Introduction to signaling during early embryonic development

Monica Bonetti and Jeroen Paardekooper Overman

1 Zebrafish gastrulation

The perpetuation of animal life across uncountable generations is dependent on the remarkable fact that from a single cell, a complex organism is formed. How is it possible that from a single cell, then a lump of cells, finally all these dividing cells know where to go and what to do to form a new viable generation? According to Lewis Wolpert, "it is not birth, marriage or death but gastrulation which is truly the most important time in your life". Indeed, gastrulation, the process of cellular arrangements (epiboly, internalization, convergence and extension) after an initial stage of cell division following fertilization is a complex event that sets the stage for further developmentin all vertebrates. Embryonic development is highly predictable and can be studied more easily in some animal models than others. Compared to mammals which develop *in utero*, fish embryos are staged and imaged easily during development [1].

Primary cell divisions

Fish development starts with a large yolk syncytium with the oocyte sitting at the animal pole. After fertilization, the zygote starts to divide and initially forms a large lump of cells on top of the yolk known as the blastoderm. The blastoderm consists of three cell layers: mesenchymal deep cells that will give rise to the embryo *proper*. The deep cells are surrounded by a protective layer of epithelial enveloping layer cells (EVL). The most vegetal cells of blastoderm fuse with the yolk to form a multi-nucleated structure known as the yolk syncytial layer (YSL). The EVL acts as a protective layer for the deep cells, which are tightly attached to it. Likewise, the YSL is attached to the EVL. Initially, messenger RNA is maternally provided, yet after 2.5 hours post fertilization (hpf), zygotic transcription is initiated and maternal mRNA is cleared by miR-430 at 4hpf [2].

Epiboly

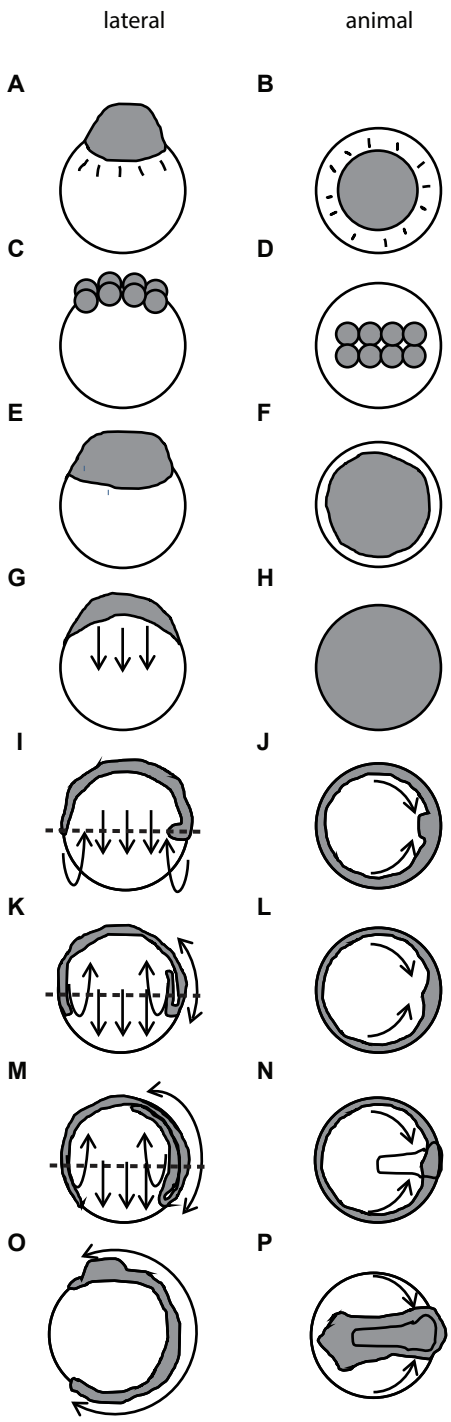
At sphere stage, or four hpf, a process called epiboly causes the deep cells to move over the yolk towards the vegetal pole, much like a knitted hat being pulled over your head (figure 1). First, intercalation of the deep cells to more superficial layers cause a thinning and spreading of the tissue. Not only cell migration but also changes in cell shape drive this process. These initial cell movements cause the embryo to shape itself from a 'sphere' towards the 'dome' stage. Further vegetal movement of the deep cells is caused by the pulling of the YSL and the attached EVL. This is partially mediated by microtubuli, which are attached to an actin meshwork at the vegetal pole of the yolk. Additionally, massive endocytosis in the YSL vegetal of the EVL attachment also mediates epiboly independent of microtubuli [3].

