Noonan and LEOPARD syndrome in zebrafish : molecular mechanisms and cardiac development

Bonetti, M.

Noonan and LEOPARD syndrome: an overview

Monica Bonetti[1] and Jeroen den Hertog[1,2]

1.Hubrecht Institute-KNAW and University Medical Centre Utrecht, the Netherlands
2. Institute of Biology Leiden, Leiden University, the Netherlands

Noonan syndrome (NS) (OMIM:163950) is a common (1:2000) heterogeneous syndrome that is related to other clinical syndromes associated with the RAS-MAPK pathway, including LEOPARD syndrome (LS) (OMIM:151100), cardio-facio-cutaneous (CFC) syndrome (OMIM:115150), neurofibromatosis type 1 (NF1) (OMIM:162200) and Costello syndrome (CS) (OMIM:218040). Collectively, these syndromes are termed RASopathies.

1. Symptoms

Noonan syndrome is characterized by short stature, congenital heart defects, including pulmonary stenosis, low set ears, webbed neck, hypertelorism, low set eyelids and chest deformities [1] (Figure 1). Whereas these features are prominent in a large group of NS patients, some patients may either be of normal height, or have less prominent craniofacial features. Other features include scoliosis, deafness and an increased propensity to develop leukemia. Whereas some patients exhibit cognitive defects as well, many NS patients have normal intelligence [2]. Noonan patients have a higher chance of developing juvenile myelomonocytic leukaemia (JMML), and mice carrying the D61G NS mutation develop myeloproliferative disease [3]. Moreover, Gab2, a downstream effector of Shp2, is essential for the aetiology of the myeloproliferative disorder, since double-mutant mice lacking Gab2 are less likely to develop myeloproliferative disease than Shp2 D61G knock-in mice [3]. A paternal origin for *de novo* mutations has been observed in NS, with a possible link to the age of the father as well [4]. Additionally, transmission of NS is skewed maternally, that may be attributed to the reduced fertility in male NS patients [4].

LEOPARD syndrome, an acronym of Lentigines, Electrocardiographic conduction anomalies, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retarded growth and sensorineural Deafness is clinically very similar to NS. Patients exhibiting LS are often initially diagnosed with NS. Only when certain 'café au lait' spots or lentigines appear during early adolescence a clinical distinction is often made between NS and LS. Whereas NS and LS share many common characteristics, several clinical features are unevenly distributed among NS and LS patients. For instance, pulmonary stenosis (PS) is more common in NS, whereas hypertrophic cardiomyopathy (HCM) is more prevalent in LS [5]. Additionally, craniofacial defects are more prominent in NS than in LS patients and LS patients exhibit less height deficits than NS. Postnatal growth retardation is a clinical manifestation of NS and LS patients. Approximately 70% of adults with NS are below the 10th percentile for height [6] and 85% of adults with LS are below the 25th percentile [7]. The short stature in NS patients is generally due to a decrease in responsiveness to growth hormone (GH), as evidenced by reduced levels of insulin-like growth factor 1 (IGF-1) [8-11]. A Ptpn11 Noonan syndrome mouse model displays increased ERK activation in response to growth hormone; blocking MAPK signaling restores IGF-1 levels, thus reducing the postnatal growth retardation in these mice [8]. The molecular basis of growth defects in LS is less well understood.

2. Genetics

Roughly 50% of all cases of NS are caused by mutations in *PTPN11* while heterozygous missense mutations in *PTPN11* are observed in up to 90% of LS cases. Other mutations have been identified in genes encoding for proteins along the RAS-MAPK pathway. Mutations were identified in *MEK1*, *MEK2*, *KRAS*, *NRAS* and in *SHOC2* [12-16]. Also *RAF1* and *BRAF* gain of function mutations were identified in patients with NS and LS [17,18] (Figure. 2). Mutations in *CBL*, an E3 ubiquitin ligase with tumour suppressing activity, are causative of Noonan-like syndrome [19]. Moreover, Noonan-

like symptoms are associated with mutations in the histone methyltransferase, *MYST4*, resulting in enhanced MAPK signalling [20]. The genetic cause of some 25% of all Noonan cases still remains to be determined. It is evident that these patients do not have mutations in any of the known Noonan syndrome-associated genes.

