.02.007) and the Human Frontier Science Program (Research Grant RGP9/2003).

MATERIAL AND METHODS

Trangenic lines and heat-shock experiments

Fish were kept under standard conditions as previously described (Westerfield, 1995). *lost-a-fin/alk8* mutant alleles used in this study are *laf* ^{tm110b} (Mullins, 1996) and *laf* ^{gl2} (Hogan, 2006).

To generate transgenic lines with inducible Noggin and Bmp expression we cloned zebrafish *noggin3* or tagged *bmp2b*, a FLAG tag is located at the N-terminus of the mature protein, downstream of the hsp70 promoter (Halloran, 2000). DNA constructs were injected at the 1-cell stage to generate *tg(hsp70:noggin3)* and *tg(hsp70:bmp2b)* carriers. Embryos collected from crosses between heterozygous transgenic carriers and wild type fish were heat-shocked by transferring the embryos to E3 medium preheated to 37°C and incubation at 37°C for 30 min. In case of the *tg(hsp70:bmp2b)* line, ectopic *bmp2b* mRNA was induced 10 min after the heat-shock using PCR primers on the FLAG tag. When crosses between a wt fish and a heterozygous *tg(hsp70:noggin3)* or a *tg(hsp70:bmp2b)* carrier fish were heat-shocked at shield stage, 50% of the embryos displayed a C5 dorsalization or a V4 ventralization respectively, showing that both transgenes are very efficient. For genotyping transgenic embryos or fish the following primers were used that amplify a specific 500 bp fragment when the transgene is present; forward 5'-cgcaggaaagaacatgtgagc-3', reverse 5'-cgggttggactcaagacgatag-3'. PCR conditions used; 25 cycles and 55°C during annealing.

Morpholino injections

Morpholino oligonucleotides (MOs; Gene Tools) were dissolved in water to 4 mM. For injection (1 nl per embryo), MOs were diluted in 1x Danieu's buffer (Nasevicius and Ekker, 2000). Sequences of used MOs were: *bmp4* splice MO 5'- ggtgtttgattgtctgaccttcatg-3', Fluorescein modified *bmp4* ATG MO 5`-GTCTCGACAGAAAATAAAGCATGGG-3` (Leung, 2005), standard control morpholino 5'-CCTCTTACCTCAGTTACAATTTATA-3'. *Southpaw* morpholinos used were described previously (Long, 2003). To specifically target dorsal forerunner cells embryos were injected with 0.8 ng *bmp4* splice MO and 1 ng fluorescein modified *bmp4* ATG MO at the 512-cell stage (3-4 hpf). At 80% epiboly embryos were selected for specific uptake of the MOs by the DFCs using a fluorescence stereo microscope (Leica) and were fixed at the appropriate stage in 4% pfa.

Generation of constructs, mRNA synthesis and RT-PCR

Zebrafish bmp4 was cloned into the BamHI and EcoRI sites of pCS2+ using the following PCR primers; forward 5'-cgggatccatgattcctggtaatcgaatgctg-3', reverse 5'-cggaattcttagcggcagccacaccc-3'. Capped mRNA was prepared with the Message Machine kit (Ambion, Austin, TX). To analyse the effect of bmp4 splice MO injections on mRNA splicing the following primers were used on cDNA derived from uninjected and injected embryos at 25 somite stage (see also fig. 5 for details); primer#1 (exon1) forward 5'-gcgtcggctcttcacctg-3', primer#2 (intron1) reverse 5'-cgagtgaacaaacctcttaaagc-3', primer#3 (exon2) reverse 5'-tggcgcctttaacacctcata-3'.

In situ hybridization

In situ hybridization was carried out as previously described (Thisse, 1993). Embryos were cleared in MetOH and mounted in benzylbenzoaat/benzylalcohol (2:1) before pictures were taken. Riboprobes were generated by transcription in the presence of digoxigenin-11–UTP from linearized templates of *cmlc2, amhc, vmhc* (Yelon, 1999), *foxa3* (Odenthal and Nusslein Volhard, 1998), *lefty1, lefty2* (Bisgrove, 1999), *spaw* (Long, 2003), *ntl* (Schulte Merker, 1994), *bmp4* (Chen, 1997), *ptc1* (Concordet, 1996), *spry4* (Furthauer, 2001), *nkx2.5* (Chen and Fishman, 1996).

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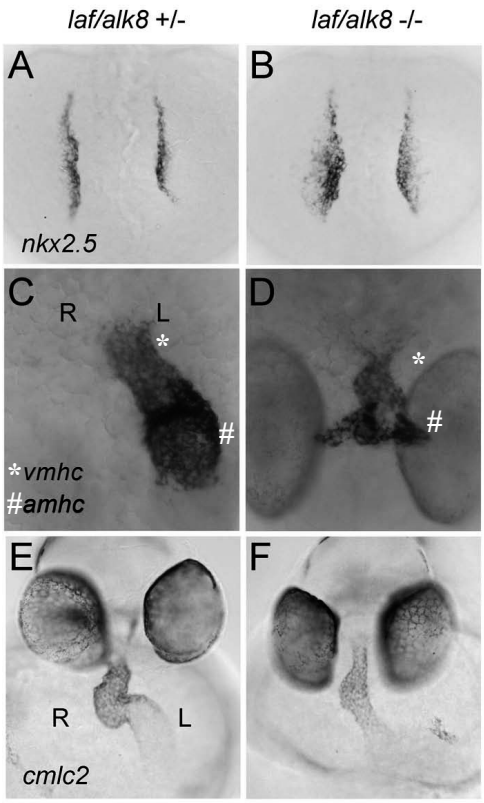
SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 1 Comparable *nkx2.5* expression in *laf/alk8* siblings (A) and mutant (B) embryos at the 15-somite stage. Heart tube in *laf/alk8* siblings jogs to the left (C) whilst it remains in the middle in mutant embryos (D). Embryos at 28 hpf double stained with *vmhc* (*) and *amhc* (#). *laf/alk8* sibling (E) and mutant (F) embryos show absence of cardiac looping in the mutant embryo. (A-D) Dorsal views with anterior to the bottom of the panel. (E,F) ventral views.

Supplement Table 1: Altered cardiac jogging in *Tg(hs:noggin3)*

transgene	*HS@*	*genotype*	*n*	*jogging (%)*		
				left	*right*	*no*
hs:noggin3	10-somite	sib	34	100	0	0
		transg.	15	13	0	87
hs:noggin3	15-somite	sib	20	100	0	0
		transg.	21	14	0	86
hs:noggin3	18-somite	sib	18	94	0	6
		transg.	17	24	0	76
hs:noggin3	22-somite	sib	12	100	0	0
		transg.	12	83	0	17
hs:noggin3	25-somite	sib	24	100	0	0
		transg.	22	100	0	0
hs:noggin3	28-somite	sib	26	96	4	0
		transg.	26	100	0	0

Supplement Table 2: Injection of wt *bmp4* and *bmp4^{splice}* mRNA

mRNA injected		*observed phenotypes**					
		n	wt	V2	V3	V4	†
wt *bmp4*	1,6 pg	116	9	5	7	32	47
,,	8 pg	64	0	0	0	0	100
bmp4^{splice}	1,6 pg	28	100	0	0	0	0
,,	8 pg	122	100	0	0	0	0
,,	40 pg	54	100	0	0	0	0

* Ventralization was scored as previously described in Kishimoto (1997) *Development* 124; 4457-4466.

Supplement Table 3: Effect of Bmp4 knock down on cardiac jogging

morpholino	*n*	*jogging (%)*		
		left	*right*	*no*
wt	124	94	2	4
bmp4 ATG MO (2 ng)	249	38	19	43
bmp4 splice MO (0.8 ng)	96	32	18	50

Chapter 4

TGFβ signaling and left-right patterning: a story of positive and negative feedback loops

Manon Verhoeven
Jeroen Bakkers

ABSTRACT

Left-right (L/R) identity is first established within and surrounding the node, Kupffer's vesicle (KV) in zebrafish. For correct L/R patterning of different organs it is crucial that the L/R information is transferred from the node to the LPM and maintained there. Specifically left-sided Nodal expression in the LPM has been reported essential. Here, we studied the regulation of the nodal-related gene *spaw* and in particular the role of Bmp signaling therein. We found that *spaw* expression in the LPM and the expression of its inhibitor *lefty1* in the midline are both downstream of Nodal signaling. Furthermore, our data shows that Bmp signaling acts upstream of Spaw and is required to repress *spaw* expression in the right LPM and around the KV. We demonstrated that Bmp signaling works synergistically with Spaw signaling to induce *lefty1* expression in the midline and regulates its propagation into an anterior direction. We therefore suggest a model in which Bmp signaling directly restricts Spaw around the KV and represses Spaw in the LPM via induction of Lefty1 antagonism. Our results shed light on the complex regulation of transferring L/R identity from the node to the LPM in the developing embryo.

INTRODUCTION

Vertebrate species display an external bilateral symmetry while internally different organ systems develop asymmetrically. Left-right (L/R) identity is first established within the node, or Kupffer's vesicle (KV) in the zebrafish. During early stages of L/R determination Shh and Notch signaling have been reported to induce *Nodal* expression in the node (Pagan-Westphal and Tabin, 1998; Krebs, 2003; Raya, 2003) Because most organs that display L/R asymmetry are derivatives of the lateral plate mesoderm (LPM), it is essential that the L/R information is transferred from the node to the LPM and maintained there. Although there have been some recent reports on how this information is transferred in mouse embryos, we do not know whether these mechanisms are mouse specific or can be applied to other vertebrate systems as well.

In mice, active Nodal signaling results in the onset of downstream targets, like Lefty 1 and 2, Pitx2, Cyclops and Nodal itself. After *Nodal* expression is initiated in the node and in close vicinity around the node, a second wave of *Nodal* expression spreads through the left LPM in an anterior-posterior fashion. Nodal is currently the only signaling gene whose function in the node has been found essential for subsequent *Nodal* expression in the left LPM, thereby transferring L/R information to the rest of the embryo (Brennan, 2002; Saijoh, 2003). Importantly, Nodal regulates its own expression through an auto-regulation mechanism (Osada, 2000; Saijoh, 2000; Norris, 2002; Long, 2003). Lefty, induced by Nodal signaling, antagonizes Nodal and dampens the activity or the response to Nodal signals in surrounding cells. The antagonizing activity of Lefty represses right-sided *Nodal* expression (Chen and Schier, 2002). *Lefty* expression in the midline may function as a physical barrier to prevent the spread of asymmetric signals in the LPM to the inappropriate other side, thereby avoiding the right LPM to acquire a left-sided identity. It has also been suggested that the midline may function as a source of molecules that either direct or repress gene expression in adjacent tissues (Lohr, 1997; Lohr, 1998). The interaction between Nodal and Lefty1 and 2 in mouse was shown based on a self-enhancement, lateral-inhibition (SELI) mechanism (Nakamura, 2006). According to this model, a small difference in Nodal signaling between the left and right side is amplified. Central in this model is the observation that Nodal induces both its own expression as well as the expression of its inhibitor (Lefty). Due to the different diffusion properties of the nodal and Lefty proteins (Lefty can diffuse over very large distances while Nodal diffusion is more restricted) Nodal signaling is induced locally (self-enhancement) and repressed further away from the source (lateral-inhibition). It is currently unknown whether other factors are involved in this tightly regulated relationship between Nodal and Lefty1 and whether this model can also be applied to other organisms. Reports on the role of Bmp signaling in the regulation of asymmetric L/R information have been inconsistent. Studies in chick and mouse suggest that Bmp signaling acts as a positive facilitator of the left-sided molecular cascade, and is required for Nodal induction and its maintenance in the left LPM

(Fujiwara, 2002; Piedra and Ros, 2002; Schlange, 2002). In contrast, a repressive function of Bmp signaling was described initially in chick and later supported by studies in other organisms (Rodriguez Esteban, 1999; Yokouchi, 1999; Zhu, 1999; Chocron, 2007; Furtado, 2008; Mine, 2008). This paradox mainly indicates the complexity of the regulatory mechanisms in establishing and maintaining left-right patterning.

Here, we use transgenic lines to either overactivate or inhibit Bmp signaling and analyze maternal-zygotic (MZ) *sfw* mutants to shed light on the complex triangular regulation between *spaw, lefty1* and *bmp4*. We show that *lefty1* expression in the midline is dependent not only on Nodal signaling but also Bmp signaling. Our results demonstrate that Bmp signaling represses *spaw* expression in the KV as well as in the right LPM. These results help understand the complex relationship between different members of the TGFβ superfamily in their task to transfer the L/R identity from the node to the LPM in the developing embryo. Furthermore, we investigate the time frame in which Nodal signaling establishes L/R information, regulating cardiac laterality and visceral laterality. This reveals Spaw is required up until the 11-15 somite stage for correct L/R patterning and is dispensable after that.

MATERIALS & METHODS

Transgenic lines and heat shock experiments

Fish were kept under standard laboratory conditions. To induce *noggin* and *bmp* expression, we used *tg(hsp70:noggin3)* and *tg(hsp70bmp2b)* transgenics, respectively. Embryos collected from between heterozygous transgenic carriers and wild-type fish were heat-shocked by transferring the embryos to E3 medium preheated to 37°C and incubate them for 30 minutes at 37°C. Efficiency of the transgenic lines as well as the genotyping have been described in Chocron 2007. Transgenic embryos tend to display a minor delay in development, we therefore staged siblings and transgenic always based on the number of somites. MZ*sfw* is described in chapter 2.

In situ hybridization

Embryos were fixed in 4% paraformaldehyde/4% sucrose in PBS with 10% Tween o/n at 4°C. Whole-mount *in situ* hybridization was performed as described (Thisse1993). Embryos were subsequently cleared in Murray's (benzylalcohol:benzylbenzoate 1:2) and stored at 4°C.

SB treatment

Solid anhydrous SB431542 was dissolved at a concentration of 10 mM in DMSO. SB431542 was added to embryos in a concentration of 100µM in E3 medium without any additives. Up to 50 wild-type embryos were treated in a 12-well plate in a volume of 2.5 ml/well. Albino wild-types were used to prevent pigment formation. Control embryos were treated with a corresponding dilution of DMSO in E3 medium. To remove the inhibitor or DMSO treatment, embryos were washed with E3 medium three times.

RESULTS

spaw, lefty1 and bmp4 are dynamically expressed throughout embryonic development

To assess the relationship between the expression patterns of *spaw, lefty1* and *bmp4* and to investigate their regulation we first determined the expression of *spaw, lefty1* and *bmp4* in wild-type embryos. To determine the roles of *spaw, lefty1* and *bmp4* specifically with respect to L/R patterning, we evaluated their expression during somitogenesis. Starting at the 5-somite stage, just before the Kupffer's vesicle (KV) is present, and ending our time line at the 20-somite stage, just before the morphological break in left-right symmetry (cardiac jogging) can be observed. We anticipate that L/R patterning by that time will be established and interpreted by the heart. The expression of the three genes was very dynamic, specifically during somitogenesis. *spaw*, the zebrafish ortholog of nodal, is not expressed before the onset of somitogenesis (Long2003). We detected the earliest *spaw* expression at the 3-somite stage (data not shown). At the 5-somite stage, *spaw* was bilaterally expressed in two stripes surrounding the Kupffer's vesicle (fig.1A). Between 3 and 7 somites this expression starts weak and rapidly gets more profound. We observed that when the expression levels increase, the domain remains restricted to the close surrounding of the KV. In all time points investigated, *spaw* expression was retained around the KV. At the 10-somite stage, *spaw* was also expressed in the LPM (fig.1B). Initially spaw expression was bilateral, but *spaw* was rapidly expressed stronger in the left LPM. This expression in the LPM is very characteristic in shape; it does not spread laterally, but progresses in the anterior direction. At the 15-somite stage *spaw* expression had extended approximately halfway the A-P axis (fig.1C). In addition, at the 20-somite stage we observed that when *spaw* expression propagates more anterior and reaches the LPM surrounding the future heart, the expression expands slightly to the lateral sides (fig.1D). After the 22-somites stage, *spaw* expression was rapidly downregulated, and completely absent at later stages of development (data not shown).