Involution

At about 50% epiboly, cells start to move inwardly in a process called internalization or emboly. This will form the germ ring. Notably, at the ventral and medial parts of the embryo this occurs via involution of sheets of cells, while at the dorsal region this occurs via the ingression of individual cells. These processes give rise to the different cell layers endoderm, mesoderm and ectoderm. The internalizing mesendodermal cells move inwardly towards the animal pole, forming the hypoblast. Meanwhile, the overlaying cells that do not move inwardly (epiblast) will form the ectoderm and continue epiboly.

Convergence and extension

At 6hpf, cells accumulate dorsally from the lateral and ventral regions of the embryo in a process called convergence. Meanwhile, lengthening of the embryonic axis occurs by cells intercalating and moving anteriorly in a process called extension. Together, these processes are termed



convergence and extension (C&E) which mediates the anterio-posterior lengthening and medio-lateral thickening of the embryo forming the longitudinal axis. Additionally, cells moving inwardly at the dorsal side of the embryo form a thickening called the shield, which functions in a similar way as the Spemann-Mangold organizer in other vertebrates like *Xenopus* and mouse, thus calling this stage the 'shield' stage.

Cells more lateral to the shield move slower than cells close to the shield. Several cellular movements can be distinguished in several areas of the embryo. From more ventral to dorsal these are: 1) vegetal pole migration in the ventral mesoderm, 2) slow dorsal convergence along irregular paths, 3) cell packing (fast and efficient convergence) in the lateral mesoderm, 4) medial planar intercalations and 5) polarized radial intercalations in the medial presomitic mesoderm. Most dorsally in the axial mesoderm, cells show 6) anterior-directed migration and 7) mediolateral intercalations [4].

Figure 1. Schematic representation of zebrafish development from the Zygote Period to the end of the Gastrulation Period.

Following the brief zygote period (a/b), when the embryo is at the single-cell stage, the cleavage period (c-d) runs from the two-cell to the 64-cell stage (i.e. 0.75–2.25 h post-fertilization (h.p.f). (h–p) The blastula period follows the cleavage period and runs from the 128-cell stage to the 50% epiboly stage (i.e. from 2.25 to 5.3 hpf). (e-f) Formation of the enveloping layer (EVL) and yolk syncytial layer (YSL). (g–p) The gastrula period then runs from the end of the blastula period at 50% epiboly (i.e. 5.3 hpf) through to the bud stage at 10 hpf, after which the segmentation period begins. Cell movements of convergence and extension (indicated with the arrows) start during the gastrulation period.