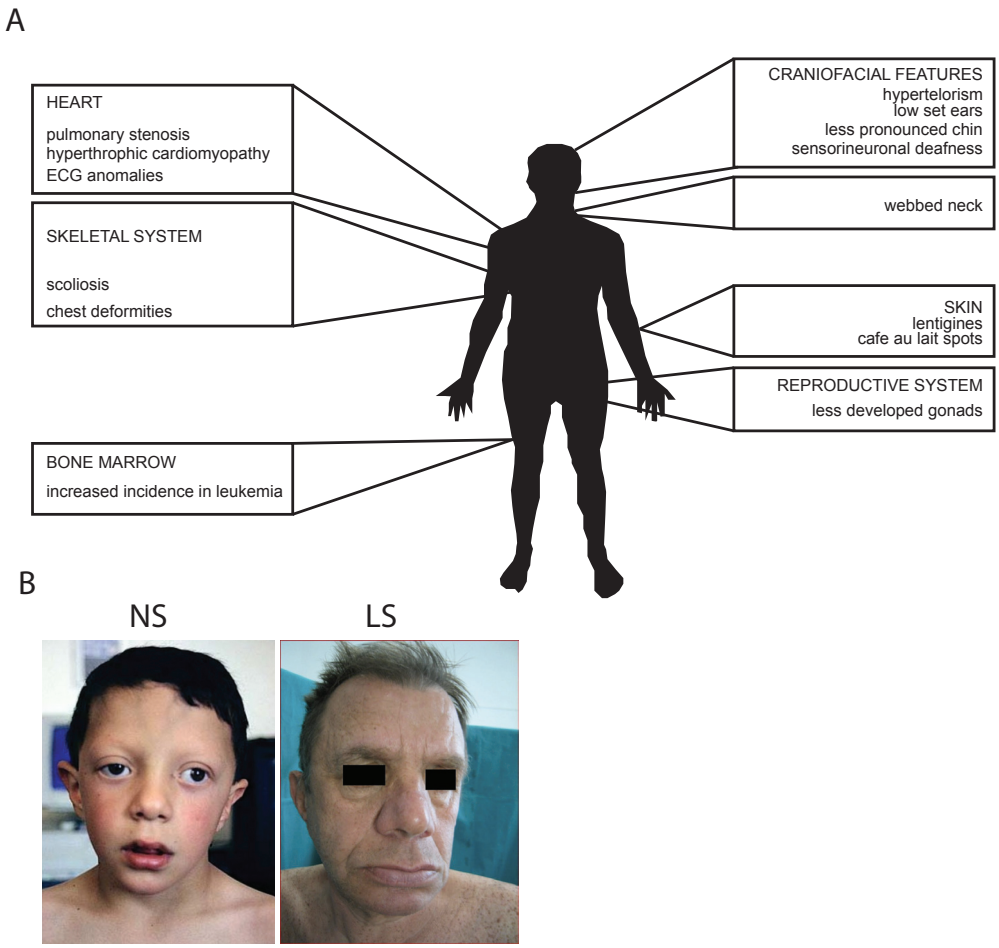

Figure 1. Clinical characteristics and facies of Noonan and LEOPARD syndrome patients. A) Diagnosis of Noonan and LEOPARD syndrome is primarily dependent on clinical features, including short stature, typical face dysmorphology, and congenital heart defects. Diagnosis can be difficult due to the wide spectrum of clinical features, not all of which need to be present for diagnosis. Cardinal features of Noonan and LEOPARD syndrome include hypertelorism, ptosis, and low-set, posteriorly rotated ears with a thickened helix. Cardiac abnormalities most commonly associated with the two syndromes include pulmonary stenosis (NS) and hypertrophic cardiomyopathy (LS). Other associated features include the presence of a webbed neck, chest deformity, mild intellectual deficit, cryptorchidism, poor feeding in infancy, bleeding tendencies, and lymphatic issues. B) Photographs of families diagnosed with Noonan [81] and LEOPARD [82] syndrome with mutations in *PTPN11*.

3. Mechanism

Whereas mutations that cause NS all result in a phosphatase hyperactive form of Shp2, LS causing mutations in Shp2 all display strongly reduced phosphatase activity [21,22]. How is it possible that two seemingly opposite mutations cause similar clinical features? Examination of the structural mechanism of Shp2 revealed a gain-of-function in NS forms of Shp2, giving rise to a hyperactive form [23]. NS mutations predominantly reside in the interface between the N-SH2 domain and the PTP domain, resulting in the impairment of the closed conformation and enhanced catalytic activity of NS-Shp2 [24,25]. By contrast, most LS mutations reside close to the active site and result in strongly reduced, yet detectable, catalytic activity [24,26,27] (Chapter 1). Phosphatase assays have shown a catalytic defect in LS Shp2 [24,26,28]. LS-Shp2 mutations are mostly located in the protein-tyrosine phosphatase (PTP) domain, and the local conformational changes induced by LS-Shp2 mutations impair the catalytic properties of Shp2 [29]. However, in vivo, LS Shp2 may act as a gain-of-function as well [30]. In flies, LS mutations give rise to gain-of-function defects that cause upregulation of MAPK and require residual PTP activity of Shp2 [30]. LS-Shp2 displays residual phosphatase activity [26,27]. LS mutations cause loss of phosphatase activity and at the same time result in enhanced binding to Gab1 and PI3K [26]. Further investigation showed that LS, unlike NS causes activation of the PI3K-AKT pathways upon growth factor stimulation [31]. This is due to enhanced phosphorylation of Gab1 due to the loss of phosphatase activity of LS Shp2 [31]. Enhanced PI3K activation likely contributes to the discrepancies between NS and LS. PI3K/AKT signaling is upregulated in hearts of LS/+ knock-in mice [22] as well as in fibroblasts isolated from LS patients [31]. Despite these explanations it remains unclear how activating and inactivating mutants of Shp2 can generate overlapping syndromes. Most probably, NS and LS share some of their targets in order for them to generate the overlapping clinical features.

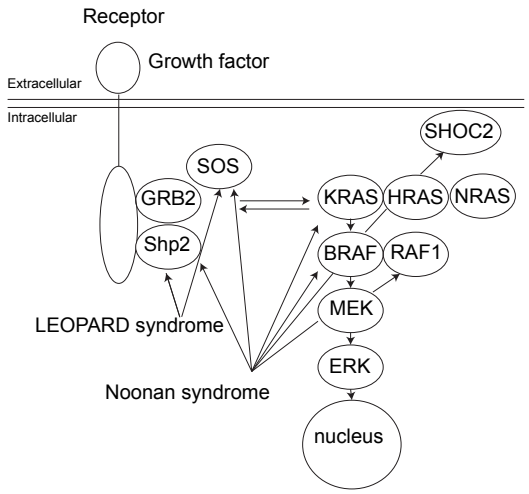

Figure 2. Overview of the RAS-MAPK pathway associated with NS/LS disorders. Syndromes caused by mutations in genes of RAS-MAPK signalling proteins are indicated.