The Nodal antagonist *lefty1* was detected at the 5-somite stage in two separate domains; the posterior notochord, just anterior to the KV, as well as anterior in the prechordal plate (fig.1E). Up to the 10-somite stage the expression in the prechordal plate did not change while the notochord expression extended more anterior (fig.1F). At the 15-somite stage expression of *lefty1* in the prechordal plate was lost while the midline expression remained, reaching approximately halfway the embryo (fig.1G). Although the midline expression remained at the 20-somite stage we often observed a gap in the *lefty1* expression resulting in separated posterior and anterior midline expression domains (fig.1H). At the 22-somite stage, *lefty1* expression was also observed in the left cardiac field, as well as in the left side of the epithalamus (data not shown). Both *spaw* and *lefty1* displayed a dynamic expression pattern in a posterior-to-anterior fashion. To visualize the relationship between the dynamic expression patterns of *spaw* and *lefty1*, we performed a

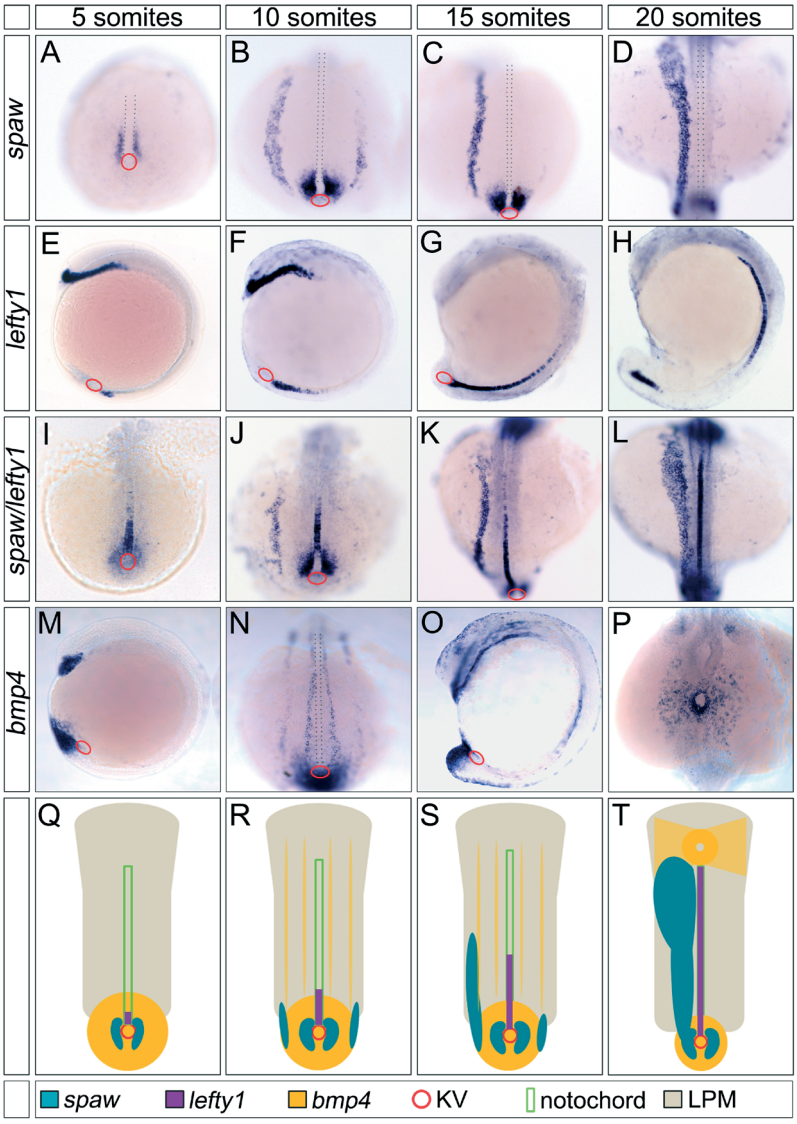


Figure 1 The expression of *spaw*, *lefty1* and *bmp4* is highly dynamic. ISH of *spaw* (A-D), *lefty1* (E-H), *spaw*/*lefty1* (I-L) and *bmp4* (M-P) in wild-types at stages indicated above. (Q-T) are schematic representations of expression patterns of the three genes. *spaw* is expressed primarily in the peri-nodal area, a domain that remains until the 22-somite stage (A-D). *spaw* is expressed in the LPM from the 12-somite stage on (B), first also on the right side, later only on the left side(C,D). *lefty1* is expressed in the posterior notochord and anterior prechordal plate (E). The notochord expression expands in the anterior direction (F-H). Expression of *lefty1* precedes *spaw* expression in the LPM (I), during later stages, *spaw* expression in the LPM always precedes *lefty1* in the notochord (J-L). *bmp4* is expressed in the tailbud and anterior prechordal plate (M), and at 10- and 15-somite stages also bilaterally in the LPM (N,O). At the 20-somite stage *bmp4* is predominantly expressed in the LPM surrounding the cardiac field (P). (A-D), (I-L), (N) and (O) are dorsal views, (E-H), (M) and (O) are lateral views. KV is indicated in red in all stainings of 5 to 15 somites stages.

double *ISH*, with probes against both mRNAs. We observed at the 5-somite stage that *lefty1* is expressed in the posterior notochord prior to expression of *spaw* in the LPM (fig.1I). We observed that at later stages the *spaw* expression domain in the left LPM was slightly more anterior than *lefty1* expression in the notochord at all time points (fig.1J-L). This could suggest that *spaw* expression in the LPM induces *lefty1* expression in the midline after the 5-somite stage.
During early somitogenesis, around the 5-somite stage, separate domains of *bmp4* expression could be distinguished. Expression was observed in the prechordal plate as well as in the tailbud, in a relative large domain surrounding the KV (fig.1M). Around the 10-somite stage an additional expression domain was observed in the LPM. Two bilateral stripes of *bmp4* expression were located in close vicinity of the embryonic midline, and two bilateral stripes were located more laterally in the LPM (fig.1N). We observed some variation in staining of this particular domain. Starting at the 15-somite stage *bmp4* expression was also observed specifically in the LPM surrounding the heart primordium (fig.1O). This specific expression domain remained during late somitogenesis, while the expression in other domains regressed (fig.1P).

lefty1 expression in the notochord and spaw expression in the LPM are dependent on spaw

To investigate the triangular relationship between *spaw*, *lefty1* and *bmp4* expression, we analyzed their expression in mutants lacking a functional spaw gene. We used an MZ*sfw* mutant, which was isolated in a screen for laterality mutants (described in chapter 2), since reports describing results using spaw morpholino knock downs were inconsistent (Long, 2003; Wang and Yost, 2008). The *sfw* mutation results in an inactivating missense mutation in the C-terminus of the Spaw protein. In MZ*sfw* mutant embryos *spaw* was detected in the area around the KV at the 5-somite stage (fig.2A) and this expression remained in all following stages (fig.2B,C,D), although it is much weaker compared to wild-type expression (fig.1B,C,D). The expression of *spaw* in the LPM of MZ*sfw* mutants was however not initiated at the 10-somite stage and was never observed in any of the following stages (fig.2B,C,D). This demonstrates that *spaw* is required to induce its own expression in the LPM. The remaining weak expression of *spaw* around the KV suggests that *spaw* is induced there by upstream factors, but regulates its own expression to increase expression levels. It is possible that the amplitude of the expression in the nodal area is crucial for propagating spaw expression to the LPM.
In MZ*sfw* mutants, *lefty1* expression in the midline was never observed in all stages analyzed (fig.2E-H). In addition, the *lefty1* expression in the left cardiac field and left epithalamus was not visible at the 22-somite stage (fig.1H). This demonstrates that lefty1 expression in these domains dependents on intact Spaw signaling. The *lefty1* expression in the prechordal plate was however present at the 5-somite stage (fig.2E), demonstrating that this expression is regulated independent of *spaw*.
The expression of *bmp4* was found largely unaffected at all stages in MZ*sfw*

mutants. At the 5-somite stage, *bmp4* was expressed in both prechordal plate as well as tail bud (fig.2I). The bilateral stripes were also observed flanking the midline, although again some variation was observed (fig.2L), and expression in the LPM surrounding the cardiac field is present from the 15-somite stage on (fig.2K,M). This demonstrates that all the different expression domains of *bmp4* are regulated independent of *spaw*. In conclusion, using the MZ*sfw* mutant we confirm that *spaw* regulates its own expression in the LPM, while the expression of *spaw* surrounding the KV can be initiated independent of Spaw function. Our date demonstrates that *bmp4* expression in the LPM is independent from Spaw function. In addition we have shown that *spaw* expression is required for the expression of *lefty1* in the midline and thereby creates a negative feedback loop.

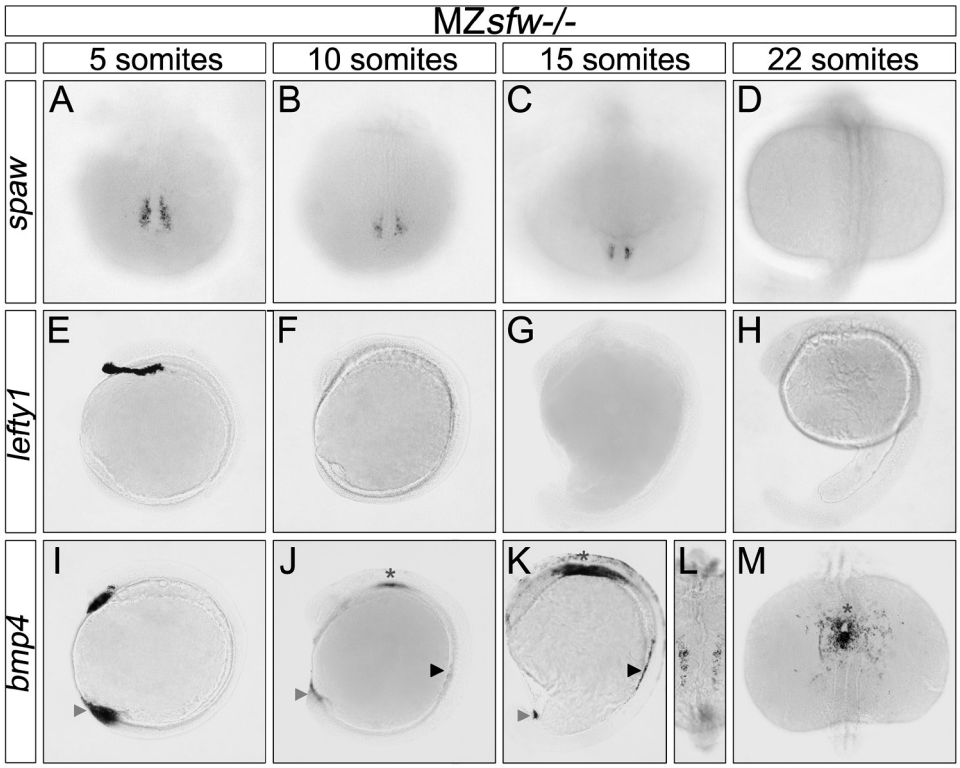


Figure 2 The expression of *lefty1* but not the expression of *bmp4* is dependent on nodal signaling. ISH of *spaw* (A-D), *lefty1* (E-H), *bmp4* (I-M) in *MZsfw* mutants at stages indicated above. Compare expression in *MZsfw* mutants to expression in wild-types in figure 1. *spaw* is expressed normally in the peri-nodal area (A), although significant weaker during later stages (B-D). *spaw* is never expressed in the LPM in *MZsfw* mutants at any of the stages analyzed (B-D). *lefty1* is expressed in the anterior prechordal plate as normal (E), but is never detected in the notochord at any stage, or in the cardiac field and epithalamus (F-H). *bmp4* is expressed in all domains (I-M). Grey arrowheads indicate tailbud expression, black arrowheads indicate expression in the LPM and the asterisk indicates the pericardial expression. (A-D), (L) and (M) are dorsal views, (E-H) and (I-K) are lateral views.

Bmp signaling is both required and sufficient to induce lefty1 expression in the midline

Our earlier results showed that Bmp signaling is required at two different time points, early and late somitogenesis, to regulate L/R patterning. During early somite stages Bmp signaling is required for repressing *spaw* expression on the right side of the embryo, while during late somite stages Bmp signaling is necessary for inducing *lefty1* and *lefty2* expression in the left cardiac field and anterior notochord (Chocron, 2007). To address how Bmp signaling during early somite stages might regulate L/R patterning we analyzed the effect of Bmp signaling on the early expression of both *spaw* and *lefty1*. We used two transgenic lines, *tg(hsp70:noggin3)* and *tg(hsp70:bmp2b),* which upon heat shock inhibit or activate Bmp signaling respectively in the whole embryo. When *noggin* expression was induced at tailbud stage we observed no significant differences in *spaw* expression at the 5-somite and 10-somite stage. Both in the *tg(hsp70:noggin3)* embryos as well as in the wild-type siblings *spaw* was expressed around the KV (fig.3A,D,B,E and supplementary table 1). The *lefty1* expression at the 5-somite stage was unaffected in the prechordal plate of heat shocked embryos, but absent or strongly reduced in the posterior

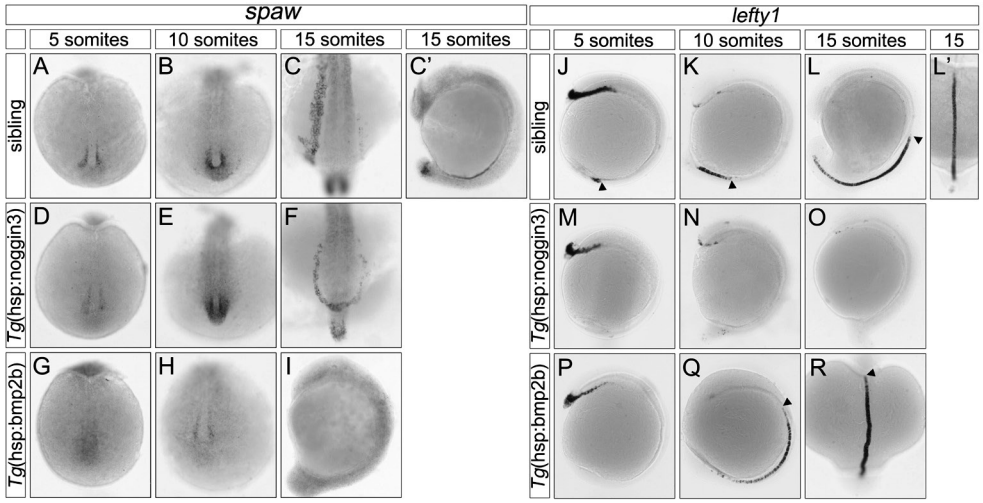


Figure 3 Bmp signaling represses *spaw* expression in the left LPM and peri-nodal area. (D-F) Heat shocked *tg(hsp70:noggin3)* embryos display unaffected *spaw* expression around the node (D,E), but *spaw* is bilaterally expressed in the LPM at the 15-somite stage (F). (G-I)Heat shocked *tg(hsp70:bmp2b)* transgenic embryos lack or have reduced peri-nodal *spaw* expression (G,H). No *spaw* expression was detected at later stage of development (I). (C' and I) are lateral views, the rest of the *spaw* stainings are dorsal views. Bmp signaling induces *lefty1* expression in the midline. (M-O) Heat shocked *tg(hsp70:noggin3)* embryos specifically loose *lefty1* expression in the posterior midline (M), and no *lefty1* expression was detected in later stages (N,O). (P-R)Heat shocked *tg(hsp70:bmp2b)* transgenic embryos lack *lefty1* expression in the posterior midline at the 5-somite stage (P), but display ectopic *lefty1* expression in the midline at later stages (Q,R). Undulating notochords were observed as a side effect of manipulating Bmp signaling (I). (L' and R) are dorsal views, the rest of the *lefty1* stainings are lateral views.

notochord (fig.3M). At later stages, the *lefty1* expression in the notochord, that normally expands anterior, remained absent in transgenic embryos (fig.3N,O). Similar to our previous observation, from the 15-somite stage onward the induction of *noggin* in transgenic embryos resulted in a bilateral *spaw* expression in the LPM (fig.3F), instead of being restricted to the left side (fig.3C)(Chocron, 2007).
Ectopic Bmp signaling had an opposite effect on the expression of *spaw* and *lefty1*. *Tg(hsp70:bmp2b)* heat-shocked at tailbud stage displayed a reduction of *spaw* expression at 5 and 10-somite stages (fig.3G,H and supplementary table 1) and lacked *spaw* expression completely at the 15-somite stage, both in the nodal area and the LPM (fig.3I). The *lefty1* expression at the 5-somite stage was unaffected in the prechordal plate, but absent or strongly reduced in the posterior notochord (fig.3P). At the 10-somite stage however, *lefty1* was ectopically expressed throughout the notochord and reached a more anterior domain than normally observed at this stage (fig.3Q). At the 15-somite stage *lefty1* expression remained more anterior and stronger compared with wild-type siblings (fig.3R). The notochord was slightly undulated in the transgenic embryos, indicative of an extension defect caused by overactivated Bmp signaling. Together, these results indicate that both Bmp signaling and Spaw signaling are necessary for induction of *lefty1* expression in the posterior notochord and suggest that the repression of *spaw* expression in the right LPM by Bmp signaling is, in part, mediated via the induction of *lefty1* in the notochord.

spaw is necessary L/R patterning during a defined period throughout somitogenesis

The expression of *spaw* is very dynamic during development and present at various stages during somitogenesis. During that period *spaw* is responsible the correct L/R patterning, indicated by the complete disturbance of asymmetric organ morphogenesis in *sfw* mutants. To address the question at which time points *spaw* is necessary to regulate the laterality of the different organs we used the chemical inhibitor SB431542 that specifically blocks Nodal signaling by inhibiting the Alk4 and Alk7 receptor (Sun, 2006). The inhibitor specifically acts on signaling of nodal-related genes and not signaling via other members of the TGFβ superfamily, like Bmp signaling components (Sun, 2006). To determine the time frame when *spaw* is required for correct L/R patterning, SB431542 was added to the embryos at specific time points. We started adding the inhibitor no earlier than at 90% epiboly to avoid affecting mesoderm formation. Since *spaw* is not expressed before the onset of somitogenesis, we do expect to manipulate the entire time frame of *spaw* expression. Adding the inhibitor at specific time points resulted in a dramatic reduction of *spaw* and *lefty1* expression compared to control embryos treated with DMSO (data not shown). Since these effects are similar to those observed in the *spaw* mutant, we concluded that treating embryos with SB431542 results in reduced Spaw signaling. Next, we addressed how inhibition of Spaw signaling at various time points would affect organ laterality. Heart laterality was evaluated by the direction of the cardiac jogging 28 hpf and cardiac looping at 48 hpf (fig.4A,B). We observed that when

SB431542 was added as early as 90% epiboly and as late as the 2-somite stage, cardiac jogging and looping were completely compromised. Addition of SB431542 at the 6- and 10 somite stage partially affected the jogging as well as the looping of the heart, while addition of SB431542 after the 10 somites stage had little to no effect on the cardiac laterality. To assess when Spaw signaling is required for correct L/R patterning of the intestine, embryos exposed to SB431542 were evaluated for visceral laterality by staining the treated embryos for *foxa3* expression at 48