2 Signalling in gastrulation

Non canonical Wnt signalling

Convergence and extension cell movements are mediated by multiple signalling pathways that drive cell migration, adhesion and cell polarization. A principal pathway regulating C&E is the non-canonical Wnt/planar cell polarity (PCP) pathway [5]. The PCP pathway was first discovered in *Drosophila*, where mutants for components of the, then unknown, PCP pathway showed unorganized (unpolarized) wing epithelia. Later, disruption of the PCP pathway showed reduced body axis and neural tube defects in vertebrates [5]. In zebrafish, the core non-canonical Wnt/PCP pathway consists of extracellular Wnt glycoproteins, Wnt5 and Wnt11. Mutants for these Wnts (*pipetail* and *silberblick,* respectively) were identified in the late 90's [6,7] having defects in forebrain development and tail formation, respectively. The Wnts are able to bind the Frizzled 2/7 and the co-receptor glypican 4/Knypek a heparan sulphate proteoglycan [4]. Binding of Wnt to Frizzled causes intracellular recruitment of Dishevelled by its PDZ domain. Dishevelled in turn recruits Daam1, which causes RhoA, a small GTPase, activation. Additionally, Dishevelled activates the other small GTPases Rac1 and Cdc42. RhoA and Rac1 elicit activation of other downstream kinases, ROCKII and Jun kinase, respectively, which cause cytoskeletal rearrangements while Rac1 exerts its function on protein kinase C [4]. Other non-canonical Wnt pathways may regulate gastrulation as well. Ror2, the tyrosine kinase receptor for Wnt5, is able to modulate expression of paraxial protocadherin (PAPC) in *Xenopus* [8]. PAPC is an important regulator of cell adhesion and gastrulation [9]. Convergence and extension cell movements are also mediated through the Src family kinases Fyn and Yes, which are controlled upstream by Csk [10]. Interestingly, Fyn/Yes signalling acts parallel to non-canonical Wnt signalling, both of which act on RhoA downstream [11]. Additionally, maternally contributed canonical Wnt signalling activates Stat3 on the dorsal side of developing embryos [12]. Stat3 morphant embryos show C/E defects without affecting cell fate. More recently, *ofd1*, a gene associated with oral-facial-digital type 1 syndrome, which is required for ciliary motility and function was shown to interact genetically with components of the non-canonical Wnt pathway. Loss of *ofd1* results in shorter and wider embryos, indicating a role for cilia in C/E movements as well [13].

Cell-cell adhesion

Epiboly movements are severely impaired in zebrafish embryos of the E-cadherin mutant half baked. Cells deficient for E-cadherin are able to migrate, but are unable to undergo mesenchymal to epithelial transition necessary for proper intercalation movements during epiboly [14]. The cell adhesion molecule PAPC also regulates C/E by activation of RhoA and JNK [9]. Moreover, the trimeric G-proteins Gα12 and Gα13 mediate Rho activation, and mutants show C/E defects while their cells exhibit a rounder shape [3].

MAPK signalling

The Src family kinases Fyn and Yes not only control non-canonical Wnt signalling, but also control MAPK signalling. Mitogen activated protein kinase (MAPK) signalling is crucial for zebrafish development. For example, while partial knockdown of Erk1 causes convergence defects, partial loss of Erk2 results in extension defects, showing distinct functions of these kinases [15]. Notably, while more complete loss of Erk1 only mildly affects epiboly, Erk2 knockdown results in a developmental arrest at the onset of epiboly. Interestingly, loss of Erk1 is rescued by expression of Erk2, but not *vice versa*, indicating a prerequisite function of Erk2 during gastrulation [15].
Interestingly, also expression of activating forms of NRas, BRaf and Mek induce gastrulation