4. Spatiotemporal control

Cardio-facio-cutaneous (CFC) syndrome is one of the RASopathies, and is caused by mutations in *BRAF*, *MEK1* or *MEK2*. Like NS, activating mutations give rise to short stature, heart defects and craniofacial defects arising from hyperactivation of the MAPK pathway. In zebrafish, NS and CFC induced gastrulation defects can be rescued by treatment with the MEK inhibitor CI-1040 [14,32]. Importantly, inhibitor treatment is only required during a small temporal window prior to shield formation [14,32]. Not only temporal control but also spatial control (membrane-bound versus cytoplasmic, or lipid-raft versus non-lipid raft localization) of protein signalling is likely a vital aspect in the aetiology of RASopathies. SHOC2 is a scaffolding protein that binds RAS and RAF. Under normal stimulation, SHOC2 is

targeted to late endosomes and activates ERK1/2 [33]. An interesting finding was that a mutation in SHOC2 that causes Noonan-like syndrome with loose anagen hair results in N-myristoylation of SHOC2. This causes aberrant translocation of SHOC2 to the cell membrane instead of late endosomes, which results in enhanced MAPK activation in a cell-type specific manner [13,33]. Interestingly, whereas NS mutations in Shp2 and SHOC2 are additive [34], NS and LS mutations in Shp2 are not [28]. Moreover, NS associated mutations in *RAF1* cause enhanced heterodimerization with BRAF which also results in enhanced ERK activation [35]. More importantly, not only kinase-impaired D486N RAF1 but also kinase-activating NS forms of RAF1 required heterodimerization with BRAF to enhance MEK/ERK activation [35]. Interestingly, RAF1 mutations have been found in NS and LS patients, suggesting a common mechanism for NS and LS [17].

Targeting of Shp2 to lipid rafts results in integrin clustering, Focal Adhesion Kinase phosphorylation, Rho and ERK activation, all of which are hallmarks of fibronectin attachment [36]. Shp2 is targeted to lipid rafts upon adhesion, and dominant negative RhoA blocks Shp2 induced signalling. In addition, Shp2 phosphatase activity is essential for negative feedback to Rho [36]. The downstream target of Shp2, RhoA, also prevents apoptosis by activating the MAPK pathway and expression of *bcl-2*, in zebrafish [37]. A phosphatase independent, but SH2 domain dependent role for LS Shp2 was described in preventing p53 mediated apoptosis in the brain and neural crest, suggesting that the SH2 domain of Shp2 may be essential for RhoA activation [37,38]. An emerging model is that under basal conditions, Shp2 binds Abl, which activates p190 RhoGAP, which in turn inactivates Rho. In its active state, Shp2 inactivates p190 RhoGAP by dephosphorylation. In addition, Shp2 dephosphorylates an inhibiting tyrosine phosphorylation site on ROCKII, causing activation of the pathway [39]. It is noteworthy that NS Shp2-mediated activation of ROCKII causes heart defects in Xenopus [40].

Recently, a role for Ephrin signalling has been implicated in Shp2 mediated ERK activation [41]. Hepatic growth factor (HGF) induced ERK activation is dependent on EphA2 phosphorylating the C-terminal tyrosines, Y542 and Y580 of Shp2, thereby mediating prolonged Grb2 binding and ERK activation. Interestingly, ERK activation is independent of Shp2 phosphatase activity, since expression of T468M (LS) or a N308D (NS) variant enhanced ERK activation. Mutation of the C-terminal tyrosines in the LS background suppress ERK activation as well, suggesting an essential role for these tyrosines in mediating ERK activation. Comparison of Shp2 knockdown embryos and zebrafish embryos expressing LS-Shp2 allowed assessment of a phosphatase and ERK dependent role for Shp2 in zebrafish neural crest specification and cell migration [38].

Shp2 phosphatase activity plays a role in cell migration. IGF1, which cooperates with integrins to promote cellular migration and invasion [42,43] , mediates IRS1 binding to a complex containing FAK, paxilin and Shp2. This results in the dephosphorylation of FAK and paxilin. Interestingly, Shp2 phosphatase activity is essential for integrin deactivation since Shp2 C460S expressing cells show higher numbers of focal adhesion contacts [44]. This is likely independent of ERK activation since treatment with a MEK inhibitor did not attenuate this effect [44]. Shp2 also mediates activation of Fyn kinase at focal adhesion sites in a catalytic activity independent manner, where Shp2 binds to a6b4 integrin via its N-SH2 domain and to Fyn with its C terminal tyrosine Y580, thereby acting as a docking protein [45]. Additionally, Fyn phosphorylates Shp2 Y542, thereby providing a positive feedback loop [45]. Also Src kinase is known to be activated by, and bound to Shp2 in a catalytically independent manner [46]. Whereas the SH3 domain of Src was essential for this binding, a specific Src binding site has not yet been identified in Shp2 [46]. Other kinases also influence Shp2 function, as Abl phosphorylates Shp2, at Y580. Interestingly, Abl indirectly causes

phosphorylation of target sites in Shp2 that are located at or near residues often mutated in NS and LS respectively, namely Y63 and Y279 [47]. Phosphorylation of Y279 caused a decrease in growth factor mediated ERK activation [47]. Other pathways than the Rho, MAPK and Src pathways are also controlled by Shp2, like the JAK/STAT pathway. In contrast to these other pathways, Shp2 plays an inhibiting role [48]. Interestingly, down regulation of Stat3 by activating variants of Shp2 may contribute to pulmonary stenosis in NS and myeloproliferative disorder in JMML [49]. Indeed, phosphorylated Stat3 is inhibited by active Shp2, and expression of active Stat3 rescues the activating Shp2 defects in bone marrow cells [49].

Several attempts have been made to find an inhibitor for Shp2 for treatment and use in biochemical assays [50]. One of these, NSC-87877 binds to the catalytic site of the PTP domain and is able to inhibit Shp2 mediated, growth factor induced ERK activation [50]. In addition, phenylhydrazonopyrazolone sulfonate (PHPS1) was identified by an *in silico* screen, and further tested *in vitro* for its phosphatase inhibitory capacity [51]. PHPS1 was able to inhibit HGF induced cell scattering that is mediated by Shp2 phosphatase activity [51]. However, these compounds only target the phosphatase dependent function of Shp2, while Shp2 also exhibits phosphatase independent mechanisms.