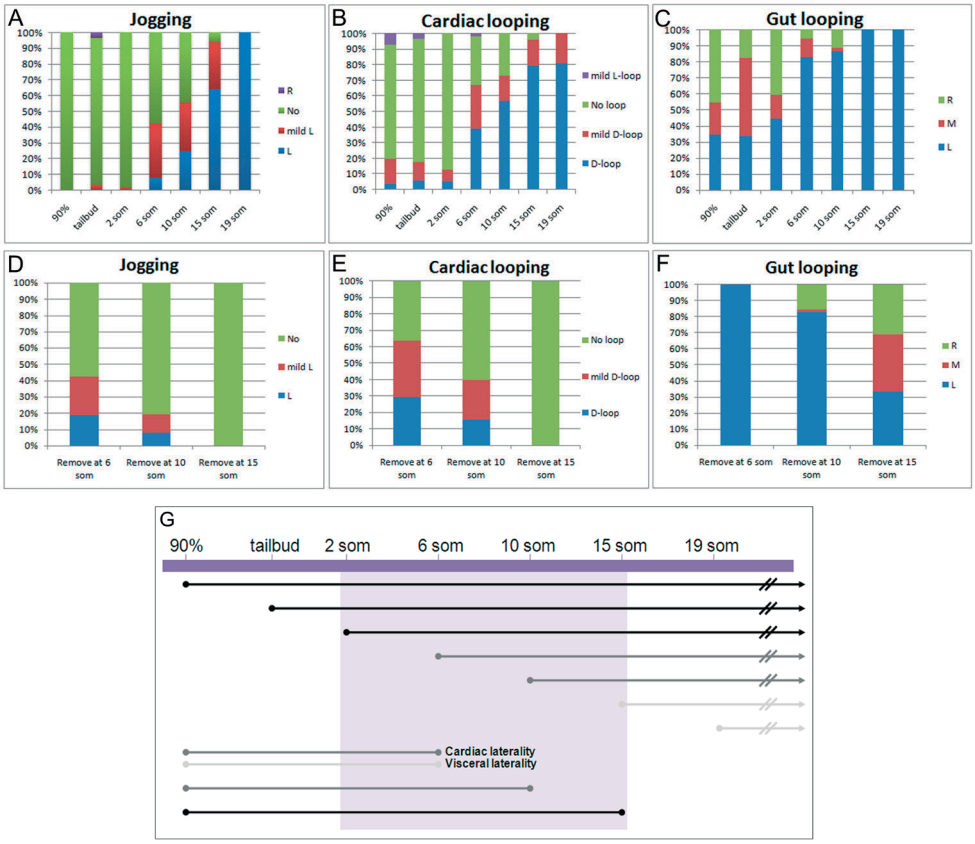


Figure 4 Nodal signaling is required for organ laterality between 2 and 15-somite stages. SB431542 nodal inhibitor was added at indicated stages and was continuously present until phenotypes were scored (A-C). Cardiac jogging was evaluated live at 28 hpf (A), while cardiac looping and gut looping were assessed by ISH stained for *cmlc2* (B) and *foxa3* (C) respectively. Addition of Sb431542 up until the 2-somite stage resulted in complete disturbance of organ laterality, both heart and gut. Addition of SB431542 at 6 and 10 somites led to partial compromised laterality, while addition at later stages had little to no effect (A-C). SB431542 was added at 90% epiboly and removed at indicated stages (D-F). Removing SB431542 at the 6-somite stage resulted in a partial disturbance of cardiac laterality, but rendered visceral laterality normal. Removal at the 10-somite stage led to affected laterality in both heart and visceral organs, while removing SB431542 as late as 15 somites completely compromised laterality in all organs (D-F). (H) Bars represent the treatments with SB431542 at indicated stages, black bars indicate completely affected laterality, and medium grey bars suggest partial effects and light grey bars indicate there was no significant effect on the laterality.

hpf. We observed that when SB431542 was added before or at the 2-somite stage the looping of the gut and the positioning of the liver was randomized. Addition of SB431542 at the 6- and 10-somite stage resulted in only a mild effect on the visceral laterality, while adding the inhibitor after the 10-somite stage had no effect at all on visceral laterality (fig.4C). To define the window in which Spaw signaling is required for patterning the L/R axis, we added the SB431542 at 90% epiboly and washed it away again at specific time points. First we checked what the effect of the temporal incubation with SB431542 was on the expression of *spaw* and *lefty1* at the 22-somite stage. We observed that when removing SB431542 at the 6-somite stage, the expression of both genes was partially restored to normal. The majority of the treated embryos displayed a delay in expression pattern of *spaw* and *lefty1*. *Spaw* was often expressed bilaterally, although left-sided *spaw* was often expanded more anterior. The delay was even more apparent in embryos treated with SB431542 until 10- to 15-somite stages. In addition, the expression domains of both *spaw* and *lefty1* extended less anterior (data not shown). We observed with respect to cardiac laterality, based on both cardiac jogging and subsequent looping, treating the embryos with SB431542 from 90% epiboly up until the 6 or 10-somite stage resulted in a partial disturbance. Cardiac laterality was only completely affected when the SB431542 was present until the 15-somite stage (fig.4D,E). Incubating the embryos with SB431542 from 90% epiboly to the 6-somite stage had no effect on the laterality of the visceral organs. Incubating the embryos with SB431542 until the 10-somite stage affected gut looping and the position of the liver partially. For a complete randomization of the visceral laterality, we needed to incubate the embryos with SB431542 until the 15-somite stage (fig.4F).

Overall, from these observations we can conclude that for both cardiac and visceral laterality there is not a specific time point at which Spaw signaling is required. We observed a broad window (2-somite stage until the 15-somite stage) during which Spaw signaling needs to be active for correct laterality of the cardiac and visceral organs (fig.4G). Furthermore, the heart and visceral organs display differences regarding the time frames when they are most sensitive to Spaw inhibition.

DISCUSSION

Left-sided Nodal expression in the LPM is essential for correct L/R patterning. Here we studied the regulation of *spaw* expression and in particular the role of Bmp signaling therein. We found that *spaw* expression in the LPM and *lefty1* expression in the midline are both downstream of Nodal signaling. Furthermore, our data shows that Bmp signaling acts upstream of Spaw since it is required to repress *spaw* expression in the right LPM and around the KV. In addition, we demonstrated that Bmp signaling works synergistically with Spaw signaling to induce *lefty1* expression in the midline and regulates its propagation into an anterior direction. This implies a regulation of Nodal signaling by Bmps at two levels, direct via repression of spaw and indirect via induction of Lefty1 antagonism.

lefty1 expression and spaw expression in the LPM are dependent on spaw

Here we have used the MZ*sfw* loss of function mutant to study Spaw requirement in regulating *spaw*, *lefty1* and *bmp4* expression. Although *spaw* knock down experiments have been reported previously, we already showed in chapter 2 that the phenotype with respect to cardiac laterality differs between *spaw* morpholino injected embryos and MZ*sfw* mutants, suggesting only a partial knockdown of *spaw* by the morpholino. We observed that *spaw* expression around the KV is independent on Spaw activity while spaw expression in the LPM is entirely dependent on Spaw activity. Using *spaw* morpholino knock down experiments other researchers reported that *lefty1* expression in the posterior notochord is either independent or dependent on Spaw signaling (Long, 2003; Wang and Yost, 2008). Using a *spaw* mutant allele (*sfw*$^{t30973)}$ we found that the expression of *lefty1* in the notochord during all stages was dependent on Spaw activity, resolving this controversy. In addition we observed that *bmp4* expression in the tailbud was largely unaffected in the MZ*sfw* mutant embryos. The weaker staining for bmp4 observed in the mutant embryos was likely due to some variation in the staining pattern, which we have also observed in batches of WT embryos.

Bmp signaling restricts spaw expression around the KV and in the right LPM by inducing lefty1 in the notochord

Left-sided expression of *Nodal* in the LPM appears to be a prerequisite for establishing the normally invariant pattern of left-right asymmetries. Any variation from this normal *Nodal* expression pattern, be it bilateral, absent, or on the opposite (right) side, has a dramatic effect on the *situs* of several internal organs. Inappropriate *Nodal* expression in the LPM thus leads to severe laterality defects, underscoring the importance of determining how the asymmetric expression of *Nodal* is regulated. A key factor in initiating *Nodal* expression in the left LPM is *Nodal* expression around the node (Brennan, 2002; Saijoh, 2003). Our data demonstrates that Bmp signaling represses *spaw* expression in the perinodal area in zebrafish (Fig.5). This correlates with findings in the mouse, where Bmps are expressed around the node and Bmp

antagonists Noggin and Chordin were found necessary to promote perinodal Nodal expression. Ectopic Bmp also markedly decreased perinodal *Nodal* expression (Mine, 2008). We hypothesize that the broad expression of *bmp4* in the tailbud of zebrafish embryos functions to repress nodal, while this repression is relieved in the perinodal area by Bmp antagonism. Bmp signaling in the tailbud thereby functions to restrict *spaw* expression to the perinodal area. Therefore, although *spaw* positively regulates its own expression, around the node this only results in a higher level of expression and not in expanded expression. This suggests local differences in Bmp signaling around the KV, which should be addressed in the future.

The local expression of *spaw* around the node needs to be transferred to the LPM. It is currently unknown by what mechanism perinodal Spaw in zebrafish is transferred to the LPM and if and how Bmp signaling affects this process. Spaw, as a secreted ligand, could migrate itself to the LPM, but relaying the L/R information through other signaling components is also possible. In mouse, Nodal is transferred directly from node to LPM guided by interaction with sulfated glycosaminoglycans (Oki, 2007). Additional experiments, e.g. by expressing an eGFP-Spaw fusion protein, could provide new insights in the dynamics and possible migration of perinodal Spaw and the transfer of Spaw to the LPM.

Once the signal is transferred to the LPM, small differences between levels in the left and right LPM, created by nodal flow are converted into a robust difference by a SELI mechanism. By self-enhancement locally and lateral inhibition at long-range, Nodal (Spaw in zebrafish) and Lefty1 respectively, would amplify left-side specific Nodal activity. We indeed observed that both *spaw* and *lefty1* expression depend on Spaw activity, which would support a SELI mechanism in zebrafish (Fig.5). Also supporting this model is the recent finding that embryos injected with a *lefty1* morpholino displayed bilateral *spaw* expression (Wang and Yost, 2008). However, our data now demonstrates that in addition to Spaw activity, also Bmp signaling is necessary for the expression of *lefty1* in the notochord. This suggests that there is not a simple two-component system in zebrafish that is sufficient to propagate the left signal in the LPM. One explanation for this could be that that unlike in mice, zebrafish *lefty1* is not expressed in the LPM overlapping with *spaw* expression but its expression is restricted to the notochord. Mathematical studies will be required to test whether this model can be applied to zebrafish embryos.

We observed that overactivated Bmp signaling induces ectopic *lefty1* expression in the notochord at the 10 somite stage and later. At the 5 somite stage, however, overactivated Bmp signaling did not result in *lefty1* induction in the posterior part of the notochord, reflecting that *lefty1* induction is dependent on both Bmp signaling as well as Nodal signaling. The fact that we do see an induction of *lefty1* in the notochord upon overactivation of Bmp signaling at the 10 somite stage, might suggest the presence for a co-factor, that is not yet expressed at the 5-somite stage. Another possibility is that responding cells start expressing appropriate receptors after the 5-somite stage, that change the efficiency of Bmp signaling. The effect we see at the 10 somite stage and later could be either a direct effect of Bmp signaling

on *lefty1* expression or a more indirect effect. Besides specifying ventral cell fate, members of the Bmp family of signaling molecules have recently been assigned a role in specification of dorsal cell fate as well (Esterberg, 2008). These authors showed that inhibiting Bmp signaling activity at 80% epiboly differentiates the notochord prematurely (Esterberg, 2008). In our experiments, when we inhibited

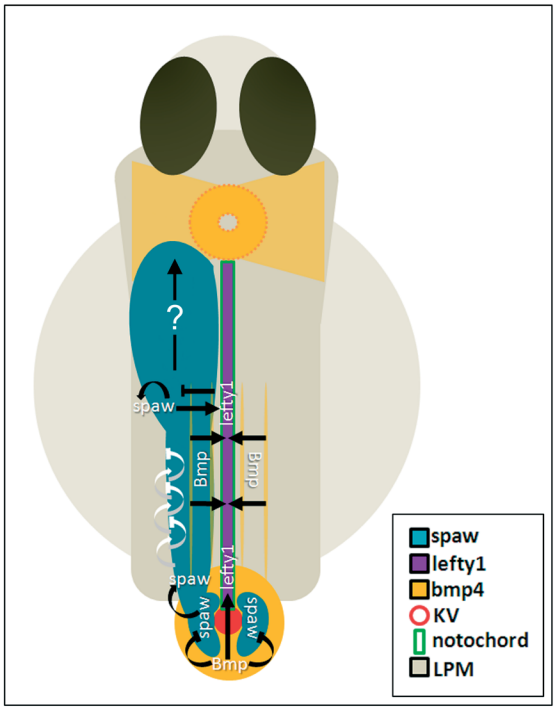


Figure 5 Cartoon of the model proposed of the regulation of left-sided *spaw* expression during early L/R patterning. *Spaw* is expressed around the KV, and this expression is negatively regulated and thereby restraint by Bmp signaling. Bmp signaling and perinodal Spaw signaling in the tailbud synergistically induce *lefty1* expression in the notochord, and this notochord expression is propagated in anterior direction positively regulated by both Bmp and Spaw in the LPM. Spaw is induced by early L/R signals (includes Shh and Notch), while concomitantly escaping repression by Bmp signaling by local Bmp antagonism. According to the SELI model, Spaw is transferred to the LPM bilaterally. Through positively inducing its own expression, while laterally *spaw* expression is inhibited by the long-range acting inhibitor *lefty1*, an initial small difference in *spaw* between the left and the right side is converted into specific expression on the left side. By inducing *lefty1* expression in the notochord in collaboration with Spaw, Bmp signaling represses Spaw on the right side and secures left side-specificity.

Bmps signaling at the tailbud stage, we never observed any *lefty1* expression in the notochord. This suggests that the loss of *lefty1* expression or the ectopic *lefty1* expression in the notochord upon inhibiting or ectopic Bmp signaling respectively cannot be simply attributed to an effect on notochord differentiation.

We hypothesize that Bmp signaling is necessary for inhibition of *spaw* expression on the right side of the embryo, most likely by inducing *lefty1* in the posterior midline. To validate this theory, additional experiments are necessary including analysis of

spaw expression in *tg(hsp70:bmp2b)* embryos injected with a *lefty1* morpholino. Together, our data demonstrates that Bmp signaling and Nodal signaling are required synergistically for the induction of l*efty1* in the posterior midline (summarized in figure 5).

spaw is necessary for L/R patterning for a defined period throughout somitogenesis

Spaw regulates the laterality of multiple organs. The dynamic expression pattern in a posterior-anterior fashion combined with the position of the different organs in the LPM could indicate that Spaw regulates the laterality of different organs sequentially. We have determined, by using the nodal inhibitor SB431542, that Spaw in zebrafish is required for correct L/R patterning of the heart and visceral organs concomitantly from the 2-somite stage until the 15-somite stage at the latest. SB431542 inhibits signaling of nodal-related genes, and does not affect Bmp signaling (Inman, 2002). Although there are three nodal/related genes known in zebrafish, the effect of this inhibitor can be solely attributed to *spaw*. This because *squint* is not expressed beyond gastrulation and *cyclops* is described to be involved in the laterality of the brain and not in patterning the laterality of the heart and visceral organs. The addition of the inhibitor earlier in development, just after mid-blastula transition resulted in perturbed development of many structures, like somites, notochord, blood, hatching gland (Hagos and Dougan, 2007). This phenotype reflects the inhibition of *cyclops* and *squint* signaling, and none of these phenotypes were observed when the inhibitor was added after 90% epiboly.

Manipulation of Nodal signaling during gastrulation was previously demonstrated to influence the number of DFCs and the formation of DFC clusters (Choi, 2007; Oteiza, 2008). Previous reports showed that *lrdr1* expression in the dorsal forerunner cells (DFCs) that form the KV is necessary for functional fluid flow in the KV and is thereby required for normal L/R development. *lrdr1* is already expressed in the DFCs at 90% epiboly, when we first add SB431542 to inhibit nodal signaling (Essner, 2005). We hypothesize that addition of SB431542 at 90% epiboly or later stages does not interfere with the initial formation of the KV and *lrdr1* expression, but analyzing morphology and *lrdr1* expression after addition of SB431542 in future experiments is needed to validate this. While the effect on the cardiac jogging, subsequent cardiac looping and visceral looping was very severe in 100% of the treated embryos when nodal signaling was inhibited during early somitogenesis, this was accompanied by a dramatic reduction of spaw expression in the LPM, instead of a complete absence. This suggests that under normal conditions a certain threshold of left-side specific spaw expression is necessary for asymmetric expression of downstream genes and directing the cardiac jog to the left side. Perturbed Nodal signaling has been reported previously to compromise cardiac rotation and jogging in zebrafish (chapter2 this thesis)(Long, 2003; Smith, 2008) and affected cardiac looping laterality in various model organisms (chapter2 this thesis)(Brennan, 2002; Saijoh, 2003; Yamamoto, 2003). Spaw expression reaches the cardiac field surroundings approximately at the

22 somites stage and was proposed to regulate asymmetric *bmp4* expression, which could function as a chemoattractant for the cardiomyocytes (Chocron, 2007; Smith, 2008). Strikingly, our results showed that inhibition of Nodal signaling at the 15 somites stage or later does not affect cardiac laterality, suggesting the propagation of *spaw* expression anteriorly after the 15 somites stage is not necessary for cardiac laterality. These results suggest Spaw determines cardiac laterality prior to the 15 somite stage. They are in agreement with previous reports showing that KV disruption by microsurgery at the 3-7 somites stage resulted in abnormal pitx2 expression in the LPM and perturbed heart laterality, whereas KV disruption at the 10-15 somites stage rendered *pitx2* expression and heart laterality unaffected. This indicated roughly that the KV is necessary for L/R patterning around the 5 somites stage, but dispensable around the 12 somites stage (Essner, 2005). Temporary Spaw inhibition by SB431542 treatment until the 6 somite stage or the 10 somite stage resulted in a partial recovery of the expression of *spaw* and *lefty1* as well as the cardiac laterality. This suggests a continuous function of the KV, creating nodal flow and transferring L/R information to the LPM.