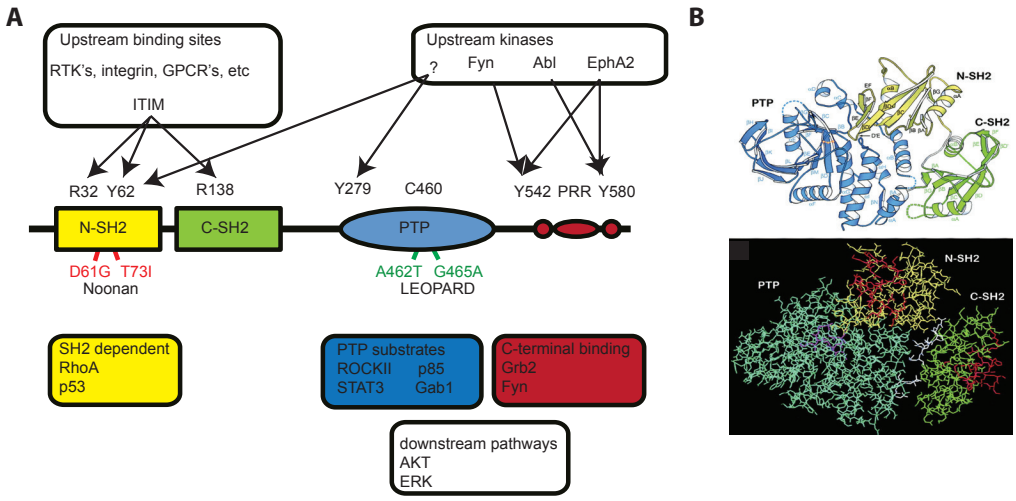


Figure 2. A Schematic representation of the Shp2, its binding sites and disease associated mutations.

A) Schematic representation of Shp2 protein, indicating the N terminal (N-SH2) and C-terminal (C-SH2) SH2 domains (yellow and green respectively), the catalytic protein-tyrosine phosphatase (PTP) domain (blue) and C-terminal tail (red). Many upstream kinases phosphorylate Shp2 on its tyrosines to regulate its activity, including the Src family kinase Fyn, Abl and EphA2. Upstream binding sites of receptors, including receptor tyrosine kinases (RTKs), Integrins and G-Protein Coupled Receptors (GPCRs), bind to the tandem SH2 domains of Shp2 when they are phosphorylated. Of particular note are Immunoreceptor Tyrosine Inhibitory Motifs (ITIMs) which are tyrosine motifs that are aptly spaced for Shp2 tandem SH2 binding. The arginines 32 and 138 are essential for the binding properties of Shp2. SH2 dependent functions of Shp2 include interactions with p53 and RhoA. The catalytic cysteine 460 is essential for the phosphatase function of Shp2, whereby mutation of C460 to serine completely abrogates its activity. Known PTP substrates are ROCKII, p85, Stat3 and Gab1. The C terminus of Shp2 contains two tyrosines and a proline rich region (PRR). Proteins known to associate with Shp2's C-terminus are Grb2 and Fyn. Activation of Shp2 is essential for downstream signaling pathways including the ERK and AKT pathways. Mutations associated with Noonan syndrome have been found mainly in the N-SH2 domain whereas LEOPARD associated mutations are found in the PTP domain, near the catalytic site. Whereas NS associated mutations result in enhanced PTP activity, LS mutations result in decreased PTP activity. In the studies described in this thesis, the NS mutations D61G and T73I, and the LS mutation A462T and G465A are mainly used. See text for further details.B) Crystal structure of Shp2. Structures in top and bottom are in similar orientations. The N- and C-terminal SH2 domains are yellow and green, respectively; the catalytic PTP domain is blue, and interdomain linkers (residues 104–111 and 217–220) are white in both panels. Top: Ribbon diagram showing secondary structure and organization of the domains. Orange, the side chain of Cys459 (the catalytic nucleophile); dashed lines, disordered loops. Bottom: All nonhydrogen atoms of SHP-2 are displayed. Although SH2 domain–bound peptides are not present in the structure, residues of both domains known to contact phosphopeptides are colored red. Note that peptide binding sites of both SH2 domains are exposed on the molecule surface. A distinct surface of the N-SH2 domain occupies the active site of the PTP domain. Magenta, residues of the PTP signature motif, HCSAGIGRS; these residues participate in catalysis and phosphate binding.[25]

defects [16,17]. Hyperactivation of the MAPK pathway results in C/E defects similar to knockdown of Erk1/2, suggesting a tight balance between activation and inactivation of this signalling pathway. Indeed, phenotypes resulting from hyperactive forms of BRaf and Mek are rescued using small molecule inhibitors of Mek, restoring the physiological levels of Erk1/2 activation [16,17]. What is more striking is that a treatment during a temporal window of 1 hour prior to shield formation is sufficient to rescue the gastrulation defect and later phenotypes. This indicates that, during shield stage, a tight balance between activation and inactivation of the MAPK pathway is important for proper development [16,17].