Thus, Shp2 is involved in a plethora of signalling mechanisms and one can imagine that biochemical changes in the enzyme may result in widespread defects. In LS patient derived induced pluripotent stem (iPS) cells, several downstream proteins are hyper- or hypo-tyrosyl phosphorylated [52]. Interestingly, although LS Shp2 is thought to result in a decreased ability to activate the ERK pathway following growth factor stimulation due to its loss of phosphatase activity, basal levels of MEK are upregulated in these cells [52]. Gene expression profiling of NS and Noonan-like syndrome patients show that many alterations are present on the transcriptional level and that NS-specific gene expression signatures are distinguished [53]. NS Shp2 expressing cells show enhanced expression of Src family kinase substrates, indicating that these proteins may play an essential role in the aetiology of Shp2 induced NS [53].

5. Cardiac defects associated with NS and LS

Noonan and LEOPARD patients share several clinical manifestations, including scoliosis, facial dysmorphic features, short stature and congenital heart defects. Despite those clinical similarities, the heart defects are quite distinct between Noonan and LEOPARD patients. The most common cardiac defect in NS is pulmonary stenosis (PS) resulting from dysplastic valve leaflets, but stenosis of mitral valve, atrial, ventricular, and atrio-ventricular septal defects, or, rarely, double-outlet right ventricle are also seen [54,55]. Moreover, hypertrophic cardiomyopathy (HCM) has been found in few NS patients without Shp2 mutations [56] and genotype-phenotype correlation studies show that only 8% of Shp2-associated NS patients present with HCM [57]. HCM is most commonly present in NS patients with mutations in *RAF1* [58]. Electrocardiogram (ECG) of NS patients often shows a characteristic pattern, with a left axis deviation, abnormal R/S ratio over the left precordium, and an abnormal Q wave. These ECG features are characteristic for NS and they are not related to a specific cardiac defect (PS or HCM) [59]. In contrast, the most common cardiac defect in LS is HCM, but similar valve anomalies to NS are also observed [60].

Although Shp2 is a major player in cardiac disease, its function in heart development in NS and LS remains poorly addressed. Several animal models have been used to investigate the role of Shp2 in NS and LS. The injection of the most prevalent human NS Shp2 mutations into *Xenopus* results

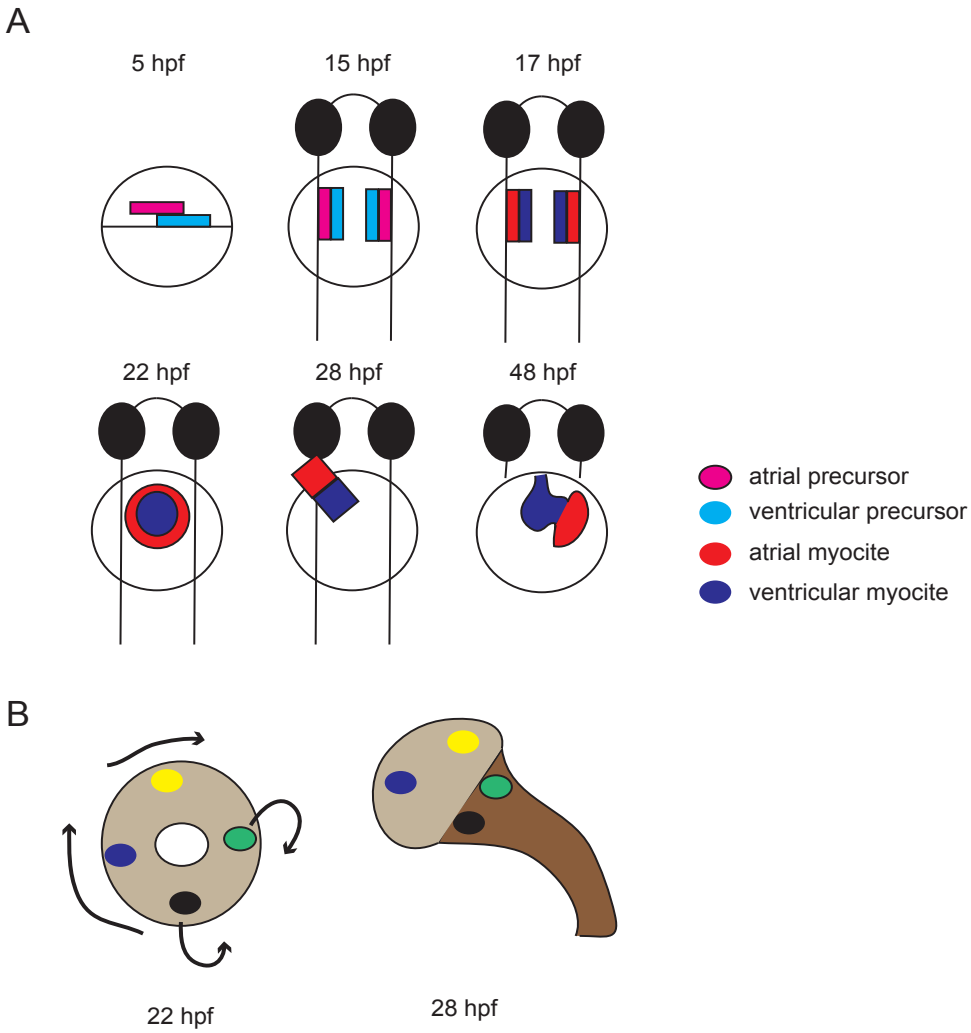

Figure 3. Stages of cardiac development.
A) At 5 h post fertilization (hpf) cardiac progenitor cells are positioned bilaterally in the lateral marginal zone of the blastula. Atrial progenitor cells (pink) are located more ventrally than the ventricle progenitor cells (light blue). During gastrulation, the cardiac progenitor cells move dorsally towards the mid-line to end up in the anterior later plate mesoderm (ALPM). At 17 hpf, the future ventricle and atrial myocardial cells differentiate because of the expression of cardiac myosins (blue/red). The bilateral heart fields fuse at the mid-line (22hpf), forming a cardiac disc structure with the ventricular myocytes internally and atrial myocytes externally of the disc. Cardiac morphogenesis transforms the cardiac disc into a cardiac tube. At 28 hpf, the linear heart tube has formed. At 36 hpf, cardiac looping has started, with a displacement of the ventricle towards the mid-line. The heart tube continues to loop and forms an S-shaped loop (48 hpf).
B) Cartoon in which the migration, rotation and involution of the cardiac tissue during heart tube formation is indicated. The color-coding of the 4 cells indicates the original and the final position of the cardiomyocytes [63, 69].