Based on our results that inhibition of nodal signaling beyond the 15-somite stage did not affect organ laterality, we divide the time frame of Spaw in two distinct phases. Between the 3-somite stage and the 12-somite stage, Spaw is expressed around the node and is required for setting up L/R patterning by transferring Spaw to the LPM. Between 12 and 15 somites Spaw regulates the induction of Pitx2. Downstream target Pitx2, responsible for transferring the L/R information to organ primordia, is first detectable at the 13-somite stage and its expression is lost after *spaw* knock down (Long, 2003). In addition, the expression pattern of *pitx2* is similar to *spaw* expression, propagating from posterior to anterior, but with a lag time of 2 somites (Wang and Yost, 2008). Interestingly, our data now suggests that after both *spaw* and *pitx2* are induced in the left LPM at the 15-somite stage, Nodal signaling is dispensable for organ laterality. In mouse, *Pitx2* expression was reported to be directly induced by Nodal, but maintained by Nkx2 (Shiratori, 2001). This explains how *Pitx2* expression persists long after Nodal signaling is gone. In zebrafish, although the regulation of Pitx2 has not been studied in depth, the propagation of *pitx2* expression in the LPM was proposed under the control of *spaw* propagation through the LPM (Wang and Yost, 2008). Our data now indicate that in zebrafish, like in mouse, *pitx2* expression is induced in the LPM by Spaw, but is maintained by another process. Whether this maintenance of *pitx2* in zebrafish is also regulated by one of the isoforms of *nkx2,* or by another transcription factor, needs to be resolved.

In conclusion, our results indicate that organ laterality is determined during early somitogenesis, driven by the action of the KV, long before actual asymmetric tissue morphogenesis occurs. Spaw is necessary to transfer the L/R information from the KV to the LPM. In the LPM other genes, like *pitx2*, are required to maintain the L/R identity of the LPM. The role of Spaw during later stages of somitogenesis has always been masked by the requirement of Spaw during early stages of somitogenesis;

perturbed Nodal signaling during all stages in all cases resulted in affected organ laterality (chapter2 this thesis) (Brennan, 2002; Saijoh, 2003; Yamamoto, 2003). This study provides fine tuning on the role of Spaw in determining organ laterality. Piece by piece, the mechanism of establishing, maintaining and interpreting asymmetric information becomes unraveled and using every model organism to its advantage will ultimately answer most, if not all, questions.

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Supplementary table 1

Spaw expression		**n**	**Normal**	**Weak**	**Absent**	**Bilateral**
5 somites						
hs:*noggin3*	sibling	24	83%	17%	-	-
	transgenic	14	93%	7%	-	-
10 somites						
hs:*noggin3*	sibling	18	100%	-	-	-
	transgenic	22	100%	-	-	-
15 somites						
hs:*noggin3*	sibling	16	100%	-	-	-
	transgenic	14	-	-	-	100%
Spaw expression		**n**	**Normal**	**Weak**	**Absent**	**Bilateral**
5 somites						
hs:*bmp2b*	sibling	24	71%	21%	8%	-
	transgenic	20	5%	35%	60%	-
10 somites						
hs:*bmp2b*	sibling	28	93%	-	7%	-
	transgenic	20	20%	30%	50%	-
15 somites						
hs:*bmp2b*	sibling	14	93%	-	-	7%
	transgenic	19	5%	-	95%	-
Lefty1 expression		**n**	**Normal**	**Weak**	**Absent**	**Overexpressed**
5 somites						
hs:*noggin3*	sibling	24	67%	25%	8%	-
	transgenic	24	4%	13%	83%	-
10 somites						
hs:*noggin3*	sibling	42	45%	29%	26%	-
	transgenic	40		47%	53%	-
15 somites						
hs:*noggin3*	sibling	41	76%		24%	-
	transgenic	23	4%	30%	66%	-
Lefty1 expression		**n**	**Normal**	**Weak**	**Absent**	**Overexpressed**
5 somites						
hs:*bmp2b*	sibling	22	100%	-	-	-
	transgenic	18	-	-	100%	-
10 somites						
hs:*bmp2b*	sibling	31	32%	26%	42%	-
	transgenic	17	-	6%	6%	88%
15 somites						
hs:*bmp2b*	sibling	48	73%	10%	17%	-
	transgenic	42	-	19%	-	81%

Chapter 5

Wnt/beta-catenin acts upstream of Bmp signaling during atrioventricular canal formation in the zebrafish heart

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Submitted

ABSTRACT

During development specific regions of the primary heart tube initiate a chamber-myocardium specific program of gene expression, which includes the expression of chamber differentiation markers like atrial natriuretic factor (Nppa) and proliferation factors like N-myc. Experiments in mice identified a specific region of the myocardium that is prevented from chamber differentiation by T-box factor Tbx2, and forms the atrioventricular canal. Here we show that in zebrafish a conserved Bmp-Tbx2 pathway controls myocardial differentiation. In the atrioventricular canal region *bmp4* and *tbx2b* are highly expressed. Using genetic mutants and transgenic lines, we provide evidence that Bmp signaling and Tbx2b are required in a linear pathway to prevent differentiation into chamber myocardium in this region. Using zebrafish with a mutation in the adenomatous polyposis coli protein, resulting in activated Wnt/β-catenin signaling, we demonstrate that Wnt/β-catenin signaling is sufficient to inhibit differentiation of chamber myocardium. Using inducible *tg(hsp70:dnTcf3)* and *tg(hsp70:axin)* lines to block Wnt/β-catenin signaling we demonstrate that Wnt/β-catenin signaling is also required to prevent myocardial differentiation in the atrioventricular canal region. Furthermore, genetic rescue experiments in adenomatous polyposis coli protein mutant embryos demonstrate that Wnt/β-catenin signaling activates and requires the Bmp-Tbx2 pathway to inhibit differentiation of chamber myocardium. Altogether, these data demonstrate that we have identified Wnt/β-catenin signaling as a novel factor upstream of the Bmp-Tbx2 pathway to control atrioventricular canal specification and to block myocardial chamber differentiation.

INTRODUCTION

During heart development, positional information along the linear heart tube is crucial to guide its compartmentalization into functional chambers. Compared to the primary myocardium of the linear heart tube, chamber myocardium acquires a more differentiated state that is adapted for a lifetime of mechanical work (Moorman and Christoffels, 2003; Stennard, 2005). The formation of chamber myocardium requires the localized initiation of a transcriptional differentiation program, leading to the initiation of the expression of several specific genes, like Nppa (ANF), Smpx (Chisel), N-myc and the gap-junction genes connexin 40 (Gja5) and connexin 43 (Gja1) (Delorme, 1997; Christoffels, 2000). The myocardium of the inflow tract (IFT), atrioventricular canal (AVC), the inner curvature and the outflow tract (OFT) retains the embryonic phenotype, which stands for low proliferation and no differentiation (Christoffels, 2004). Within the AVC, endocardial cushions give rise to cardiac valves, after the endocardial cells undergo epithelial to mesenchymal transformation (EMT), delaminate and invade the extracellular matrix (Armstrong and Bischoff, 2004; Beis, 2005). A large portion of congenital cardiac malformations arise from an incorrect development of the atrioventricular canal region (Hoffman and Kaplan, 2002). Hence, further insights regarding the mechanisms behind the regulation of the site-specific differentiation programs are essential. T-box containing transcription factors play a critical role in controlling diverse embryonic developmental processes as e.g. cell-type specification, tissue patterning and morphogenesis. A Tbx gene regulatory network has been extensively described that functions to regionally pattern the myocardium(Chapman, 1998; Russ, 2000; Naiche, 2007). Of the 18 T-box factor genes identified in mammals, at least 6 (Tbx1/2/3/5/18/20) are expressed in the developing heart (Plageman, 2005; Hoogaars, 2007). Haplo-insufficiencies for several human T-box genes have been linked to congenital anomalies including DiGeorge syndrome, Holt-Oram syndrome, ulnar-mammary syndrome and recently also septal defects (Basson, 1997; Baldini, 2004; Stennard, 2005; Meneghini, 2006; Kirk, 2007). Tbx20 has been shown to act as a transcriptional repressor during the split of the myocardium into chamber and non-chamber fates (Stennard, 2005). In zebrafish, morpholino knockdown of Tbx20 produces small, dysmorphic hearts showing upregulation of *tbx5* (Szeto, 2002), and in mouse Tbx20 null embryos cardiac development is also grossly abnormal (Cai, 2005; Singh, 2005; Stennard, 2005; Takeuchi, 2005). Tbx2 is expressed in non-chamber myocardium, most predominantly in the AVC, where it has been proposed to repress formation of chamber myocardium (Habets, 2002; Christoffels, 2004; Harrelson, 2004). Tbx2 has been shown to act as a transcriptional repressor, together with Nkx2.5, directly repressing promoter activity of the chamber-specific gene Nppa (Habets, 2002). Mice with a Tbx2 loss of function display expansion of different chamber-specific genes into the AV myocardium. Tbx5 directly activates the Nppa promoter through the same T-box binding sites, both alone and synergistically with other transcription factors. These findings indicate that activating and repressing T-box containing

transcription factors compete in the regulation of target genes e.g. Nppa (Habets, 2002). In mouse Tbx20-deficient embryos, Tbx2 was strikingly upregulated and ectopically expressed throughout the entire heart, indicating that Tbx20, directly or indirectly, represses Tbx2 and plays a major role in its regional expression (Cai, 2005; Singh, 2005; Stennard, 2005). The fact that Tbx2 and Tbx20 are co-expressed in the AVC myocardium raises the question whether a specific mechanism allows Tbx2 to escape repression by Tbx20 in the AVC. The signal to counteract the repressive activity of Tbx20 in the AV myocardium has been proposed to be Bmp2 (Yamada, 2000; Takeuchi, 2005), although the precise mechanism of this counteraction awaits further investigation. Different studies in mouse have led to conflicting evaluations of the regulatory relationship between Tbx20, Bmp and Tbx2. Tbx20 mutant hearts demonstrated expanded Tbx2 expression throughout the myocardium despite diminished *Bmp2* expression, suggesting that Tbx2 regulation is independent of Bmp2 (Cai, 2005; Stennard, 2005). However, another Tbx20 null allele displayed ectopic *Bmp2* expression together with an expanded *Tbx2* expression (Singh, 2005). Besides regulating patterning within the AV myocardium, Bmp2 has been identified as a crucial signal for AV cushion formation, by promoting Has2 expression and regulating EMT (Sugi, 2004; Takeuchi, 2005). However, nothing is known about factors regulating Bmp2 within the AV canal myocardium, creating an interesting topic for investigation. Wnt signaling can be divided into canonical signaling (via β-catenin) and alternative non-canonical signaling pathways (not β-catenin mediated). In the canonical Wnt signaling pathway adenomatous polyposis coli protein (APC) is an essential component of the axin-containing destruction complex that phosphorylates β-catenin and targets it for ubiquination and degradation by the proteasome. In the presence of Wnt ligand, β-catenin is stabilized and accumulates in the nucleus where it can bind and activate Tcf transcription factors (Fodde R, 2001). Non-canonical pathways are mediated through G-proteins and different kinases (Eisenberg, 2007). Both Wnt signaling pathways have been implicated to play an important role in the formation of cardiac tissue (Brade, 2006; Eisenberg, 2006). Initial evidence from studies with Drosophila indicated that the formation of the dorsal vessel is dependent on the expression of the canonical Wnt1 ortholog wingless (Park, 1996). Over the years functional analysis in chicken and *Xenopus* embryos indicated an inhibitory role of the canonical Wnt/β-catenin pathway during myocardial specification (Marvin, 2001; Schneider, 2001). Recent reports suggest that there might be a biphasic role for Wnt/β-catenin signaling; an early induction of cardiac specification followed by a phase of inhibition (Ueno, 2007). Moreover, Wnt/β-catenin signaling can also promote cardiogenesis by inducing proliferation of cardiac progenitor cells in the secondary heart field (Ai D, 2007; Kwon, 2007; Ueno, 2007). Although Wnts are also expressed in the myocardium at later stages of heart development only little is known about their function or the mechanism by which canonical Wnt signals regulate specific aspects of cardiogenesis.

Continuing our previous studies on the regulation of differentiation by Bmp signaling, here we demonstrate that in zebrafish there is a conserved Bmp-Tbx2

signaling cascade that controls the differentiation of the myocardium into chamber versus AVC. Expression of Bmp4 in the zebrafish heart is confined to regions of no differentiation into chamber myocardium. In addition, our data provide evidence for Wnt/β-catenin as a novel factor acting upstream in this linear Bmp-Tbx pathway to prevent cardiac chamber differentiation and control AVC formation.

MATERIALS AND METHODS

Zebrafish strains and heat shock experiment

Zebrafish were raised under standard laboratory conditions at 28.5°C. Fish lines used: *laf*^{m110} (Mintzer, 2001), *cpt*^{m169} (Kramer, 2002), *apc*^{ca50} (Hurlstone, 2003), *tg(hsp70:dnTCF*^{GFP}*)* (Lewis, 2004) and *tg(hsp70l:axin1-YFP)* (see supplement). Heat shock was applied by transferring the embryos to water prewarmed to 39.5°C followed by incubation at 37°C for 1 hour.

Establishment of a heat-shock inducible Axin1YFP transgenic zebrafish line.

YFP was fused to the C-terminus of mouse *axin1*. The *axin1* open reading frame used lacks the RGS domain at the N-terminus (the first 355 amino acids) and represents a splice variant that lacks exon 9. The fusion protein was cloned downstream of a 1.5kb fragment of the zebrafish hsp70-4 promoter (Halloran, 2000) and upstream of the SV40 polyadenylation signal of the vector pCS2+. An I-SceI meganuclease restriction site was inserted 5' of the transgene. The sequence of the construct is available upon request. A transgenic zebrafish line carrying this transgene was established using plasmid injection into fertilized eggs together with I-SceI protein using standard procedures. The line has been designated *tg(hsp70l:axin1-YFP)* w35 by the ZFIN nomenclature committee.

RESULTS

Ectopic Wnt/β-catenin signaling affects pattering of the myocardium.

Many studies have shown the importance of Wnt/β-catenin signaling during cardiac specification and cardiac progenitor cell proliferation (Cohen, 2008). At later stages of mouse heart development, several Wnts are expressed in the developing myocardium. However, only little is known about their function at that time of embryo development. In the zebrafish heart, we found *wnt11r* to be expressed in the cardiac chambers and to be excluded from the AVC, while *wnt2bb* was expressed specifically in the AVC at 48 hours post fertilization (hpf) (fig. 1A,B). Although being expressed in the heart, Wnt2bb does not seem to have an essential function in the AVC since *prt/wnt2bb* mutant embryos do not have a cardiac defect ((Ober, 2006) and data not shown).

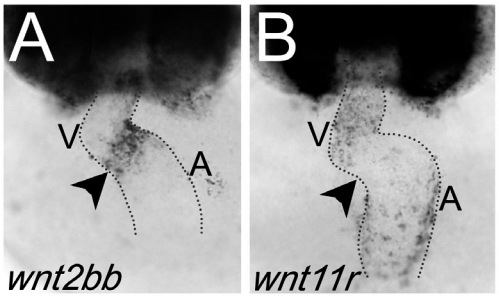

Figure 1 Expression of *wnt2bb* and *wnt11r* in the zebrafish heart. Expression pattern by whole mount in situ hybridisation of *wnt2bb* (A) and *wnt11r* (B) in wild-type embryos at 48 hpf. All pictures were taken as a ventral view. The arrowheads indicate the AVC and dashed line outlines the heart. V, ventricle; A, atrium.