4 Shp2

Several studies have shown that the non-receptor tyrosine phosphatase SHP2 plays a fundamental role during gastrulation [18-20]. In zebrafish, knockdown of Shp2 results in defective gastrulation cell movements, without affecting cell fate specification [18]. In *Xenopus*, Shp2 is required for mesoderm induction and completion of gastrulation [21]. A study in Shp2 homozygous mutant embryonic stem cells showed that Shp2 is required at the initial steps of gastrulation, and that Shp2 mutant cells do not properly respond to signals initiated by fibroblast growth factors [20].
SHP2 is encoded by the *PTPN11* gene in humans. The importance of SHP2 in human genetics arose with the discovery that mutations in the *PTPN11* gene are the main cause of Noonan and LEOPARD syndromes, two autosomal dominant disorders with overlapping clinical features [22,23]. Due to a partial genome duplication in teleosts, the zebrafish genome encodes two Shp2 genes, *ptpn11a* and *ptpn11b* coding for the proteins Shp2a and Shp2b. Both Shp2a and Shp2b are derived from a common Shp2 ancestor [24]. SHP2 consists of two Src homology 2 (SH2) domains in tandem, a tyrosine phosphatase domain and a C-terminal tail [25] (Figure 2).

The SH2 domains of SHP2 are able to bind to phosphorylated tyrosines and thereby mediate binding of Shp2 to its target sites. Under basal conditions, Shp2 is kept in an 'inactive' closed state, whereby the N-terminal SH2 domain blocks the active site of the PTP domain to prevent dephosphorylation of its target sites [25]. The C-terminal SH2 domain however, is able to bind to tyrosyl phosphorylated SHP2-specific binding sequences and promote binding. Since SHP2 contains two SH2 domains in tandem, most SHP2 target proteins have tandem tyrosyl phosphorylation sites. Once the C-SH2 domain engages the target sequence, the tandem N-SH2 domain is prone to bind the other phosphorylation site by which SHP2 adopts an 'open' structure. Opening the structure of SHP2 causes an availability of the PTP domain, which facilitates dephosphorylation of its targets (Figure 3). These do not have to be the binding partner of SHP2 itself, but may also be other proteins in the complex [26].

Dephosphorylation of SHP2 target proteins is one of the mechanisms whereby Shp2 activation exerts its activity to activate downstream signalling pathways [27]. In *Xenopus*, a mutational analysis was performed to study the role of Shp2 in mesoderm induction following basic-fibroblast growth factor (bFGF) induction. Whereas the C-terminal SH2 domain is necessary only for wild-type function of Shp2, the N-SH2 domain and PTP activity are indispensable for Shp2 function [21]. The C-terminal tail, however, appears to be dispensable for Shp2 function. Alternatively, Shp2 may exhibit adaptor like activity, since EGF induced PI3K activation is enhanced by wild type- and phosphatase dead Shp2, but not by a Shp2 mutant that is unable to bind to its target [28].

Other studies suggest a role for the C-terminal domains of SHP2 [29]. The C-terminal tail of SHP2 consists of a proline rich domain, which is able to bind a Src homology 3 (SH3) domain, flanked by two tyrosines (Y542 and Y580 in zebrafish), which may be bound to SH2 domains. This suggests a role for SHP2 as an adaptor protein, while the C-terminus has also been opted to play a role in the conformational 'opening' and 'closing' of the phosphatase [30]. The upstream growth factor signalling pathway seems to have a decisive role in Shp2-mediated signaling, since C-terminal phosphorylation is mediated by FGF and PDGF, but not by EGF and IGF [29].