in hearts that failed to complete looping or undergo chamber formation coupled with a delay or arrest of the cardiac cell cycle in M-phase [40]. Similarly, the overexpression of NS and LS Shp2 mRNA in zebrafish embryos at the 1-cell stage result in heart displacement defects at 24 hpf [28]. Moreover, NS and LS Shp2 induce convergence and extension cell movement defects at the end of gastrulation, followed by the development of craniofacial defects and reduced body axis length at 4dpf [28].

The mouse model expressing the NS-associated mutant D61G develops short stature, craniofacial abnormalities and multiple cardiac defects, including pulmonary stenosis and double outlet of the right ventricle (DORV). Their endocardial cushions show an increased Erk activation. When homozygous, the D61G mutant is embryonic lethal [61]. Similarly the knockin mice harboring the LS *Ptpn11* mutation Y279C recapitulates the human disorder, showing short stature, craniofacial dysmorphia, and HCM. LS/+ mice also exhibit increased Akt/mTOR activity and the inhibition of mTOR signaling with rapamycin completely normalized and reversed the LS cardiac defects [62]. Conclusively, LS models support the conclusion that excessive AKT/mTOR activity, not MAPK, is essential for the development and maintenance of HCM.

6. Zebrafish as a model to study heart development.

The role of NS and LS Shp2 variants in the development of cardiac defects is still unclear. Zebrafish has become a powerful model to study cardiac development in recent years [63,64]. Moreover, the transparency of zebrafish embryos facilitates time-lapse analysis of the onset and nature of the cardiac defects *in vivo*. The heart is the first organ to form and function during zebrafish embryo development. Heart development begins with the specification of myocardial and endocardial progenitor cells. Before gastrulation, 5 h post fertilization (hpf), the heart progenitor cells are formed and placed in the ventral and lateral regions of the embryo. Atrial progenitor cells are located more ventrally in the lateral marginal zone when compared with ventricular progenitor cells [65]. By 14 somites (17hpf), the cardiac progenitors cells appear as two bilateral pools on the lateral plate mesoderm (LPM) [66]. Next, by 22 somites (22 hpf), these pools combine at the LPM to form the heart disk. This disk moves anteriorly and to the left and forms the heart tube (24-28 hpf). Afterwords, by 30 hpf, ventricular and atrial chambers form. By 36 hpf, the heart undergoes looping morphogenesis and, by 48 hpf, functional valves are formed (Figure 3A)[66]. Up to 22 somites, the heart morphogenesis is a symmetrical process because there are no morphological differences between the left and right cardiac fields. However, after the cardiac disc formation, the myocardial tissue originating from the right cardiac field involutes ventrally and moves towards the anterior/left (Figure 3B). In this way, the left–right organization of the cardiac disc change into a dorsal–ventral organization of the cardiac tube. Four-dimensional confocal miscroscopy and the automated cell tracking showed that co-occurring with right-field involution, the myocardial disc rotates in a clockwise direction [67-69]. As a consequence of these moviments, the cardiac tube is created, with its venous pole located at the left side and its arterial pole located at the mid-line. Breaking of the heart symmetry is initiated by a transient ciliated organ called Kupffer's vesicle that is derived from the dorsal forerunner cells (DFCs) [70,71]. Cilia within the KV rotate in a counter-clockwise direction, thus generating a fluid flow in the same direction [72,73]. The directional fluid flow in the KV induces asymmetric expression of two conserved L/R signaling genes *pitx2* (a *bicoid*-related transcription factor) and *southpaw* (*spaw*, a Nodal-related gene) in the LPM. The bilateral expression of *spaw*/*nodal* starts around the KV at the 4 somite stage and its expression resides in the perinodal area [74]. At around the 10–12 somite stage, *spaw*/*nodal* expression starts in the LPM. The left-sided *spaw*/*nodal* expression moves to the

anterior part of the embryo [74]. At late somite stages, the leftward expression of *southpaw* overlaps with the left-sided expression of other genes, such as *lefty2*. *Spaw*/*nodal* is proposed to be a significant player in the diffusion of the asymmetric expression in the left LPM, together with the activation of its own antagonists, Lefty1 and Lefty2 [75]. In particular, Lefty1 has the function to block the nodal expression on the right-side of the LPM. *Lefty1* expression acts in the midline as physical barrier to avoid the diffusion of asymmetric signal in the LPM to the right side, preventing the right LPM to acquire a left-sided identity [76]. Impairment of the L/R patterning determination can cause either a randomized positioning of organs (*situs ambiguus*) or a mirror-image duplication of organs (*situs inversus*). In humans, clinically significant laterality defects occur in 1 in 10,000 births [77]. The mortality associated with laterality defects is always ascribed to complex congenital heart defects. In fact the heart morphogenesis is very susceptible to impairments in the left-right (LR) body plan. The heart tube undergoes looping morphogenesis quite early in vertebrate development and before the heart tube has completed its formation. Because cardiac looping is a highly conserved process, the directionality of the heart loop can predict the body *situs* [78]. As the heart tube proceeds in the elongation, it develops a dextral loop ("d" loop). The process of looping and the directionality of the loop are important for normal heart development. Because most cardiac structures originate from cells derived from more than one area of the heart tube, the significant function of looping is to rearrange regions of the heart tube so they are appropriately positioned for proper formation and alignment of chambers, valves, and septa. Thus, the directionality of looping establishes once the left ventricle underlies the left atrium, and the right ventricle beneath the right atrium [79]. The heart shows an atrio-ventricular (A-V) concordance only when the position of the ventricles is correct. Moreover, L/R chambers division is fundamental for the functions of the heart and if L/R asymmetry is abnormal, one or more congenital defects may arise. These defects range from atrial septal defects (ASD) or ventral septal defects (VSD) to the double outlet of the right ventricle (DORV) and transposition of the great arteries (TGA). It is noteworthy that laterality disease is frequently associated with congenital heart defects (CHD) [80]. However, cardiac defects associated with laterality diseases are also reported in Shp2-D61G knock-in mice. A proportion of the heterozygous Shp2-D61G mice displays septal defects and DORV [61]. However, laterality defects are not the only cause of septation defects or DORV. Hence, it remains to be determined whether laterality defects have a role in human NS and LS. Overall the zebrafish model is becoming a very powerful model to study cardiac development. By evolving new and more specific assays, the zebrafish model can be used to study mechanisms leading to human cardiac diseases and to model human congenital cardiac diseases.