To address the role of Wnt/β-catenin signaling in the myocardium we analyzed a previously identified zebrafish mutant deficient in the Adenomatous polyposis coli protein (Apc) (Hurlstone, 2003). In *apc* mutants Wnt/β-catenin signaling is ectopically activated in both the endocardium as well as the myocardium. While WT embryos at 48 hpf have a looped heart with a constriction at the AV boundary, the hearts in *apc* mutants failed to loop and the chambers are not well separated by an AV constriction (suppl. fig. 1). In addition we observed that the expression of *bmp4* (equivalent to Bmp2 in mouse and chick) and *tbx2b,* which are both normally expressed in the AVC myocardium and excluded from chamber myocardium (fig. 2A,H), are both expanded into the chamber myocardium in *apc* mutants (*bmp4*; 17 out of 20 (17/20) embryos) (*tbx2b*; 13/13 embryos) (fig. 2B,I). The ectopic expression of these AVC genes was most prominently observed in the ventricle myocardium. Conversely, we observed a very strong down regulation of the chamber specific *nppa* expression in *apc* mutants (30/31 embryos) (fig. 2P) compared to WT embryos (fig. 2O). The down regulation of *nppa* expression in the ventricle could already be observed at the linear heart tube stage when *nppa* expression is independent

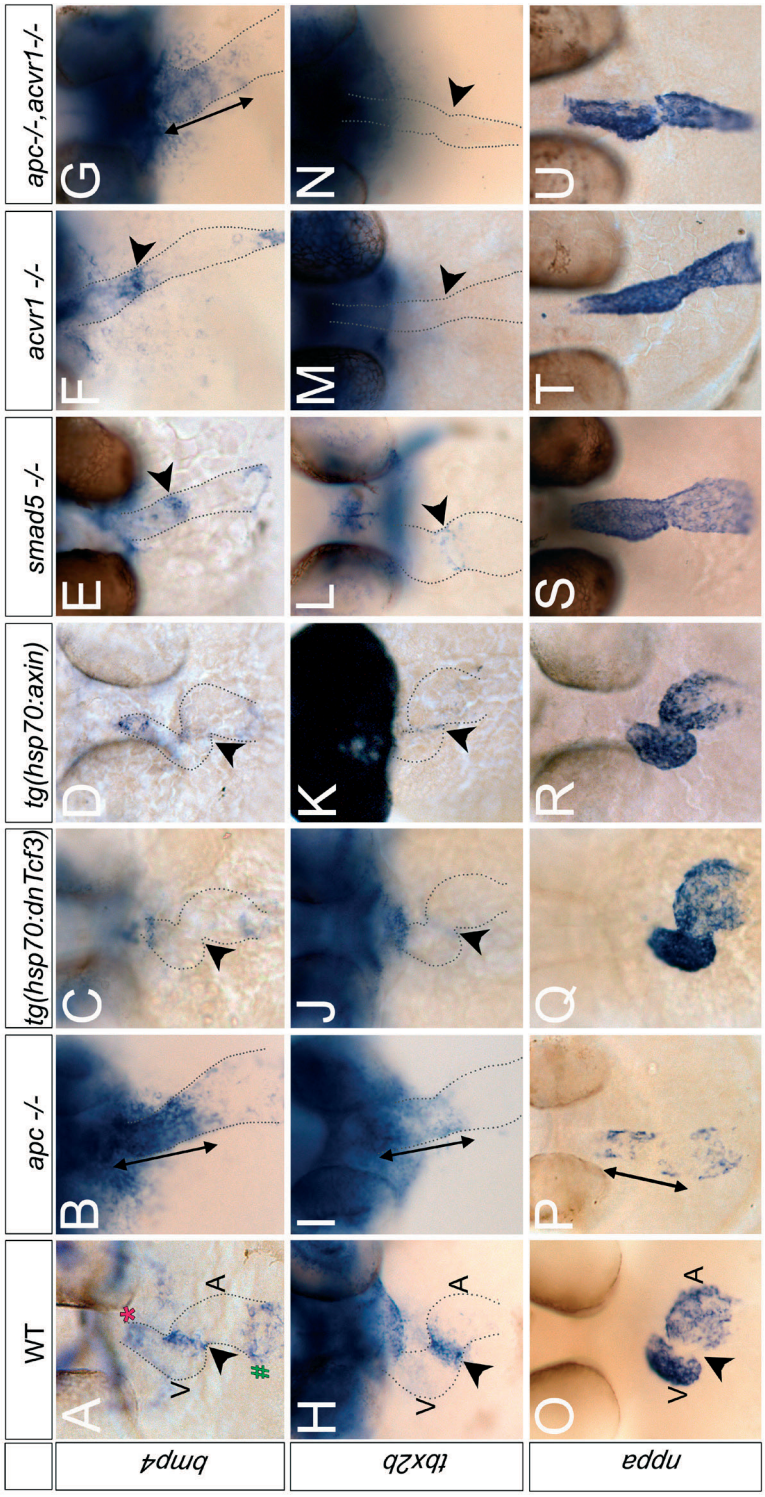

Figure 2 Wnt/β-catenin signaling is sufficient and required for *bmp4* and *tbx2b* expression in the AVC. In situ hybridization showing expression of *bmp4* (A-G), *tbx2b* (H-N) and *nppa* (O-U) in wt control (A,H,O), *apc* mutant (B,I,P), *tg(hsp70:dnTcf3)* (heat shocked at 48 hpf) (C,J,Q), *tg(hsp70:axin)* (heat shocked at 36 hpf) (D,K,R), *cpt/smad5* mutant (E,L,S), *laf/acvr1* mutant (F,M,T) and *apc;acvr1* double mutant (G,N,U) embryos. Embryos were fixed at 48-52 hpf and all pictures were taken as a ventral view. The arrowheads indicate the AVC and dashed line outlines the heart. V, ventricle; A, atrium. In panel (A) # (in green) indicates *bmp4* expression at the inflow and * (in red) indicates *bmp4* expression at the outflow pole of the heart tube.

from cardiac function (fig. 3A,B) (Auman, 2007). Other myocardial genes such as *tbx5* and *tbx20* that are known regulators of *nppa* and *tbx2b* respectively are still expressed in *apc* mutants (fig. 4A-D), suggesting no defects in differentiation of the myocardium. From these and previous results we can conclude that ectopic Wnt/β-catenin signaling in the heart not only results in the expansion of AVC endocardium (Hurlstone, 2003), but also results in the expansion of the AVC myocardium at the expense of chamber myocardium.

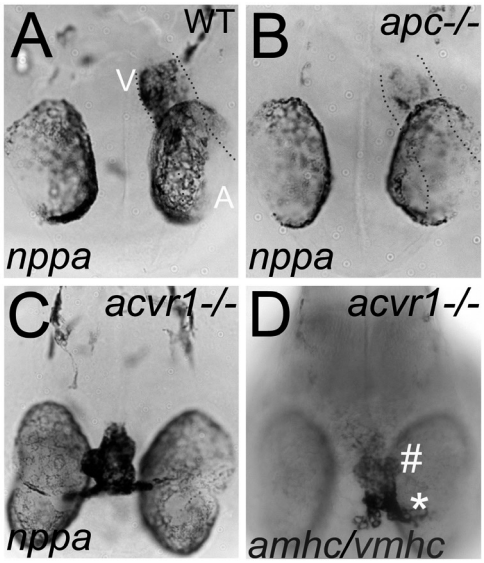

Figure 3 Altered *nppa* expression in *apc* and *acvr1* mutant embryos at the linear heart tube stage. Expression of *nppa* in wt (A), *apc* mutant (B) and *acvr1* mutant (C) embryos at 30 hpf. At this stage normal *nppa* expression is largely confined to the future ventricle and independent of heart function. Note the reduced *nppa* expression in the *apc* mutant embryo and ectopic *nppa* expression (in the future atrium) in the *acvr1* mutant embryo. (D) Double *in situ hybridization* using antisense probes for *atrial myosin heavy chain* (*amhc*, *) and *ventricle myosin heavy chain* (*vmhc*, #) to indicate that in the *acvr1* mutant *nppa* is ectopically expressed in the future atrium. Note that *amhc* and *vmhc* expression patterns are not specific for chamber myocardium since these genes are expressed long before chambers emerge and are also expressed in the AVC of wt embryos(Yelon, 1999). V, ventricle; A, atrium.

Endogenous Wnt/β-catenin signaling is required for AVC formation

Since in *apc* mutants Wnt/β-catenin signaling is ectopically activated, we next wanted to investigate the effect of repressing Wnt/β-catenin signaling during patterning of the myocardium. To do so we used *tg(hsp70:dnTcf)* embryos, in which a dominant negative Tcf3 (dnTcf3) is rapidly induced upon a 1 hour heat shock, resulting in the down regulation of Wnt/β-catenin target genes (Lewis, 2004). While gene expression was not affected in WT embryos upon heat shock, *tg(hsp70:dnTcf)* embryos heat shocked at 48 hpf and analyzed 4 hours later showed a loss of *bmp4* expression in the AVC myocardium (8/8 embryos)(fig. 2C). Interestingly, *bmp4*

expression was only down-regulated in the AVC, but hardly affected elsewhere in the embryo suggesting that dnTcf3 does not directly bind to the promoter of *bmp4* to repress its expression. Besides *bmp4* also *tbx2b* expression was down regulated in the AVC after induction of the dnTcf3 (9/11 embryos) (fig. 2J). Although we did not observe any visible defect in blood flow or heart contractility, *nppa* expression was upregulated and inappropriately expressed across the AVC (8/10 embryos)(fig. 2Q), while the expression of other T-box genes, like *tbx5* and *tbx20,* was not affected when dnTcf3 was induced (fig. 4E,F). To confirm that *bmp4* and *tbx2b* expression in the AVC is regulated by Wnt/β-catenin signaling we made a transgenic zebrafish line expressing Axin1, a β-catenin binding protein and a component of the β-catenin destruction complex, under control of the heat shock promoter *tg(hsp70l:axin1-YFP)*. Induction of the transgene in early embryos efficiently inhibits Wnt/β-catenin signaling (suppl. fig. 2). Overexpression of Axin1 resulted in a reduction of *bmp4* (8/12 embryos) and *tbx2b* (12/12 embryos) expression in the AVC and a concomitant expansion of *nppa* expression throughout the myocardium (8/10 embryos)(fig. 2D,K,R). This effect was most pronounced when ectopic Axin1 was induced at 36 hpf compared to Axin1 induction at 48 hpf (data not shown). Together, these results demonstrate that between 36 hfp and 48 hpf a Wnt/β-catenin signal regulates *bmp4* and *tbx2b* expression in the AVC.

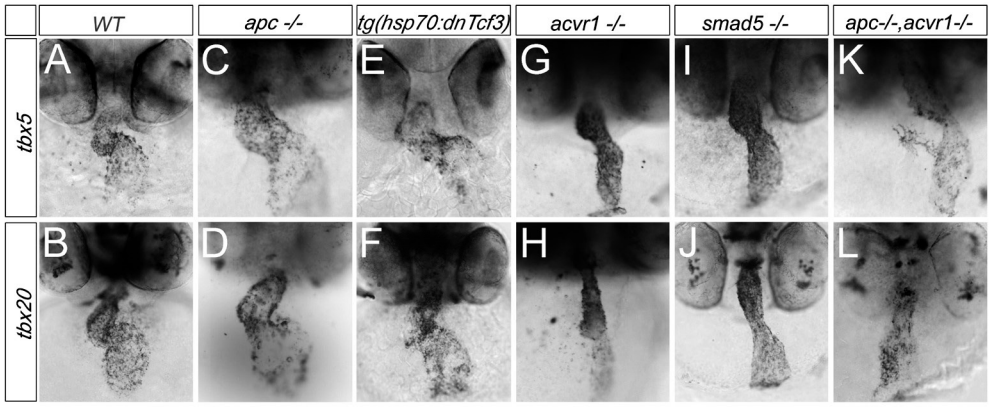

Figure 4 Aberrant Wnt and BMP signalling do not affect *tbx5* and *tbx20* expression. Whole mount in situ hybridisation for *tbx5* (A,C,E,G,I,K) and *tbx20* (B,D,F,H,J,L) in wild type (A,B), *apc* mutant (C,D), *tg(hsp70:dnTcf3)* heat shocked 4 hours prior fixation (E,F), *laf/acvr1* mutant (G,H), *cpt/smad5* mutant (I,J), and *apc;acvr1* double mutant (K,L) embryos. Embryos were fixed at 48-52 hpf and all pictures were taken as a ventral view.

Bmp signaling is required to mediate Wnt/β-catenin signaling in the AVC

Next we wanted to determine whether the patterning of the myocardium by Wnt/β-catenin signaling is dependent on the activation of Bmp signaling. Therefore we first determined whether loss of function mutants for Bmp signaling components have similar AVC defects as we observed after inhibiting Wnt/β-catenin signaling. In *lost-a-fin (laf)* mutants, Bmp signaling is reduced due to in inactivating mutation in

the Activin A receptor type I (*acvr1*, previously named *alk8*) (Bauer, 2001; Mintzer, 2001). The *captain-hook (cpt)* mutant allele *cpt*^{m169} has reduced Bmp signaling activity due to an inactivating mutation in the *smad5* gene (Kramer, 2002). Although *bmp4* expression was not affected in both *cpt/smad5* mutants (8/8 embryos) and *laf/acvr1* (13/15 embryos) (fig. 2E,F), we indeed observed a reduction of *tbx2b* expression in *cpt/smad5* mutants (3/4 embryos) and a complete loss of *tbx2b* expression in the *laf/acvr1* mutants (32/34 embryos) (fig. 2L,M). In addition, *tbx5* and *tbx20* expression was unaffected in the mutants (fig. 4G-J). These results are in agreement with earlier reports demonstrating a requirement for Bmp signaling in regulating *Tbx2* expression in the AVC of mouse embryos (Ma, 2005). As a consequence, in both the *laf/acvr1* and *cpt/smad5* mutants, *nppa* was no longer exclusively expressed within the developing chambers but showed an inappropriate expression in the AVC (in 52/54 *laf/acvr1* mutant embryos and in 30/32 *cpt/smad5* mutant embryos) (fig. 2S,T). The upregulation of *nppa* expression was already apparent at the linear heart tube stage, when *nppa* expression is independent from heart function (fig. 3C,D). We also observed an ectopic *nppa* expression in the AVC after morpholino knockdown of *bmp4* (data not shown).

Second, we wanted to determine whether Bmp signaling is required for the myocardial defects resulting from ectopic Wnt/β-catenin signaling. Therefore we generated double mutants for both *apc* and *acvr1* and analyzed expression of AVC and chamber specific genes. In *apc/acvr1* double mutants, *bmp4* was still ectopically expressed (8/8 embryos), similar to what we had observed in the *apc* single mutant embryos (fig. 2B,G). Intriguingly, while *tbx2b* was ectopically expressed in the *apc* mutants, *tbx2b* expression was completely absent (15/16 embryos) from *apc/acvr1* double mutants (fig. 2I,N). As a result, *nppa* expression was either restored (9/17 embryos) or even ectopically expressed (8/17 embryos) in the AVC of *apc/acvr1* double mutants (fig. 2P,U). Other T-box genes that also affect *nppa* expression were still expressed in *apc/acvr1* double mutants (fig. 4K,L). All together, our data demonstrate that the observed myocardial patterning defects due to the activation of the Wnt/β-catenin pathway in *apc* mutants are mediated through activation of the Bmp signaling pathway.

DISCUSSION

Wnt/β-catenin signaling has various functions during the earlier cardiac specification process ((Cohen, 2008) and references therein). Although also the myocardium of mouse and zebrafish embryos expresses several Wnts and components of the canonical Wnt signaling pathway, their role in regulating the specification of AVC versus chamber myocardium has remained elusive thus far.
AVC formation depends on the proper interaction between the myocardium and endocardium in which various signaling pathways are involved. Tbx2 is a central regulator in this process by repressing and thereby preventing Nppa expression in the AVC (Chi, 2008; Christoffels, 2004; Habets, 2002; Harrelson, 2004; Yamada, 2000). Bmp signaling regulates Tbx2 expression in the mouse AVC and our data demonstrate a similar relation in zebrafish. Recently, FoxN4 has been identified as an additional factor that may also regulate Tbx2 expression in the AVC (Chi, 2008). In addition, Notch signaling and the hairy and enhancer of split (Hes) family of genes can repress Tbx2 expression in the chambers to restrict its expression (Rutenberg, 2006; Kokubo, 2007). Our data introduce an extra layer of gene regulation and support a model in which Wnt/β-catenin signaling acts upstream of the conserved Bmp-Tbx2b pathway during the formation of AVC versus chamber myocardium. Wnt/β-catenin signaling may either directly regulate Bmp2/4 expression in the AVC region or alternatively, could do so more indirect by affecting Notch or other signaling pathways.

Conservation of BMP-Tbx2 pathway

We observed a conserved role for the Bmp-Tbx2 pathway in the myocardium to repress chamber differentiation in the AVC. The initial broad expression of Bmp4 in the linear heart tube has also been observed in the Xenopus heart (Breckenridge, 2001). In the chick, Bmp2 is the most prominent Bmp ligand to be expressed at the linear heart stage and this expression remains present in the myocardium of the AVC and IFT during looping stages (Yamada, 2000). In mouse embryos, Bmp2 is expressed predominantly in the AVC and is not present in the OFT or IFT where Bmp4 is expressed (Christoffels, 2004). Therefore, we hypothesize that in zebrafish *bmp4* expression in the heart represents the combined expression of both Bmp2 and Bmp4 as seen in mouse and chick hearts. Acvr1 (Alk2) and Bmpr1a (Alk3) are the major type I receptors that can transduce signaling of the Bmp2 and Bmp4 ligands. Indeed, conditional inactivation in mouse embryos of Acvr1 or Bmpr1a in endocardial cells results in specific AV valve defects suggesting non-redundant functions (Takeuchi, 2005; Wang, 2005). The role of Acvr1 and Bmpr1a in myocardial patterning had not been studied so far. A zebrafish Acvr1 receptor has been identified (Bauer, 2001), however its function during specification of the AVC region remains to be studied. Here we show that the inactivation of the zebrafish Acvr1 in all tissues results in the loss of myocardial AVC identity (fig. 2). Our results demonstrate that in the zebrafish heart Acvr1 is an important type I receptor required for patterning of the AVC myo-

cardium and the underlying endocardial cushions. The involvement of Bmpr1a (chapter 2) remains to be determined.