Despite the crucial role of SHP2 during gastrulation cell movements, little is known about the implication of the gastrulation defects on later development. For example, knockdown zebrafish embryos for Shp2 show defective gastrulation cell movements at 10 hpf and craniofacial/heart defects at 4 dpf [18]. However, it is poorly understood if the gastrulation cell movement defects are the direct cause of the later developmental defects and how the aberrant behavior of cells during gastrulation contribute to the impairment of development of other organs.

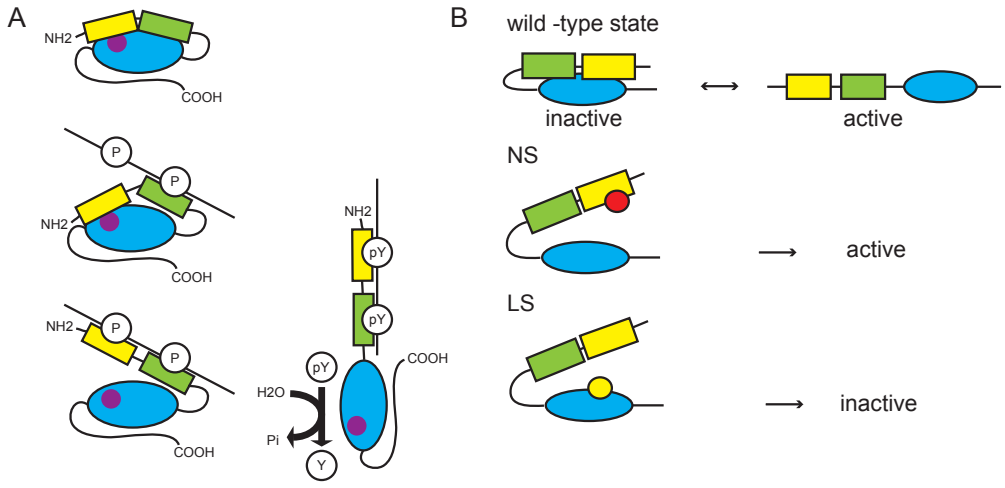


Figure 3. Regulation of Shp2 in the wild-type state and in Noonan and LEOPARD syndrome.

A) In the basal state, Shp2 is largely inactive, because the backside loop of the N-SH2 is inserted into the catalytic cleft. This results in mutual allosteric inhibition, with the N-SH2 inhibiting the PTP domain and the PTP domain contorting the pTyr peptide-binding pocket of the N-SH2 on the opposite surface. Shp2 activation starts when the two pTyr sites (pY), one that binds the C-SH2 and the other that binds the N-SH2, contacts Shp2. The C-SH2 is engaged first by its pTyr ligand. The resultant increase in local concentration of the ligand for the N-SH2 overcomes mutual allosteric inhibition, resulting in binding of the N-SH2 to its pTyr ligand, thus opening the enzyme into an active conformation. This mechanism of activation is strongly supported by crystal structure data and multiple enzymological studies [25]. Activation of Shp2 results in substrate dephosphorylation with release of phosphate. B) Regulation of Shp2 activity is, in part, achieved by an intramolecular interaction between the PTP domain of the protein and the N-SH2 domain leading to a "closed" protein conformation and autoinhibition. In the wild-type situation, "opening" of the N-SH2 and PTP domains is required for the protein to become active. NS mutations affect the interaction between the N-SH2 domain and the PTP domain, resulting in a protein that is in an "open" conformation and always active. LS mutations reside in the PTP domain and impair the catalytic activity of Shp2, resulting in a protein that is catalytically inactive.

Interestingly, not only knockdowns of Shp2 induce gastrulation cell movement defects, but also expression of mutant Shp2 with mutations that were identified in human patients with Noonan Syndrome or LEOPARD syndrome. These embryos show defects at later stages that are a phenocopy of the symptoms observed in human patients, including short stature, hypertelorism and cardiac defects [18]. Moreover, the contribution of Shp2 on gastrulation signaling pathways and its direct substrates still need to be addressed. Detailed analysis of the role of Shp2 during gastrulation may provide insight into the function Shp2 in Noonan and LEOPARD syndrome.