References

1. Tartaglia M, Gelb BD (2005) Noonan syndrome and related disorders: genetics and pathogenesis. Annu Rev Genomics Hum Genet 6: 45-68.
2. van der Burgt I (2007) Noonan syndrome. Orphanet J Rare Dis 2: 4.
3. Xu D, Wang S, Yu WM, Chan G, Araki T, et al. (2010) A germline gain-of-function mutation in Ptpn11 (Shp-2) phosphatase induces myeloproliferative disease by aberrant activation of hematopoietic stem cells. Blood 116: 3611-3621.
4. Tartaglia M, Cordeddu V, Chang H, Shaw A, Kalidas K, et al. (2004) Paternal germline origin and sex-ratio distortion in transmission of PTPN11 mutations in Noonan syndrome. Am J Hum Genet 75: 492-497.
5. Sarkozy A, Conti E, Seripa D, Digilio MC, Grifone N, et al. (2003) Correlation between PTPN11 gene mutations and congenital heart defects in Noonan and LEOPARD syndromes. J Med Genet 40: 704-708.
6. Noonan JA, Raaijmakers R, Hall BD (2003) Adult height in Noonan syndrome. Am J Med Genet A 123A: 68-71.
7. Gorlin RJ, Anderson RC, Blaw M (1969) Multiple lentigenes syndrome. Am J Dis Child 117: 652-662.
8. De Rocca Serra-Nedelec A, Edouard T, Treguer K, Tajan M, Araki T, et al. (2012) Noonan syndrome-causing SHP2 mutants inhibit insulin-like growth factor 1 release via growth hormone-induced ERK hyperactivation, which contributes to short stature. Proc Natl Acad Sci U S A 109: 4257-4262.
9. Binder G, Neuer K, Ranke MB, Wittekindt NE (2005) PTPN11 mutations are associated with mild growth hormone resistance in individuals with Noonan syndrome. J Clin Endocrinol Metab 90: 5377-5381.
10. Limal JM, Parfait B, Cabrol S, Bonnet D, Leheup B, et al. (2006) Noonan syndrome: relationships between genotype, growth, and growth factors. J Clin Endocrinol Metab 91: 300-306.
11. Noordam C, Peer PG, Francois I, De Schepper J, van den Burgt I, et al. (2008) Long-term GH treatment improves adult height in children with Noonan syndrome with and without mutations in protein tyrosine phosphatase, non-receptor-type 11. Eur J Endocrinol 159: 203-208.
12. Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, et al. (2006) Germline KRAS mutations cause Noonan syndrome. Nat Genet 38: 331-336.
13. Cordeddu V, Di Schiavi E, Pennacchio LA, Ma'ayan A, Sarkozy A, et al. (2009) Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. Nat Genet 41: 1022-1026.
14. Runtuwene V, van Eekelen M, Overvoorde J, Rehmann H, Yntema HG, et al. (2011) Noonan syndrome gain-of-function mutations in NRAS cause zebrafish gastrulation defects. Dis Model Mech 4: 393-399.
15. Carta C, Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, et al. (2006) Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. Am J Hum Genet 79: 129-135.
16. Dentici ML, Sarkozy A, Pantaleoni F, Carta C, Lepri F, et al. (2009) Spectrum of MEK1 and MEK2 gene mutations in cardio-facio-cutaneous syndrome and genotype-phenotype correlations. Eur J Hum Genet 17: 733-740.
17. Pandit B, Sarkozy A, Pennacchio LA, Carta C, Oishi K, et al. (2007) Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat Genet 39: 1007-1012.
18. Sarkozy A, Carta C, Moretti S, Zampino G, Digilio MC, et al. (2009) Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. Hum Mutat 30: 695-702.
19. Martinelli S, De Luca A, Stellacci E, Rossi C, Checquolo S, et al. (2010) Heterozygous germline mutations in the CBL tumor-suppressor gene cause a Noonan syndrome-like phenotype. Am J Hum Genet 87: 250-257.
20. Kraft M, Cirstea IC, Voss AK, Thomas T, Goehring I, et al. (2011) Disruption of the histone acetyltransferase MYST4 leads to a Noonan syndrome-like phenotype and hyperactivated MAPK signaling in humans and mice. J Clin Invest 121: 3479-3491.
21. Tartaglia M, Martinelli S, Stella L, Bocchinfuso G, Flex E, et al. (2006) Diversity and functional consequences of germline and somatic PTPN11 mutations in human disease. Am J Hum Genet 78: 279-290.
22. Kontaridis MI, Swanson KD, David FS, Barford D, Neel BG (2006) PTPN11 (Shp2) mutations in LEOPARD syndrome have dominant negative, not activating, effects. J Biol Chem 281: 6785-6792.
23. Darian E, Guvench O, Yu B, Qu CK, MacKerell AD, Jr. (2011) Structural mechanism associated with domain opening in gain-of-function mutations in SHP2 phosphatase. Proteins 79: 1573-1588.
24. Keilhack H, David FS, McGregor M, Cantley LC, Neel BG (2005) Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. J Biol Chem 280: 30984-30993.
25. Nakamura T, Gulick J, Pratt R, Robbins J (2009) Noonan syndrome is associated with enhanced pERK activity, the repression of which can prevent craniofacial malformations. Proc Natl Acad Sci U S A 106: 15436-15441.
26. Hanna N, Montagner A, Lee WH, Miteva M, Vidal M, et al. (2006) Reduced phosphatase activity of SHP-2 in LEOPARD syndrome: consequences for PI3K binding on Gab1. FEBS Lett 580: 2477-2482.
27. Yu ZH, Xu J, Walls CD, Chen L, Zhang S, et al. (2013) Structural and mechanistic insights into LEOPARD syndrome-associated SHP2 mutations. J Biol Chem 288: 10472-10482.
28. Jopling C, van Geemen D, den Hertog J (2007) Shp2 knockdown and Noonan/LEOPARD mutant Shp2-induced gastrulation defects. PLoS Genet 3: e225.
29. Qiu W, Wang X, Romanov V, Hutchinson A, Lin A, et al. (2014) Structural insights into Noonan/LEOPARD syndrome-related mutants of protein-tyrosine phosphatase SHP2 (PTPN11). BMC Struct Biol 14: 10.