Wnt/β-catenin signaling and myocardial differentiation

Besides the role of the endocardium in regulating *bmp4* expression, we observed that Wnt/β-catenin signaling is required and sufficient to promote *bmp4* expression in the AVC region. Similarly, in colon cancer cells that express an oncogenic activated β-catenin, *Bmp4* expression is highly upregulated (Kim, 2002). Wnt/β-catenin signaling has various functions during the earlier cardiac specification process (Brade, 2006) and references therein). Early activation of Wnt/β-catenin signaling in zebrafish embryos (before gastrulation) or in ES cells results in an increase of cardiac specification, possibly by mesoderm induction. Activation of Wnt/β-catenin at later stages (during gastrulation in zebrafish embryos) results in a repression of cardiac specification (Ueno, 2007). In the secondary heart field, containing undifferentiated cardiac progenitor cells, Wnt/β-catenin can promote proliferation and thereby growth (Ai, 2007; Kwon, 2007) possibly by activating FGF signaling (Cohen, 2007). So far, to our knowledge, no function has been attributed to Wnt/β-catenin signaling in the myocardium of the developing vertebrate heart. Our data suggest that Wnt/β- catenin signaling is required to prevent cardiomyocytes to initiate a chamber specific differentiation program and thereby keeping cardiomyocytes in the AVC region in an undifferentiated state. Wnts and components of the canonical Wnt signaling pathway are expressed in the embryonic heart (Ai, 2007; Brade, 2006; Cohen, 2007). Here we showed zebrafish *wnt2b* expression in the AVC region (Fig. 1A). Injection of a *wnt2b* morpholino, which affects liver specification (Ober, 2006), however, had no effect on *bmp4* and *tbx2* expression in the AVC (M.V. and J.B. unpublished observation) suggesting multiple Wnts to be involved in their regulation. In addition, we showed expression of *wnt11-related (wnt11r)*, a non-canonical Wnt, specifically in the differentiating chamber myocardium (Fig. 1B). Wnt11 promotes cardiogenesis and can also repress canonical Wnt signaling (reviewed in (Brade, 2006), suggestive for a model in which non-canonical Wnt signaling promotes myocardial differentiation and inhibits canonical Wnt signaling whereas canonical Wnt signaling prevents cardiac differentiation in the primitive heart tube by activating Bmp signaling.

To summarize, we have shown that in zebrafish a conserved Bmp-Tbx2 pathway prevents differentiation of the primitive myocardium into more mature chamber myocardium. In addition we have identified Wnt/β-catenin signaling as a novel component acting upstream in a linear Wnt/β-catenin-Bmp-Tbx2 pathway to control myocardial differentiation. Studying the role of Wnt/β-catenin signaling in AVC specification in other organisms will be important to translate these results obtained in zebrafish to mammalian AVC specification and congenital heart disease.

ACKNOWLEDGEMENTS

We are very grateful to D. Zivkovic for providing the *apc*^{ca50} fish, S. Chocron and J. Korving for technical assistance, D. Yelon, R. Ho, E. V. Korzh, E. Weinberg, E. Ober, D. Kimmelman and D. Zivkovic for sending plasmids and members of the Bakkers laboratory for stimulating discussions. Work in J.B.'s laboratory was supported by the Royal Dutch Academy of Arts and Sciences, by EU FP6 grant LSHM-CT-2005-018833, EUGeneHeart, by the Netherlands Science Organization (ALW Grant #814.02.007) and the Human Frontier Science Program (Research Grant RGP9/2003).

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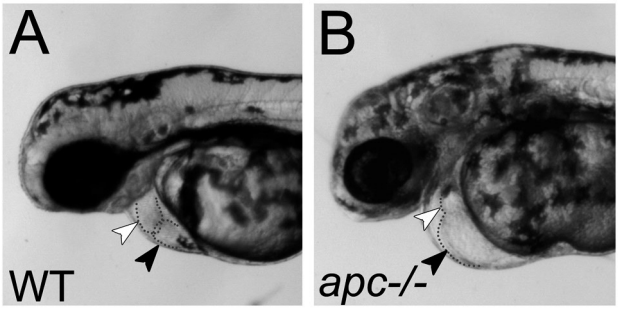
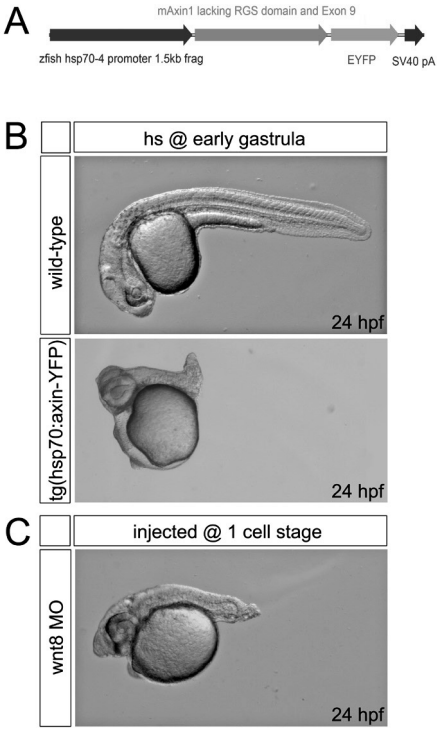


Supplementary Figure 1 Altered heart morphology in apc mutant embryos. Live images of a wt (A) and *apc* mutant (B) embryos at 48 hpf. While in a wt embryo the ventricle (orange arrowhead) is well separated from the atrium (black arrowhead), this separation is less apparent in the *apc* mutant embryo. Also notable are a dilated atrium and an unlooped morphology of the *apc* mutant heart.



Supplementary Figure 2 Transgenic zebrafish carrying a heat shock inducible mouse Axin1 as a tool to inhibit Wnt/β-catenin signaling. (A) Map of the transgene used. (B) Overexpression of Axin1YFP after heatshock in *hs:axin1YFP* transgenic embryos specifically inhibits Wnt/β-catenin signaling. Heterozygous carriers of *hs:axin1YFP* transgenes were outcrossed to wild-type fish, the progeny heat shocked at the developmental stages indicated and sorted into wild-type and transgenic embryos 2 hours after heat shock by fluorescence. Heat shocked wild-type siblings and transgenic embryos were photographed at the times indicated. (C) Loss of Wnt8 as achieved by injection of morpholino inhibiting translation of *wnt8* RNA. Note that overexpression of *axin1YFP* at early gastrula stages produces very similar phenotypes as the effects caused by loss of Wnt8 namely enlargement of anterior neural structures, most prominently eyes, and deletions of posterior trunk and tail.

Chapter

6

General Discussion

Bone morphogenetic protein (Bmp) signaling is vitally important in many aspects of cardiac development. These include cardiac induction and differentiation and establishing the L/R axis. In this thesis, we focus on the role of Bmp signaling in securing proper cardiac asymmetry, by (1) establishing correct L/R information that affects all organs, (2) directing asymmetric morphogenesis of the heart tube and (3) patterning the AVC myocardium to guarantee correct endocardial cushion formation. We demonstrated that Bmp signaling is required for determination of laterality by regulating asymmetric Nodal signaling (Spaw in the zebrafish) (chapter 3 and 4). This left side-specific *spaw* expression is a fundamental feature of L/R asymmetry and we found that Bmp signaling controls Spaw in two ways. Perinodal Spaw signaling is required for *spaw* expression in the LPM and Bmp signaling limits *spaw* expression, restricted around the node, where it is within reach of the nodal flow. We also show that Bmp signaling secures *spaw* expression specifically on the left side of the LPM, by repressing *spaw* expression of the right side, most likely by the induction of Lefty1 in the embryonic midline. Furthermore, chapter 3 appoints an additional role for Bmp signaling during later segmentation specifically in cardiac laterality, regulating cardiac jogging. We performed forward genetics screens to identify novel genes involved in the regulation of cardiac laterality specifically (described in chapter 2). Besides recovering known players in the TGFβ superfamily, we have identified a potential new gene that will provide new evidence on the interpretation of L/R information at the level the heart specifically. Moreover, in chapter 5 we provide evidence for a conserved mechanism in zebrafish in which Bmp signaling acts upstream of Tbx2b in maintaining and restricting the undifferentiated state within the AVC, crucial for correct valve formation. We also show that Wnt signaling acts upstream of Bmp signaling, leading to model of a linear pathway of Wnt-Bmp-Tbx2 in proper AVC differentiation. Altogether, governing correct Bmp signaling proves to be fundamental in constructing a functional heart.

BMP SIGNALING IS INVOLVED IN MULTIPLE ASPECTS OF CARDIAC DEVELOPMENT

Bmps can regulate a wide range of biological processes such as cell proliferation, apoptosis, differentiation and morphogenesis. During development, Bmps are critically involved in the differentiation and formation of nearly all organs and tissues, including the heart, nervous system, somites, lungs, kidneys, teeth, gonads and the gut, as well as establishing the basic body plan, including dorsal-ventral determination of the germ layers and L/R patterning (Hogan, 1996). We will focus here on the function of Bmp signaling in cardiac development.

1. Cardiac induction and cardiac differentiation

During gastrulation, cardiac progenitor cells are located in a domain where cardiac gene expression is inhibited by active canonical Wnt signaling. As development progresses, the progenitors migrate anterolaterally, where canonical Wnt signaling

is inhibited by both Dkk and Frzb2, relieving the block on the initiation of the cardiac gene program. This creates the opportunity for Bmp signaling, expressed in the overlaying endoderm and ectoderm, to induce the cardiac gene program (Schultheiss, 1997). Consequently, although Bmps are expressed along the entire anterior-posterior axis, cardiogenesis is restricted to the anterior-lateral region where Bmps and Wnt inhibitors overlap.

Several lines of evidence show that Bmps are indispensable for cardiomyocytes differentiation and induce this differentiation through two cardiac transcription factors, Nkx2-5 and Gata-4. *Bmp2* is expressed in the promyocardium and adjacent mesodermal cells. *Bmp2* deficient mouse embryos display abnormal cardiac development in the exocoelomic cavity (Zhang and Bradley, 1996), and zebrafish *bmp2b* (*swirl*) mutants completely lack heart precursors (Kishimoto, 1997). Ectopic expression of Bmp2 in the anterior mesoderm of chick embryos induces differentiation of non-precardiac mesodermal cells into beating cardiomyocytes (Schultheiss, 1997). The role of Bmp signaling in cardiac induction as well as differentiation determines the approach of studies focusing on the role of Bmp signaling at later stages of development. Using for instance conditional mouse knockouts or chimeras, chemicals that can manipulate pathways at certain time points or inducible zebrafish heat shock transgenic lines can circumvent the early effects on cardiac development allowing analysis at later stages.

2. Setting up embryonic L/R patterning

After correct cardiac induction and differentiation of precursors into cardiomyocytes, two bilateral heart regions are formed, that subsequently fuse to form one. This premature heart structure is then subjected to proper L/R patterning for correct positioning and morphogenesis. L/R asymmetry is setup in the posterior nodal region, around the Kupffer's vesicle (KV) in zebrafish. Directional nodal flow is hypothesized to create an asymmetric distribution of several key players, potentially including Nodal, (Spaw in zebrafish) (Hamada, 2002). The transfer of Spaw from the region surrounding the KV to the lateral plate mesoderm (LPM) is a crucial event for delivering the L/R information to the internal organs positioned in the LPM. The role of Bmp signaling in this latter event has been a much debated issue over the last years, resulting in opposing theories, implying positive as well as a negative regulation of Nodal signaling. Studies in both chick and mouse embryos demonstrated Bmp signaling required for inducing Nodal on the left side. Bmp signaling was found necessary for induction of Cfc/Cryptic, a positive co-factor for Nodal signaling (Fujiwara, 2002; Schlange, 2002), and exogenous applied Noggin to cultured mouse and chick embryos resulted in the absence of *Nodal* expression in the LPM, while exogenous Bmp positively affected *Nodal* expression in the LPM (Fujiwara, 2002; Piedra and Ros, 2002; Schlange, 2002). However, other studies have reported a negative regulation of Nodal expression by Bmp signaling (Chocron, 2007; Furtado, 2008; Mine, 2008; Rodriguez Esteban, 1999; Yokouchi, 1999; Zhu, 1999).

Two related genes, Cerberus and Caronte, were both found to activate left-side specific *Nodal* expression in chick by antagonizing the repressive activity of Bmp signaling on Nodal (Rodriguez Esteban, 1999; Yokouchi, 1999; Zhu, 1999). Recently, however, the role of Cerberus as a Bmp antagonist was challenged when Cerberus was reported as a Bmp agonist and together, Bmps and Cerberus induced Nodal expression (Yu, 2008). This discrepancy could be solved by additional molecular studies on the activation or repression of downstream targets of Bmp signaling. Now, Cerberus is defined as an antagonist or agonist of Bmp signaling, respectively, based on its ability to bind Bmp proteins and its receptors as well as downstream effect on Nodal (Yu, 2008; Zhu, 1999). Phosphorylation of Bmp specific receptor Smad proteins (Smad1,5 and 8) would be a valid readout for activation or repression of Bmp signaling.

In this thesis, we provide additional data in the zebrafish that supports a repressive function of Bmp signaling on Nodal expression and we propose that Bmp exerts the inhibition of Nodal signaling on the right side by inducing Lefty1 in the midline (chapter4). Lefty1 effectively inhibits Nodal, even at long distances, to secure left-side specific Nodal signaling.

A recent study reported that there might also be a direct effect for Bmp signaling on the repression of Nodal expression in the LPM. Bmp signaling via Smad1 was proposed to set a critical threshold for Nodal-dependent Nodal activation in the LPM (Furtado, 2008). Cells of the left and right LPM have to discriminate between subtle differences in morphogen levels created by the nodal flow and convert this into a robust all-or- nothing output (Nakamura, 2006). The threshold model requires that Bmp signaling represses Nodal bilaterally, not just on the right side. The threshold was set in part by limiting the availability of the common TGFβ/Nodal/Bmp pathway effector Smad4 (Furtado, 2008).

The regulation of asymmetric Nodal by Bmp signaling is beyond all doubt very complex and instead of being either a Nodal inducing factor in the left LPM or a Nodal repressing factor in the right LPM, both models might be accurate. Bmp signaling exerting opposing functions during L/R patterning reflects tissue- and/or temporal specific regulatory mechanisms. Responding cells could possibly express different receptors, co-factors and/or modulators in time. Additional genetic studies are required to unravel this complex mechanism in more detail.

Besides from the much debated role of Bmp signaling in regulating left-side specific Nodal signaling, we report in this thesis also a role for Bmp signaling in repressing Nodal signaling (Spaw) around the node (KV). We hypothesize that this is necessary for restricting Spaw around the KV, and a Bmp antagonist will be required in or around the KV to relieve the Bmp repression for proper *spaw* expression. This data was supported by results obtained in the mouse, where both Bmp antagonists Noggin and Chordin are required for proper *Nodal* expression around the node (Mine, 2008). Furthermore, extra-embryonic Bmp signaling was also reported necessary for normal formation of the node (Fujiwara, 2002). Obviously, the involvement of Bmp signaling at different levels of L/R patterning significantly complicates the

analysis its precise function. Dissecting the mechanism will require more subtle genetic studies, focusing on inducible systems and avoiding general manipulations throughout the whole embryo.

3. Directing cardiac jogging

The heart is the first organ to display asymmetric morphogenesis and has been studied extensively over the years in various model organisms. Correct cardiac looping is a prerequisite for correct positioning of the different chamber and vessels with respect to each other and proper development of the valves and septa (Ramsdell, 2005). The initial break in the symmetric heart morphogenesis occurs prior to cardiac looping, at least in zebrafish, and involves a leftward displacement of the linear heart cone (cardiac jogging), which is simultaneously extending into a tube. This movement in the anterior-lateral direction is accompanied by a series of complex movements of cardiomyocytes. The posterior cardiomyocytes were reported to migrate at higher speed than their anterior counterparts and as a result of that the heart cone rotates clockwise (Smith, 2008). Concomitantly, an asymmetric involution of the right-posterior heart field synergistically with the rotation converts the initial left-right polarity of the heart into dorsal-ventral polarity, creating torsion on the developing heart tube (Rohr, 2008; Smith, 2008). Although it is unclear if these morphogenetic processes can be extrapolated to higher vertebrates, in mouse a similar leftward displacement of the linear heart tube was observed prior to the looping process, but never studied in detail (Biben and Harvey, 1997).

The acceleration of the posterior cells in the cardiac cone, while migrating to the anterior-left side of the embryo, suggests that these cells move towards a chemoattractant. The rotation of the heart cone was dependent on active Bmp signaling and when endogenous *bmp4* was blocked, Bmp4 was shown sufficient to redirect the linear heart tube to the opposite side when ectopically introduced on the right side in the LPM surrounding the cardiac field (Smith, 2008). Bmp4 has been reported previously to possess the capacity to induce attractive chemotaxis and thereby promote condensation of the mesenchymal cells along the ureter epithelium (Miyazaki, 2003). Bmp2 has been reported to induce similar chemotaxis in human monocytes (Cunningham, 1992), vascular aortic smooth muscle cells (Willette, 1999), and corneal fibroblasts (Kim, 1999). Furthermore, during gastrulation Bmp signals guide the migration of lateral cells by regulation of cell-cell adhesion (von der Hardt, 2007). Asymmetric *bmp4* expression was reported in the left side of the cardiac cone at the 22-somite stage, persisted throughout the jogging process and the sidedness of *bmp4* expression in the tube was correlated to the direction of the cardiac jog (Chen, 1997). We reported an asymmetric expression of Bmp4 in the left LPM surrounding the cardiac field more than in the right LPM, which was dependent on Nodal signaling (Chocron, 2007). However, both groups have found the asymmetric *bmp4* expression at the level of the heart extremely difficult to analyze (chapter 3, JB and JC, personal communication), most likely due to its very dynamic expression pattern. In addition to the asymmetric bmp4 expression, also

an asymmetric activation of Smad1,5,8 proteins in the left LPM surrounding the heart was observed (Smith, 2008). This supports the model that Bmp signaling regulates asymmetric morphogenesis of the heart and thereby determines cardiac laterality. We show in chapter 3 that for proper cardiac jogging a Bmp signal is required up to the 22 somite stage. Our results based on the *bmpr1a* mutant and the *acvr1/alk8* mutant (chapter 2 and 3, respectively) showed that Bmp signaling regulates cardiac laterality specifically and thus independent of other organs. The largely unaffected laterality of the visceral organs most likely reflects the effect of maternally supplied mRNA, and indicates that Bmp signaling is required for correct laterality of other organs during early stages of development. Together, our data demonstrated that cardiac laterality can be regulated independently in time from visceral organ laterality although both require Bmp signaling for appropriate L/R regulation.