Outline of this thesis

Recent complementary *in vivo, in vitro* and *ex vivo* experiments reveal new insights into the function of Shp2 in NS and LS. However, it remains unclear how GOF and LOF mutants of Shp2 cause overlapping disease. In this thesis we investigate the role of NS and LS Shp2 using zebrafish as an animal model and the cellular and molecular mechanisms by which biochemically opposite Shp2 mutations result in two genetic disorders with overlapping features. The main theme of our projects was on NS and LS Shp2 in zebrafish, with Paardekooper Overman focusing more on the underlying molecular mechanism and Bonetti more on developmental aspects. We have indicated below who of the two of us is the main contributor to the different chapters.

In ***Chapter two*** (Paardekooper Overman) we describe several methods that are used to study phosphatases using zebrafish and techniques that we and others have developed to assess gastrulation defects and to assess changes in the phosphoproteome of developing zebrafish.

Chapter three (Bonetti) is an overview of the genetics of Noonan and LEOPARD syndrome and of molecular mechanisms that regulate these two genetic disorders. Moreover, we introduce the cardiac defects associated with Noonan and LEOPARD syndrome and the use of zebrafish as a model to study heart development.

In ***Chapter four*** (Bonetti) we study the phenotype of knock-out zebrafish embryos for both *ptpn11* genes. Shp2 has an essential role in embryonic development. Homozygous *Ptpn11-/-* mouse embryos die pre-implantation due to defective Erk activation and trophoblast stem cell death, precluding the possibility to study Shp2 function in differentiated cell types in adult animals [20]. Zebrafish embryo development occurs externally and the embryos are transparent, allowing the study of gene function *in vivo.* Due to partial genome duplication in teleosts, the zebrafish genome encodes two genes, *ptpn11a* and *ptpn11b* coding for the proteins Shp2a and Shp2b. We identified stop mutations in the N-terminal SH2 domain of each of the two *ptpn11* genes and we study the phenotype of the single homozygous mutants and of double homozygous mutant embryos.

In ***Chapter five*** (Paardekooper Overman) we describe the identification of Fer kinase as a candidate downstream factor in NS and LS signaling using a phosphotyrosine immunoprecipitation and mass spectrometry approach. Following that, we investigated the role of Fer kinase in the regulation of gastrulation and its role in the pathogenesis of NS and LS in zebrafish.

In ***Chapter six*** (Paardekooper Overman), we identify PZR, an important binding partner and substrate of Shp2 involved in adhesion and cell migration, as a hyperphosphorylated protein in NS and LS in both mouse and zebrafish. We show that PZR is required for normal embryonic development in zebrafish. Moreover, we provide evidence that PZR exerts its effects by changing the flux of Src activation in NS and LS.

Heart defects are the main cause of death in NS and LS patients. In ***Chapter seven*** (Bonetti) we investigate the role of NS and LS Shp2 in zebrafish cardiac development. We found that impaired heart function and morphogenesis are highly similar in NS- and LS-Shp2 expressing embryos. NS and LS-Shp2 both lead to up-regulation of Erk phosphorylation at the end of gastrulation, indicating a common biochemical pathway for NS and LS syndrome in zebrafish development.

The genetic cause of some 25% of all Noonan cases still remains to be determined. In ***Chapter eight*** (Bonetti and Paardekooper Overman), we describe the identification of mutations in Alpha-2-Macroglobulin-Like-1 (*A2ml1*) as cause of a disorder clinically related to NS. We identified *de novo* mutations in *A2ML1* using family-based whole exome sequencing of a case-parent trio with a clinical diagnosis of NS. Moreover, A2ML1 mutants were functionally characterized in zebrafish, showing that disease associated A2ml1 induces developmental defects, reminiscent of the developmental defects induced by a NS-variant of Shp2.
Finally the findings and the implications described in this thesis are discussed in ***Chapter nine*** (Paardekooper Overman and Bonetti).

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