30. Oishi K, Zhang H, Gault WJ, Wang CJ, Tan CC, et al. (2009) Phosphatase-defective LEOPARD syndrome mutations in PTPN11 gene have gain-of-function effects during Drosophila development. Hum Mol Genet 18: 193-201.
31. Edouard T, Combier JP, Nedelec A, Bel-Vialar S, Metrich M, et al. (2010) Functional effects of PTPN11 (SHP2) mutations causing LEOPARD syndrome on epidermal growth factor-induced phosphoinositide 3-kinase/AKT/glycogen synthase kinase 3beta signaling. Mol Cell Biol 30: 2498-2507.
32. Anastasaki C, Estep AL, Marais R, Rauen KA, Patton EE (2009) Kinase-activating and kinase-impaired cardio-facio-cutaneous syndrome alleles have activity during zebrafish development and are sensitive to small molecule inhibitors. Hum Mol Genet 18: 2543-2554.
33. Galperin E, Abdelmoti L, Sorkin A (2012) Shoc2 is targeted to late endosomes and required for Erk1/2 activation in EGF-stimulated cells. PLoS One 7: e36469.
34. Ekvall S, Hagenas L, Allanson J, Anneren G, Bondeson ML (2011) Co-occurring SHOC2 and PTPN11 mutations in a patient with severe/complex Noonan syndrome-like phenotype. Am J Med Genet A 155A: 1217-1224.
35. Wu X, Yin J, Simpson J, Kim KH, Gu S, et al. (2012) Increased BRAF heterodimerization is the common pathogenic mechanism for noonan syndrome-associated RAF1 mutants. Mol Cell Biol 32: 3872-3890.
36. Lacalle RA, Mira E, Gomez-Mouton C, Jimenez-Baranda S, Martinez AC, et al. (2002) Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation. J Cell Biol 157: 277-289.
37. Zhu S, Korzh V, Gong Z, Low BC (2008) RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway. Oncogene 27: 1580-1589.
38. Stewart RA, Sanda T, Widlund HR, Zhu S, Swanson KD, et al. (2010) Phosphatase-dependent and -independent functions of Shp2 in neural crest cells underlie LEOPARD syndrome pathogenesis. Dev Cell 18: 750-762.
39. Tandon P, Conlon FL, Taylor JM (2012) ROCKs cause SHP-wrecks and broken hearts. Small GTPases 3: 209-212.
40. Langdon Y, Tandon P, Paden E, Duddy J, Taylor JM, et al. (2012) SHP-2 acts via ROCK to regulate the cardiac actin cytoskeleton. Development 139: 948-957.
41. Miura K, Wakayama Y, Tanino M, Orba Y, Sawa H, et al. (2013) Involvement of EphA2-mediated tyrosine phosphorylation of Shp2 in Shp2-regulated activation of extracellular signal-regulated kinase. Oncogene.
42. Akiyama SK, Olden K, Yamada KM (1995) Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev 14: 173-189.
43. Felding-Habermann B, Mueller BM, Romerdahl CA, Cheresh DA (1992) Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. J Clin Invest 89: 2018-2022.
44. Manes S, Mira E, Gomez-Mouton C, Zhao ZJ, Lacalle RA, et al. (1999) Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. Mol Cell Biol 19: 3125-3135.
45. Yang X, Dutta U, Shaw LM (2010) SHP2 mediates the localized activation of Fyn downstream of the alpha6beta4 integrin to promote carcinoma invasion. Mol Cell Biol 30: 5306-5317.
46. Walter AO, Peng ZY, Cartwright CA (1999) The Shp-2 tyrosine phosphatase activates the Src tyrosine kinase by a non-enzymatic mechanism. Oncogene 18: 1911-1920.
47. Mitra S, Beach C, Feng GS, Plattner R (2008) SHP-2 is a novel target of Abl kinases during cell proliferation. J Cell Sci 121: 3335-3346.
48. You M, Yu DH, Feng GS (1999) Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway. Mol Cell Biol 19: 2416-2424.
49. Zhang W, Chan RJ, Chen H, Yang Z, He Y, et al. (2009) Negative regulation of Stat3 by activating PTPN11 mutants contributes to the pathogenesis of Noonan syndrome and juvenile myelomonocytic leukemia. J Biol Chem 284: 22353-22363.
50. Chen L, Sung SS, Yip ML, Lawrence HR, Ren Y, et al. (2006) Discovery of a novel shp2 protein tyrosine phosphatase inhibitor. Mol Pharmacol 70: 562-570.
51. Hellmuth K, Grosskopf S, Lum CT, Wurtele M, Roder N, et al. (2008) Specific inhibitors of the protein tyrosine phosphatase Shp2 identified by high-throughput docking. Proc Natl Acad Sci U S A 105: 7275-7280.
52. Carvajal-Vergara X, Sevilla A, D'Souza SL, Ang YS, Schaniel C, et al. (2010) Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. Nature 465: 808-812.
53. Ferrero GB, Picco G, Baldassarre G, Flex E, Isella C, et al. (2012) Transcriptional hallmarks of Noonan syndrome and Noonan-like syndrome with loose anagen hair. Hum Mutat 33: 703-709.
54. Marino B, Digilio MC, Toscano A, Giannotti A, Dallapiccola B (1999) Congenital heart diseases in children with Noonan syndrome: An expanded cardiac spectrum with high prevalence of atrioventricular canal. J Pediatr 135: 703-706.
55. Yoshida R, Hasegawa T, Hasegawa Y, Nagai T, Kinoshita E, et al. (2004) Protein-tyrosine phosphatase, nonreceptor type 11 mutation analysis and clinical assessment in 45 patients with Noonan syndrome. J Clin Endocrinol Metab 89: 3359-3364.
56. Tartaglia M, Kalidas K, Shaw A, Song X, Musat DL, et al. (2002) PTPN11 mutations in Noonan syndrome: molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. Am J Hum Genet 70: 1555-1563.
57. Sznajer Y, Keren B, Baumann C, Pereira S, Alberti C, et al. (2007) The spectrum of cardiac anomalies in Noonan syndrome as a result of mutations in the PTPN11 gene. Pediatrics 119: e1325-1331.
58. Roberts AE, Allanson JE, Tartaglia M, Gelb BD (2013) Noonan syndrome. Lancet 381: 333-342.