4. Patterning AVC myocardium and regulating endocardial cushion formation

The correct differentiation of the individual chambers and the atrioventricular canal (AVC) is vitally important for proper septation and valve formation. In the AVC, the myocardium produces extracellular matrix and induces the overlaying endocardium to loosen from the epithelial connection and transform into mesenchymal cells. These mesenchymal cells migrate into the developing cushion and extracellular matrix. The cushions subsequently fuse and contribute to the developing valves (Armstrong and Bischoff, 2004; Beis, 2005). We describe in chapter 5 that Bmp4 is required to induce the transcription factor Tbx2b in the AVC, which represses the chamber differentiation factor Nppa/Anf and keeping the AVC in an undifferentiated state. We observed a lack of endocardial cushion tissue when Bmp signaling was perturbed in *avcr1* mutants (chapter5). Besides patterning of the AV myocardium, Bmp signaling is also required for the induction of epithelial-to-mesenchymal transition (EMT) and formation of cardiac jelly, by direct signaling to the AV endocardium (Ma, 2005; Rivera-Feliciano and Tabin, 2006). In the primitive heart tube, *bmp4* expression was initially observed throughout the myocardium to become restricted only later during looping stages to regions of undifferentiated myocardium (AVC, IFT, OFT). Our findings demonstrated that Wnt/β-catenin signaling regulates the restricted expression of *bmp4* in the AV myocardium. Furthermore we demonstrated that the regulation of AV myocardium patterning by Wnt/β-catenin signaling was dependent on BMP signaling activity. We thereby identified Wnt/β-catenin signaling as a novel component acting upstream in a linear Wnt/β-catenin-BMP-Tbx2 pathway to control myocardial differentiation. Active Wnt signaling was detected in both AVC myocardium and endocardial cushions (Hurlstone, 2003), and further experiments are required to determine the exact Wnt signal responsible for the restriction of Bmp4 and whether it originates from myo- or endocardium.

There is a high incidence of valvuloseptal defects associated with laterality disease (Ramsdell, 2005). If L/R patterning in the heart is disrupted, cushions may be misaligned, leading to valves that do not close properly. However, although we

observed both perturbed cushion formation and cardiac looping in mutants which compromise Bmp signaling, our *hu119* mutant displays normal *has2* expression in the AV endocardium. This suggests normal cushion formation, while cardiac looping is severe affected in these mutants. Further experiments will be necessary to determine whether *hu119* mutants develop functional valves that close properly.
Overall, we have shown a conserved mechanism in which Bmp signaling regulates both the proper differentiation of the AVC and endocardial cushion formation as well as the L/R patterning in general and heart-specifically, securing impeccable valve formation from multiple angles.

THE CLINICAL SIGNIFICANCE OF ORGAN LATERALITY

Proper morphogenesis and positioning of internal organs requires delivery and interpretation of precise signals along the anterior-posterior, dorsal-ventral, and left-right axes. The complex morphogenesis of the heart and its connections to the vasculature are particularly dependent upon coordinated L/R signaling pathways. Cardiac looping is required for rearranging regions of the heart tube so they acquire appropriate positioning for proper formation and alignment of chambers, valves and septa. Also, the two atria in human derive from a common atrium that needs to be divided into two chambers with distinct L/R features. Furthermore, blood vessels that connect with the inflow and outflow of the heart regress unilaterally, requiring L/R information (Ramsdell, 2005). Therefore, the heart is especially susceptible to any defects in L/R determination and as a result, the significant mortality and morbidity in individuals afflicted by an abnormal situs almost always are attributed to complex, congenital heart defects.
Many genes involved in the determination of the L/R axis were initially identified by their asymmetric expression pattern in model organisms such as mouse, chick, zebrafish and *Xenopus.* As described throughout this thesis, members of the TGFβ superfamily, including Nodal signaling and Bmp signaling genes, play a central role in the specification of the L/R axis. Phenotypes like heterotaxia and isomerism, where the L/R axis is neither normal nor mirror-image reversed, can lead to life-threatening situations, underscoring the necessity to identify genes regulating L/R patterning at an organ-specific level. Relatively little is known about how organ primordia specifically respond to L/R positional information to subsequently generate anatomical asymmetry. Our validated way of screening specifically for cardiac laterality will provide new evidence for this in the near future. Candidate genes based on our studies in zebrafish and those of others in mice and chick could potentially be used for screening human families with a history of cardiac malformations. Prenatal diagnosis of various cardiac defects could result in better clinical status before surgery and improved postoperative outcome compared to those individuals diagnosed postnatally (Skinner, 2008).

ZEBRAFISH IS A VALUABLE MODEL SYSTEM TO DISCOVER NEW GENES INVOLVED IN (CARDIAC) LATERALITY

The chick has served as an important model system over the years to analyze mechanisms underlying the establishment and maintenance of the L/R axis, and mouse models function well to extrapolate data to the mammalian system. Zebrafish have been found extremely valuable for finding new genes involved in various developmental processes for over a decade now (Driever, 1996; Haffter and Nusslein-Volhard, 1996; Odenthal, 1996; Schier, 1996; Solnica-Krezel, 1996; Stemple, 1996; van Eeden, 1996). The strength of zebrafish as a model for cardiac laterality is based on the optical clarity combined with the possibility to perform large-scale mutant screens to identify novel genes (chapter4)(Chen, 2001; Chen, 1997). The maternal expression of many genes often allows analysis of the gene function later in development, and possibly in various different tissues. This is particularly important when studying the function of genes involved in various developmental processes, defining body axes as well functioning at the level of a specific organ, like Bmp proteins. Studying cardiac development is also facilitated in the fish, since the fish is not dependent on functional heart and circulation for survival during the first days of development. Therefore, critical genes, that lead to severe defects and perturb cardiac formation, function or circulation, can still be studied in zebrafish, while in mice this generally leads to premature death. Large numbers of progeny in zebrafish compared to the mouse make the fish much more suitable for forward genetics, but although the zebrafish genome project advances rapidly, this is still the major drawback in performing positional cloning of the responsible mutations. More knowledge and effort in reconstruction of areas of the genome that are not well annotated will overcome this handicap. Furthermore, the combination of studies in zebrafish, chick and mouse remains a necessity, using every model organism to its advantage, and enable data obtained in lower vertebrates to be extrapolated to the mammalian system.

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SAMENVATTING

Hoe een levend organisme ontstaat uit één enkele bevruchte eicel is één van de meest fascinerende processen in de biologie. Niet alleen moet deze cel zich vermenigvuldigen, maar om verschillende celtypes, weefsels en organen te vormen, moeten cellen zich ook specialiseren. Het is bijzonder te onderzoeken hoe deze cellen tijdens de embryonale ontwikkeling op het juiste moment en op de juiste plek weten wat ze moeten doen. Een kleine fout kan immers catastrofale gevolgen hebben en leiden tot verschillende aangeboren afwijkingen. De blauwdruk van alle processen die in een cel kunnen plaatsvinden, ligt opgeslagen in het erfelijke materiaal (DNA), dat is opgebouwd uit vier verschillende bouwstenen en zich opgerold in chromosomen in de kern bevindt. De volledige genetische code van de mens bestaat uit ongeveer 3 miljard bouwstenen, die naar schatting 21.500 genen coderen. Deze genen kunnen eiwitten maken, die belangrijk zijn voor de ontwikkeling en het behoud van alle processen in het lichaam. Veel van deze genen zijn ook aanwezig (geconserveerd) in lagere organismen, zoals de muis, de zebravis en de fruitvlieg. Dit laat zien dat deze genen door de evolutie heen een belangrijke en met de mens vergelijkbare rol zijn blijven vervullen. Een blijvend groeiende kennis over de genetische mechanismen die de ontwikkeling van een organisme sturen is van groot medisch belang.

Een van de grootste uitdagingen van dit moment is te bepalen welke genen een specifieke rol spelen tijdens bepaalde ontwikkelingsprocessen. Door selectief fouten (mutaties) te maken in genen van modelorganismen, zoals de muis en de zebravis, en te kijken naar de gevolgen hiervan, kunnen belangrijke inzichten worden verkregen over de functie van de coderende eiwitten. Dit kan op een gerichte manier, waarbij bekende genen worden uitgeschakeld (*reversed genetics*). Maar door willekeurig mutaties te maken in het genetisch materiaal met een materiaal dat DNA beschadigt, kunnen nieuwe genen worden ontdekt die betrokken zijn bij processen die hierdoor verstoord zijn geraakt (*forward genetics*). In het onderzoek beschreven in dit proefschrift heb ik deze laatste methode gebruikt in de zebravis om nieuwe genen te vinden die betrokken zijn bij de ontwikkeling van het hart. Zebravissen lijken qua genetische opmaak, organen en fysiologische processen erg veel op mensen, met het verschil dat dingen vaak een stuk eenvoudiger in elkaar zitten. Een menselijk hart bestaat namelijk uit twee boezems en twee kamers en de rechterhelft van ons hart pompt het zuurstofarme bloed naar onze longen, waarna het terugkomt in de linkerkant van het hart. Daarna pompt die linkerkant het zuurstofrijke bloed door de rest van het lichaam. Zebravissen hebben maar één boezem en één kamer en doordat de vis niet afhankelijk is van longen, is het onderzoeken van hartontwikkeling eenvoudiger dan in een zoogdier. Zebravissen hebben daarnaast nog het voordeel dat ze zich in grote getalen en extern (buiten de moeder) ontwikkelen, zodat de ontwikkeling van het embryo vanaf de eerste celdeling kan worden gevolgd. Zebravissen zijn ook transparant de eerste dagen van hun ontwikkeling, zodat de ontwikkeling van levende embryo's gevolgd kan

worden onder een microscoop. Het hart is een vitaal orgaan dat tijdens de ontwikkeling zeer complexe bewegingen ondergaat. In eerste instantie is het hart een rechte buis die door die bewegingen en de specialisatie van de cellen uiteindelijk een definitief orgaan vormt waarbij de verschillende onderdelen goed ten opzichte van elkaar staan en goed aansluiten op de grote vaten in ons lichaam. De ontwikkeling van ieder organisme begint symmetrisch, maar na verloop van tijd wordt ieder organisme verdeeld in een linker- en een rechterkant. Links komen dan andere genen tot expressie dan rechts, zodat cellen links zich anders gaan gedragen dan cellen aan de rechterkant. Deze asymmetrie is niet alleen van belang voor de juiste ontwikkeling van het hart, maar ook voor bijvoorbeeld onze longen, hersenen en ons hele maag-darmstelsel. Op deze manier past alles goed op elkaar en wordt er zo efficiënt mogelijk met de gegeven ruimte omgegaan. De periode van de ontwikkeling van het hart die in dit proefschrift wordt beschreven, loopt van ongeveer 20 uur tot 48 uur na bevruchting. Het hart begint als een platte schijf van cellen, die een buis vormt die onmiddellijk zowel naar voren als naar de linkerkant van het embryo beweegt. Deze beweging noemen we '*jogging*' en is de focus van dit onderzoek. De vraag is waarom de hartbuis (altijd) naar de linkerkant gaat en wat voor componenten deze beweging aansturen. Om nieuwe genen te vinden die betrokken zijn bij dit proces, hebben wij meerdere screens uitgevoerd. Daarbij gebruiken we, zoals eerder al vernoemd, vele vissen die willekeurig gemuteerd zijn in hun DNA. De nakomelingen van al die vissen hebben een grote variëteit aan defecten (mutanten), en het is zaak om uit die vele vissen juist die te vinden met het defect waarin jij bent geïnteresseerd. Voor ons waren dat vissen met het hart in het midden in plaats van aan de linkerkant. In **hoofdstuk 2** beschrijven wij de mutanten die we gevonden hebben met behulp van screens. In eerste instantie hebben we mutanten levend gescreend voor de positie van het hart. Vervolgens worden mutanten geselecteerd die alleen een defect hebben in de positie van het hart en niet van andere organen. We beschrijven één mutant met een algemeen defect in de asymmetrie van meerdere organen en twee mutanten die specifiek zijn voor het hart. Van één van die twee hebben we het gen gevonden dat verantwoordelijk is voor het defect, *bmpr1a*. De ander zal hopelijk in de nabije toekomst ook geïdentificeerd worden, maar het vinden van een verantwoordelijk gen is afhankelijk van een aantal factoren. Het is belangrijk dat de volgorde van de code bekend is in het gebied waar het gen ligt, maar dat werk is nog in volle gang. Het is belangrijk aan te tonen dat het mogelijk is deze mutanten via een screen te vinden en het kaf van het koren te scheiden. De vroege signalen die zorgen voor de verschillen tussen links en rechts zijn de afgelopen jaren ook al in andere organismen onder de loep genomen en een belangrijke rol is gevonden voor componenten van de Bmp familie. Er zijn echter zowel overeenkomsten als ook veel tegenstrijdigheden gerapporteerd. Wij laten in **hoofdstuk 3** zien dat Bmp eiwitten twee verschillende functies hebben in het bewerkstelligen van orgaanasymmetrie. Vroeg in de ontwikkeling is het eiwit '*southpaw*' cruciaal voor links-rechts asymmetrie. Het komt specifiek aan de linkerkant tot expressie en reguleert vervolgens weer andere genen. Wij laten zien

dat Bmp eiwitten belangrijk zijn om de expressie van '*southpaw*' te onderdrukken aan de rechterkant van het embryo. Zo verzekeren Bmps dat er verschil ontstaat tussen links en rechts. Later in de ontwikkeling zien we een tweede functie voor Bmp eiwitten. Die komen dan aan de linkerkant van het embryo tot expressie en reguleren daar verschillende genen en beïnvloeden specifiek de asymmetrie van het hart. We laten zien dat Bmp4 op de juiste plaats tot expressie komt om deze functie te vervullen en we bevestigen dit door Bmp4 uit te schakelen. Dus Bmp4 heeft, in ieder geval in de zebravis, zowel een onderdrukkende functie aan de rechterkant als een stimulerende functie aan de linkerkant voor links-rechts aymmetrie. We hebben deze eerste functie van Bmp4 in meer detail uitgewerkt in **hoofdstuk 4**. De verschillen tussen links en rechts worden in eerste instantie bepaald in een rond orgaan in de staartregio van het embryo, dat is vernoemd naar een knoop ('*node*'). Het is cruciaal dat die informatie correct door wordt gegeven naar de rest van het embryo om zo alle organen te bereiken en beïnvloeden. We hebben de relatie tussen Bmp4, Southpaw en een derde eiwit '*Lefty1*' onderzocht. Lefty1 wordt aangezet door Southpaw en onderdrukt vervolgens de nieuwe aanmaak van meer Southpaw (negatieve feedback). We laten zien dat Bmp4 verantwoordelijk is voor de positieve regulatie van zowel Southpaw als Lefty1. Dit leidt tot een model waarin Bmp4 Southpaw onderdrukt aan de rechterkant door het aanzetten van Lefty1. Al met al blijken Bmp eiwitten een zeer centrale rol te spelen in de bepaling van links-rechts asymmetrie, zowel algemeen als hart-specifiek. Bmp4 heeft daarnaast ook nog een andere rol in de ontwikkeling van het hart. Deze rol beschrijven we in **hoofdstuk 5**. Later in de ontwikkeling, als het hart al verschillende veranderingen heeft ondergaan, gaan de cellen zich differentiëren. Ze krijgen daardoor een specifieke functie die ze nodig hebben om een functioneel orgaan te vormen. Het hart wordt onderverdeeld in een kamer (atrium) en een boezem (ventrikel) en de cellen in het hart veranderen van structuur zodat het atrium en het ventrikel uitzetten. De regio tussen die twee delen van het hart wordt het atrioventriculair kanaal genoemd, waar later in de ontwikkeling hartkleppen worden gevormd om te voorkomen dat bloed terugstroomt in de verkeerde richting. Bmp4 komt in eerste instantie in het hele hart tot expressie, maar later voornameljk in het atrioventriculaire kanaal. Daar reguleert het de transcriptiefactor Tbx2b, die ervoor zorgt dat de cellen in het kanaal zich niet differentiëren. Dit is een belangrijke voorwaarde voor het vormen de kleppen. Wij laten zien dat deze regulatie geconserveerd is in de zebravis en beschrijven de rol van Wnt signaalcomponenten in het aansturen van Bmp4. Wnt eiwitten zijn namelijk verantwoordelijk voor de specifieke expressie van Bmp4 in het hart. Dit hebben we aangetoond met behulp van overexpressie of het uitschakelen van Wnt signalering.

De zebravis is genetisch voor 70-80% vergelijkbaar met de mens, waardoor genetische studies in de zebravis vaak vertaald kunnen worden naar aandoeningen bij de mens. De afgelopen jaren is het gebruik van de zebravis als modelorganisme voor het bestuderen van genetische processen tijdens de ontwikkeling significant gestegen en worden er in vele laboratoria grootschalige screens opgezet om te

achterhalen welke genen, op welke manier, betrokken zijn tijdens de ontwikkeling van een embryo. De resultaten beschreven in dit proefschrift dragen bij aan de kennis over de asymmetrische ontwikkeling van organen en specifiek van het hart. Op de lange termijn kan dit de diagnostiek van aangeboren hartafwijkingen ten goede komen, en mogelijk levensbedreigende situaties vroegtijdig detecteren.

DANKWOORD

Woohoo! Klaar! Mijn eindstreep is in zicht, maar eerst... op naar de bedankjes. Volledig volgens mijn eigen traditie schrijf ik dit gedeelte weer vlak voor de deadline, maar dat maakt het niet minder gemeend. Hier wordt gezegd wat gezegd moet worden. Dit boekje is namelijk niet alleen mijn eigen verdienste. Ik heb veel hulp gekregen uit verwachte en onverwachte hoeken. Zonder de hulp, expertise, inzichten, adviezen, geduld en peptalks van velen, voor en achter de schermen, was dit boekje misschien wel nooit een feit geworden.

Als allereerste natuurlijk Jeroen. Als bijna gepromoveerde ontwikkelingsbiologe is er natuurlijk niets mooiers dan naast jezelf je eigen baas te zien ontwikkelen over de jaren heen. In het begin stond je nog regelmatig op het lab, nu is delegeren je tweede natuur geworden. Lunchen met de groep heeft plaatsgemaakt voor een boterham achter je bureau. We zaten opeengestapeld met z'n 7en maar uiteindelijk, al was het maar voor eventjes, had je toch het hele domein voor jou alleen. Ik heb ontzettend veel van je geleerd deze jaren. Met veel geduld heb jij geprobeerd me het 'glas halfvol' principe eigen te maken, waardoor de wereld van de wetenschap voor mij nu minder zwart-wit is met meer grijstinten. Het project ging lang niet altijd de kant op die we allebei hadden gehoopt, maar je altijd positieve instelling tijdens onze besprekingen gaf mijn motivatie regelmatig de nodige boost. Bedankt voor al je wijsheden en heel veel succes voor de toekomst!

Sonja, van rots in de branding tot steun en toeverlaat, van labqueen tot mama, dank je voor je grenzeloze inzet. Ik zou eigenlijk beter kunnen zeggen: dank je wel voor alles en dat zou dan waarschijnlijk nog niet voldoen. Zonder jou was dit boekje er waarschijnlijk niet eens geweest. Ik ben ook blij dat ik met iemand het oog voor detail kon delen (Le-lijk!) en ik zal onze gespreken over babystuff, familieperikelen en opbloeiende ondernemingen missen. Paranimfen ging helaas niet meer, maar die kleine gaat voor, dat snap ik natuurlijk wel. Maak er maar een mooi mensje van! Emma, je luisterend oor en af en toe en een 'je kan 't' hebben me goed gedaan tijdens het schrijven van dit boekje. Zelf ben je bijna klaar en inmiddels is het vraagteken eraf, dus dat gaat ook helemaal goed komen. Die 'Goeiemorgens' waren even wennen, maar ik trek ze inmiddels prima. Dank je wel voor je confocal-expertise en je zelfgebakken muffins-on-request. Kelly, you should consider a career in teaching, you have a gift for explaining things. Thanks for the discussions as well as your help on the time-lapses. I wish you a beautiful career back home. Ruben, de herder dagen zijn voorbij, en dit schaapje is toch op het droge gekomen. We konden soms vechten als broer en zus, maar gelukkig wist jij al na twee jaar hoe mijn boekje eruit moest komen te zien. Dank je voor de peptalks! Anne, als er iemand onvoorwaardelijk hard werkt, dan ben jij het wel. Success met de rest van je PhD, dat moet goed komen, zo. Vele studenten hebben over de jaren heen natuurlijk ook het lab kleur gegeven. Van Karel, Linda, Merel, Evelien en Metamia (wat een duo!) tot Ingrid, Olaf en Sebas, studenten brengen leven in de brouwerij en veel gezelligheid. Merlijn, je hebt tijdens je stage ontzettend hard gewerkt aan de screen. Je doorzettingsvermogen is flink getest, maar we hebben er toch maar mooi een aantal

mutanten aan over gehouden. Ik ben blij dat je zo goed terecht bent gekomen, ik hoop dat je Ben beter bij kunt houden dan mij. Kris, het zonnetje van het lab, ik heb genoten van je aanwezigheid. Dank je voor al je steun, vriendschap en goede gesprekken, op het lab maar ook daarbuiten, bij jou kan ik echt m´n ei kwijt. Jouw positiviteit doet wonderen als ik het even niet meer zie zitten. Dat plekje naast mij tijdens de promotie is meer dan verdiend. Federico, you just started, I hope you tackle that *hu119* mutant, let me know how it turns out. To all de 'Bakkertjes': I enjoyed my time in the lab, thanks for all the good times! Daarnaast is onze kamer het laatste half jaar goed gevuld met allemaal nieuwe mensen. Layla (Koffie? Ja, tuurlijk!), Tom, Anko, ik wens jullie een hele mooie tijd op het Hubrecht. Layla, special thanks voor het meeleven tijdens de laatste fase van het schrijven, succes met je eigen project. Jacco, altijd geïnteresseerd, je persoonlijke benadering als groepsleider is een kwaliteit om te koesteren. Misschien een beetje misplaatst, maar ik wil toch graag alle soon-to-be papa's en mama's (voor degenen die zich aangesproken voelen) veel geluk toewensen.

Verder wil ik iedereen binnen het Hubrecht bedanken voor alle gezelligheid tijdens alle borrels en activiteiten, maar ook gewoon op de gang voor een glimlach of een praatje. Ik heb het Hubrecht een ontzettend fijne plek gevonden om te werken. Ik noem de gezelligheid maar bewust niet bij naam, bang dat ik iemand onterecht vergeet in het zonnetje te zetten, maar vele collega's (present and past) hebben bijgedragen aan een ongelooflijke werksfeer. Dank jullie allemaal! Ellen & Appie, jullie gingen mij voor en ondanks dat jullie het zelf ook vaak druk hadden tijdens de afrondingsfase van jullie eigen promotie waren jullie altijd bereid me van allerlei nuttige tips te voorzien, ik heb er veel aan gehad. Robert, thanks voor alles! ;)
Natuurlijk wil ik ook graag alle ondersteunende diensten bedanken die het werken op het Hubrecht zo zorgeloos mogelijk maken. Janny ('ik regel alles wel voor je'), Jules en Richard (kwam graag bij jullie shoppen), Romke, Eric, Thea, Sophia (kun je niet ook thuis m'n administratie bij komen houden?), en achter de schermen natuurlijk ook de mensen van de mediakeuken, de schoonmakers met de vriendelijke glimlach en de dames van de catering (Mary, you rock!). Jos, Gerard, Arjan, Walter en Jaap stonden altijd voor me klaar, ik ben echt een muts met computers! Sequencing facility, bedankt voor alle service en flexibiliteit! Harry en Jeroen, altijd gezellig bij jullie op de histologie, thanks voor alle nieuwtjes, roddels, speculaties en natuurlijk jullie hulp bij m'n coupes. Daarnaast een heel bijzondere pluim voor alle dierverzorgers, ik heb veel respect voor het werk dat jullie doen. Bert, altijd bewonderenswaardig vrolijk (al kon je me er ook gillend gek mee maken), lange Mark, koning van het opgeruimde aquarium, 'still-not-done' Erma, moeder voor alle zebravis babies en hart-op-de-tong Rob. Bedankt Mark, het moet geen makkelijke taak zijn altijd tussen de wetenschappers en de dierverzorgers te bemiddelen. Om bij de vissen te blijven, ik ben Dana en Stefan (en hun groep) dankbaar voor het delen van verschillende lijnen en reagentia.
Een groot deel van mijn promotieonderzoek draaide om forward screens en dat is natuurlijk allemaal begonnen in het pitoreske Tübingen, op het Max Planck Instituut. Ik heb de screens af en toe wel vervloekt, vooral als ik niet kon vinden wat ik zocht, maar ik ben toch heel dankbaar om betrokken te zijn geweest bij dit project en voor mijn tijd in Duitsland. Special thanks go to Muriel, Emi, Tom&Amy, Mike, Sacha, Madeleine,

Nicola, Natascia, Brigitte and the animal care takers for making my stay in Tübingen unforgettable.

Aangezien het leven zeker niet alleen draait om werken, voel ik me bevoorrecht omringd te worden door geweldige vrienden en familie, mensen op wie ik kan bouwen en die me de afgelopen jaren ontzettend moreel gesteund hebben.
M'n meisjes sinds het eerste uur, samen toegetreden tot de wondere wereld van de medische biologie, jaren lang gestreden, soms gevallen, maar nooit ten onder gegaan and still going strong. Dames, bedankt voor alle afleiding (in alle vormen en maten) en adviezen, het is altijd gezellig met jullie! Jullie krijgen me altijd weer zover dat ik zelf ook ga geloven dat ik dit allemaal best wel kan. Maaik, soms lijkt het wel of onze levens parallel lopen, echt freaky, jij begrijpt me als geen ander. Ennuh, ik heb het al vaak genoeg gezegd, maar het is vast handig om het zwart-op-wit te hebben: een wereldreis is geweldig, maar je huis is daar waar ik ook ben :). Miek, door jouw nuchtere en verhelderende blik verdwijnen problemen soms als sneeuw voor de zon. Ik voel me vereerd dat jij naast me staat om me rustig te houden tijdens m'n promotie. Als jij nou ook even je dingetje afmaakt, en niet te ver van huis gaat, hebben we met z'n vijven weer meer tijd voor de goede dingen in het leven! Samen creatief wezen, ik zie een carrière voor ons in het pimpen van schorten! Wen, dank je voor al je goede adviezen, ik ben echt jaloers op je georganiseerde pdf-jes. 't Is altijd lachen met jou, maar zodra jij de slappe lach krijgt, kom ik echt niet meer bij! San, dank je voor je lieve telefoontjes, een goed gesprek met jou doet wonderen. We moeten binnenkort eens samen gaan koken, met de rest van de Hoef-clan! Voeg je vervolgens de meisjes samen met de mannen (Steef, Jelmer, Piet, Patrick, Willem, André en wat verdwaalde zielen die zich soms bij ons aansluiten) en het feest is compleet. Menig kroeg, festival en wintersportoord is nooit meer hetzelfde als wij er zijn geweest! Rare dansjes, mojito's, rare mojito-dansjes, Hammertime, stukken zwoerd, Piet 'wat-doen-we-nu?' Paniek, project 'help-Manon-de-berg-op', lege accu's, rode banken op de piste, weerwolfsessies waar je bang van wordt.... ik zal ze niet snel vergeten. Zambezi Drive: een hele goede afleiding! Keep up the good work! Jullie zijn onwijs gegroeid het afgelopen jaar, ik blijf één van jullie grootste fans! Jo-vàn-ka, we hebben er allemaal lang op moeten wachten, maar eindelijk hoor je er echt bij, gezellig! Ik heb heel veel gehad aan al je promotie-adviezen en Indesign tips, nu kan het 'echte' leven weer beginnen voor ons. Dank jullie allemaal voor alle gezelligheid en ik hoop nog lang van jullie allemaal te kunnen genieten.

Ook mijn collega's van het Smulhuis, bedankt voor jullie interesse en steun, maar ook voor het nodige perspectief! (Wat doe je ook alweer, iets met vissen, toch?) Het is heerlijk om met een goed team te buffelen in de keuken en te werken naar een mooi eindresultaat. Diensten zijn altijd gezellig met jullie en de gasten. Chris, Juud en Joost, jullie begrijpen zowaar ook waar ik het over heb, fijn dat ik met jullie mijn frustraties kon delen over dingen en mensen die me tegenwerkten de afgelopen tijd. Chris, bedankt voor al je wijze woorden, volgende keer gaan we twee weken smullen! Juud, dank je voor je motivatiespeeches en je vastberadenheid om voor mij de ideale baan te verzinnen. Echt jammer dat ik niet bij jouw promotie kan zijn, maar misschien zien we elkaar in het Verre Oosten. Joost, we gaan die spelavondjes weer helemaal oppakken, en no worries, Manga Manga is niet voor iedereen weggelegd (van Juud valt sowieso

bijna niet te winnen). Jonathan, Anneke, Sarah en Matthijs, ver weg, maar daardoor niet minder betrokken. Een dagje bij jullie voelt zo vertrouwd, ik hoop snel weer eens jullie kant op te komen. Ester, bedankt voor je workshop, het was precies dat duwtje in de rug wat ik nodig had.

Jan, Wil, Niels en Luciënda, een lievere schoonfamilie kan ik me niet wensen, bedankt voor jullie interesse! Frank en Lotti, grote broer en schoonzus, bedankt voor alle adviezen en tips op alle vlakken. Af en toe een kritische noot, op z'n tijd een hart onder de riem ('nog effe doorbuffelen!'), en altijd het beste met me voor. Juul en Luca, nog zo jong , jullie begrijpen van dit alles nu natuurlijk nog niks, maar het is een genot om jullie op te zien groeien. Luca, je krijgt er binnenkort nog een broertje of zusje (wat zou het toch worden?) bij, dus als jullie wat ouder zijn zal tante Manon wel eens een mooi verhaal vertellen over de zebravis die zijn hart aan de verkeerde kant had zitten.
Lieve papa en mama, jullie zijn in één woord geweldig! Waar zou ik zijn zonder m'n warme nest, jullie nemen me altijd van alles uit handen zodat ik me volledig kon focussen op m'n werk en als het nodig is, ga ik nog steeds lekker een dagje naar huis om me weer eens als vanouds te laten verwennen (rooie kool met gehaktballen en/of hachee, wat een krachtvoer!). Ondanks dat jullie, zoals jullie het zelf altijd zeggen: 'het wereldje niet kennen', kan ik altijd op jullie rekenen. Bedankt dat jullie me het zelfvertrouwen geven om keer op keer m'n eigen keuzes te maken (al vraag ik jullie toch altijd graag voor een second opinion). Ik wens jullie vele mooie jaren samen toe, in binnen- en buitenland.
En dan Maarten, het mocht geen zoetsappig bedankje worden, dus misschien dan gewoon kort. Mij iedere ochtend uit bed krijgen, mijn onchristelijke werktijden en mijn 'lack of discipline', je hebt er vast je handen vol aan gehad. Dank je voor al je geduld en je zorgzaamheid (ik kan nu wel weer wat taken van je overnemen :)). Er is niks beters dan na een lange dag hard werken thuiskomen bij jou. Nu eindelijk weer meer tijd voor leuke dingen en allebei op zoek naar een nieuwe baan, samen ons hart achterna! Wie weet waar we terecht komen, maar het komt vast goed!

Dit hoofdstuk is klaar, het boek is uit… Ik heb veel mensen ontmoet en/of beter leren kennen de afgelopen jaren en aangezien ik er verstand van heb, kan ik met recht zeggen: Jullie hebben allemaal het hart op de juiste plaats zitten.

Dank jullie wel,

Manon

CURRICULUM VITAE

Manon Verhoeven werd geboren op 11 december 1979 in Oss, waar zij in 1998 haar gymnasiumdiploma behaalde aan het Titus Brandsma Lyceum. In datzelfde jaar startte zij de opleiding Medische Biologie (later hernoemd tot Biomedische Wetenschappen) in Utrecht. Gedurende deze studie volgde zij drie wetenschappelijke stages. Tijdens haar eerste stage deed ze onderzoek op het Jordan laboratorium voor Haematologie onder begeleiding van Dr. Saskia Ebeling in het Universitair medisch Centrum (UMC) in Utrecht. Dit werd vervolgd door een tweede stage bij de vakgroep Fysiologische Chemie in het UMC, onder begeleiding van Prof. dr. Hans Bos en Dr. Jorrit Enserink. Voor een extracurriculaire derde stage is zij uitgeweken naar het Beth Israel Deaconess Medical Center/Harvard Medical School in Boston, waar zijn onder leiding van Prof. dr. Steven Balk werkte op het gebied van Endocrinologie. Het doctoraal diploma werd behaald in maart 2004 wat gevolgd werd door een periode van 4 maanden in Boston om het onderzoek voort te zetten van haar derde stage. In september 2004 startte zij dit promotieonderzoek in het Hubrecht Institute for Developmental Biology and Stem Cell Research onder begeleiding van Dr. Jeroen Bakkers. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

LIST OF PUBLICATIONS

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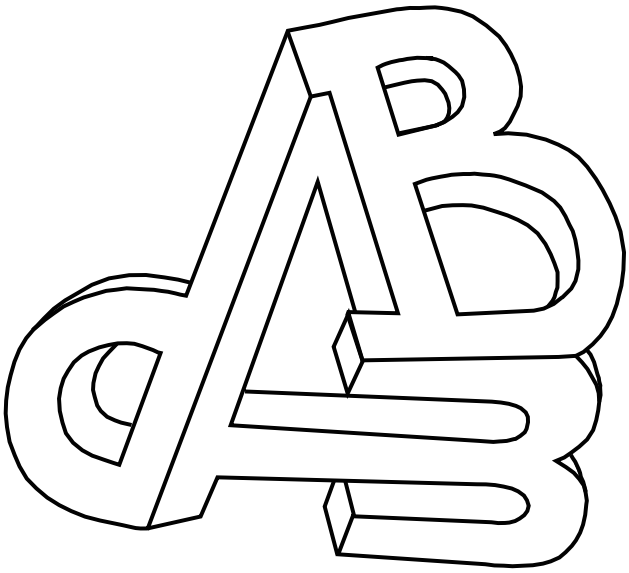

‘When science discovers the center of the universe
a lot of people will be disappointed to find they are not it.’
Bernart Bailey