59. Raaijmakers R, Noordam C, Noonan JA, Croonen EA, van der Burgt CJ, et al. (2008) Are ECG abnormalities in Noonan syndrome characteristic for the syndrome? Eur J Pediatr 167: 1363-1367.
60. Limongelli G, Pacileo G, Marino B, Digilio MC, Sarkozy A, et al. (2007) Prevalence and clinical significance of cardiovascular abnormalities in patients with the LEOPARD syndrome. Am J Cardiol 100: 736-741.
61. Araki T, Mohi MG, Ismat FA, Bronson RT, Williams IR, et al. (2004) Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat Med 10: 849-857.
62. Marin TM, Keith K, Davies B, Conner DA, Guha P, et al. (2011) Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated PTPN11 mutation. J Clin Invest 121: 1026-1043.
63. Bakkers J (2011) Zebrafish as a model to study cardiac development and human cardiac disease. Cardiovasc Res 91: 279-288.
64. Lien CL, Harrison MR, Tuan TL, Starnes VA (2012) Heart repair and regeneration: recent insights from zebrafish studies. Wound Repair Regen 20: 638-646.
65. Keegan BR, Meyer D, Yelon D (2004) Organization of cardiac chamber progenitors in the zebrafish blastula. Development 131: 3081-3091.
66. Yelon D, Horne SA, Stainier DY (1999) Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214: 23-37.
67. Baker K, Holtzman NG, Burdine RD (2008) Direct and indirect roles for Nodal signaling in two axis conversions during asymmetric morphogenesis of the zebrafish heart. Proc Natl Acad Sci U S A 105: 13924-13929.
68. de Campos-Baptista MI, Holtzman NG, Yelon D, Schier AF (2008) Nodal signaling promotes the speed and directional movement of cardiomyocytes in zebrafish. Dev Dyn 237: 3624-3633.
69. Smith KA, Chocron S, von der Hardt S, de Pater E, Soufan A, et al. (2008) Rotation and asymmetric development of the zebrafish heart requires directed migration of cardiac progenitor cells. Dev Cell 14: 287-297.
70. Cooper MS, D'Amico LA (1996) A cluster of noninvoluting endocytic cells at the margin of the zebrafish blastoderm marks the site of embryonic shield formation. Dev Biol 180: 184-198.
71. Melby AE, Warga RM, Kimmel CB (1996) Specification of cell fates at the dorsal margin of the zebrafish gastrula. Development 122: 2225-2237.
72. Essner JJ, Amack JD, Nyholm MK, Harris EB, Yost HJ (2005) Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. Development 132: 1247-1260.
73. Kramer-Zucker AG, Olale F, Haycraft CJ, Yoder BK, Schier AF, et al. (2005) Cilia-driven fluid flow in the zebrafish pronephros, brain and Kupffer's vesicle is required for normal organogenesis. Development 132: 1907-1921.
74. Long S, Ahmad N, Rebagliati M (2003) The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. Development 130: 2303-2316.
75. Branford WW, Yost HJ (2002) Lefty-dependent inhibition of Nodal- and Wnt-responsive organizer gene expression is essential for normal gastrulation. Curr Biol 12: 2136-2141.
76. Lohr JL, Danos MC, Groth TW, Yost HJ (1998) Maintenance of asymmetric nodal expression in Xenopus laevis. Dev Genet 23: 194-202.
77. Peeters H, Devriendt K (2006) Human laterality disorders. Eur J Med Genet 49: 349-362.
78. Manner J (2000) Cardiac looping in the chick embryo: a morphological review with special reference to terminological and biomechanical aspects of the looping process. Anat Rec 259: 248-262.
79. Franco D, Kelly R, Moorman AF, Lamers WH, Buckingham M, et al. (2001) MLC3F transgene expression in iv mutant mice reveals the importance of left-right signalling pathways for the acquisition of left and right atrial but not ventricular compartment identity. Dev Dyn 221: 206-215.
80. Ramsdell AF (2005) Left-right asymmetry and congenital cardiac defects: getting to the heart of the matter in vertebrate left-right axis determination. Dev Biol 288: 1-20.
81. Digilio M, Marino B (2001) Clinical manifestations of Noonan syndrome. Images Paediatr Cardiol 3: 19-30.
82. Porciello R, Divona L, Strano S, Carbone A, Calvieri C, et al. (2008) Leopard syndrome. Dermatol Online J 14: 7.